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Title: An analysis of the morphological and biological properties of a novel human leukocyte- and platelet- rich concentrate

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Abstract

Wound healing is a complex process that involves several overlapping and interacting biological pathways. The consequences of delayed or abnormal wound healing may result in tissue formation that has impaired function or structural abnormalities. As a result, clinicians have sought ways to enhance this process. Recently, the use of autologous platelet concentrates have become popular in the management of wound healing sites. However, controversy exists as to how these biomaterials should be prepared and applied. We therefore sought to investigate whether a biologically viable and clinically effective platelet concentrate could be prepared using standard laboratory equipment. The findings are presented in a series of articles that have been published in peer-reviewed journals. The results suggest that the experimental platelet concentrate produced, has a morphological structure that consists of a dense fibrin network intermingled with platelets, has the ability to accelerate cellular growth in-vitro, has no adverse effects on cells in-vitro, can concentrate and release a systemically ingested antibiotic over a period of 24 hours in-vitro, can be stored for at least 60 minutes without showing signs of deterioration, and has shown clinical evidence of accelerating wound healing in sinus augmentation and alveolar ridge preservation procedures. The reduced cost of producing such a biomaterial allows it to be available to resource poor settings and to wider range of healthcare providers as compared to standard platelet concentration techniques. Further studies are required to investigate the clinical potential of this promising biomaterial.

Keywords

Blood

Platelets

Leukocytes

Fibrin

Concentrates

Regeneration

Wound

Healing

Growth factors

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I would like to thank my parents, family and supervisors for the unconditional support and encouragement in completing this thesis. Your contribution will never be forgotten.

Declaration

I hereby declare that "An analysis of the morphological and biological properties of a novel human leukocyte- and platelet- rich concentrate" is my own work and that it has not been submitted, or part of it, for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

Mogammad Thabit Peck,   8 November 2018

Signature

Witness

………………………

Prof LXG Stephen

Thesis at a Glance

List of articles

Study	Citations	Aim	Study Design	Main Findings
I Factors affecting the preparation, constituents, and clinical efficacy of leukocyte- and platelet-rich fibrin (L-PRF)	1	To review the literature with regards to factors affecting the preparation of L-PRF	Narrative literature review	Many factors affect the preparation of platelet concentrates. The literature is contradictory with regards to standardised preparation methods for L-PRF.
II The effect of preparation type on the fibrin diameter of leukocyte- and platelet-rich fibrin (L-PRF)	0	To investigate and compare the fibrin network and fibrin fibre diameter of L-PRF prepared by two different methods	In vitro observational study	L-PRF as prepared by the author has a similar structure to that presented in the literature. Preparation methods have a direct effect on the morphology of L-PRF.
III Platelet-Rich Fibrin (PRF)-The effect of storage time on platelet concentration	6	To determine whether storage time had a significant effect on the platelet concentration of platelet-rich fibrin (PRF)	In vitro observational study	Platelet concentration does not deteriorate if L-PRF is stored for up to 60min at room temperature.
IV The in vitro effect of leukocyte- and platelet-rich fibrin (L-PRF) and cross-linked hyaluronic acid on fibroblast viability and proliferation	0	To investigate the effect of cross-linked HA and L-PRF on fibroblast viability and proliferation.	In vitro observational study	L-PRF, alone or in combination with cross-linked HA, is compatible with cell growth and proliferation. L-PRF as prepared by the author accelerates cell growth in the first 24 hours.
V Antibiotic release from leukocyte- and platelet-rich fibrin (L-PRF) – an observational study.	0	To determine whether L-PRF prepared after an oral dose of antibiotic had any significant antimicrobial effect over a 48-hour period.	In vitro observational study	Although L-PRF remains structurally intact for a few days, it does not appear to increase the duration of the release of systemically-ingested antibiotics

VI The use of leukocyte- and platelet-rich fibrin (L-PRF) to facilitate implant placement in bone-deficient sites: a report of two cases.	13	To present two cases where generic L-PRF was used to stimulate bone formation to facilitate ideal placement of dental implants	Case series	A "proof of principle" report that indicates that the L-PRF as prepared by the author, has beneficial clinical outcomes
VII Alveolar ridge preservation using leukocyte and platelet-rich fibrin: a report of a case.	45	To present a case where generic L-PRF was successfully used to preserve the alveolar ridge after tooth extraction	Case report	A "proof of principle" report that indicates that the L-PRF as prepared by the author, has beneficial clinical outcomes

This thesis is based on the following articles, which will be referred to in the main text by their Roman numerals. Reference to the articles in the text may not appear in the numerical order shown below.

I. Peck MT, Hiss D, Stephen L. Factors affecting the preparation, constituents, and clinical efficacy of leukocyte- and platelet- rich fibrin (L-PRF). *SADJ* August 2016, 71 (7). 298-302

II. Peck MT, Hiss D, Stephen L. The effect of preparation type on the fibrin diameter of leukocyte- and platelet- rich fibrin (L-PRF). *SADJ* May 2018. 73 (4). 193-197

III. Peck MT, Hiss D, Stephen L, Majeed A. Platelet- Rich Fibrin - The effect of storage time on platelet concentration. *SADJ* November 2015. 70 (10). 448-451

IV. Peck MT, Hiss D, Stephen L, Olivier A. The effect of leukocyte- and platelet- rich fibrin (L-PRF) and cross-linked hyaluronic acid on fibroblast

viability and proliferation. *SADJ* July 2018. 73 (4). Accepted for publication

V. Peck MT, Hiss D, Stephen L, Maboza E. Antibiotic release from leukocyte- and platelet- rich fibrin (L-PRF) - an observational study. *SADJ May* 2018. 73 (4).

VI. Peck MT, Marnewick J, Stephen L, Singh A, Patel N, Majeed A. The use of leukocyte- and platelet- rich fibrin (L-PRF) to facilitate implant placement in bone deficient sites: a report of two cases. *SADJ* March 2012, 67 (2). 54-59

VII. Peck MT, Marnewick J, Stephen L. Alveolar ridge preservation using leukocyte and platelet-rich fibrin: a report of a case. *Case Rep Dent*. 2011; 2011:345048

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Contribution by the candidate

The candidate was responsible for conceptualizing, planning, performing and data collection for the studies carried out. The candidate was also the main contributing author to writing of the manuscripts that were accepted for publication.

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Abbreviations and acronyms

3T3 cells   mouse fibroblast cell line

ADP   adenosine diphosphate

A-PRF   advanced Leukocyte- and platelet- rich fibrin

A-PRF+   advanced Leukocyte- and platelet- rich fibrin+

ARP   alveolar ridge preservation

ATP   adenosine triphosphate

bFGF   basic fibroblast growth factor

BMP/BMPs   bone morphogenic proteins

CD34   cluster of differentiation protein 34

CD38   cluster of differentiation protein 38

CD90   cluster of differentiation protein 90

clHA   cross-linked Hyaluronic acid

Da   Dalton

DMEM   Dulbecco's modified eagles medium

DMSO   dimethylsulphoxide

ECM   extracellular matrix

EDTA   ethylene-diamine-tetra-acetic acid

EGF   epidermal growth factor

FBS	foetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GBR	guided bone regeneration
G-CSF	granulocyte colony-stimulating factor
GF/GFs	growth factors
GTR	guided tissue regeneration
HA	hyaluronic acid
HGF	hepatocyte growth factor
HGFs	human gingival fibroblasts
HLA-DR	human leukocyte antigen-DR
HSC	heamatopoietic stem cells
HUVEC	human umbilical vein endothelial cells
IGF	insulin-like growth factor
IGF-1	insulin-like growth factor -1
IL-1β	interleukin-1β
L-PRF (C)	leukocyte- and platelet-rich fibrin (Choukroun type)
L-PRF (I/E)	leukocyte- and platelet-rich fibrin (Intraspin/EBA type)
L-PRF (O)	leukocyte- and platelet- rich fibrin (Other)

L-PRF	leukocyte- and platelet-rich fibrin
L-PRP	leukocyte- and platelet- rich plasma
ml	millilitres
MMPs	matrix metalloproteinases
mRNA	messenger RNA
MSC	mesenchymal stem cells
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
N-cm/Ncm	Newton-Centimeter
nm	nanometer
OCS	open canalicular system
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDGF-AB	platelet-derived growth factor AB
pH	power of hydrogen
PRF	platelet- rich fibrin
PRP	platelet-rich plasma
RCF	relative centrifugal force
RPM/rpm	revolutions per minute

SEM	scanning electron microscope/microscopy
Smads	small mother against decapentaplegic
TGF-beta	transforming-growth factor-beta
TGF-β1	transforming growth factor beta 1
TNF-α	tumour necrosis factor alpha
VEGF	vascular endothelial growth factor
α-SMA	alpha-smooth muscle actin

Chapter 1

1.1 Introduction

Wound healing is a complex process that involves several overlapping and interacting biological pathways which aim to reconstitute lost or injured tissue (Etulian, 2018). It is characterized by either "regeneration" (where the damaged tissue is completely restored to its original state) or "replacement" (where the damaged tissue is replaced by scar tissue that does not have the same properties of the lost tissue) (Krafts, 2010). Wound healing is often a combination of both these processes. Clinically, the consequences of delayed or abnormal wound healing may have a direct effect on the treatment, resulting in tissue that has impaired function or structural abnormalities. As a result, clinicians have sought ways to enhance wound healing to affect a better clinical outcome (Knighton et al, 1986).

Based on the biological principles of wound healing, the application of concentrated growth factors (GFs) to damaged tissue has been explored as a promising pathway to improve clinical results (Knighton et al, 1986). These GFs have traditionally been derived from exogenous sources, but recently, newer techniques have been developed so that the use of autogenous GFs has become the preferred choice. These autogenous GFs are harvested directly from the host, thereby reducing the risk of cross infection or adverse immune responses.

In recent times, autogenous platelet concentrates have become the main source of autogenous GFs associated with the clinical management of wounds (Whitman, 1997). This is due to the fact that platelets are easy to harvest, are readily available, and have a high concentration of GFs (many of which have been associated with wound healing).

Several methods have been proposed as a means of concentrating autologous platelets. One of the most common methods used, results in the formation of platelet-rich plasma (PRP) (Whitman, 1997). This is an autologous platelet concentrate that is used in the management of surgically induced wounds or in wounds where the healing is impaired. The preparation of PRP involves centrifuging blood to divide it into its constituent components, and then chemically activating the platelets to form a gel like biomaterial that could be applied to the wound site. This platelet concentrate had initially been introduced as an adjunct treatment option for maxillofacial procedures but has since been widely used for a variety of clinical treatments. However due to several factors, including the processing time, cost, materials, equipment, and the need for additive chemicals, simpler techniques were investigated to produce autologous platelet concentrates.

In 2001, Choukroun et al introduced Leukocyte- and platelet- rich fibrin (L-PRF). This was an autologous platelet concentrate that differed in its preparation method to PRP, in that was it less complex and required fewer steps, and less time. In addition, no chemicals are used during its preparation. This method has shown that L-PRF not only concentrates platelets, but also leukocytes, and embeds these in a dense fibrin meshwork, resulting in a biomaterial which is

easily manipulated for clinical use. The introduction of L-PRF saw platelet concentrates being widely used, initially in dental procedures and later expanding to several other medical disciplines. However, based on the original protocol, the preparation of L-PRF requires the use of specific equipment, which may not be easily available in resource poor settings.

Based on the positive clinical reports of L-PRF presented in the literature, we proposed modifying the original preparation protocol so that standard equipment and materials could be used to prepare an autologous biologically active leukocyte- and platelet rich concentrate. This would potentially allow platelet concentrates to be made available to a much larger patient base than was previously possible. In this context, in 2009, the author modified the classical L-PRF preparation method and introduced the following method of concentrating autologous leukocytes and platelets;

- A standard table-top laboratory centrifuge was used (PLC-03, Hicare International, Taiwan).

- Standard blood silica-coated collecting tubes were used (Greiner BioOne International AG, Germany).

- Autologous blood collected in the tubes were then centrifuged for 10 to 12 minutes at ± 400g (based on the radial diameter of the centrifuge).

- The fibrin clot was then separated from the red blood cells using surgical scissors and tissue forceps.

- This fibrin clot could then be physically manipulated for clinical use

The current thesis as presented by the candidate is presented in the form of Doctoral thesis (PhD) by publications as set out by the guidelines of The University of the Western Cape (C2012/01). This has a number of advantages in that;

- It allows students and future academics opportunities to develop a research identity early on in their studies/careers,
- contributes to the early dissemination of new knowledge produced and the impact of the research is more immediate,
- the publications become a barometer of progress towards the completion of the doctoral degree,
- external feedback as a result of the publication, will be received at a much earlier stage from independent and objective reviewers, thereby strengthening the academic quality of the research in the thesis.

The culmination of clinical reports and laboratory research has been presented in the form of seven scientific articles, presented and published in peer reviewed healthcare journals extending from 2011 to 2018. The papers are presented in the form of independent chapters, but do not appear in chronological order. They are rather presented in a sequence that introduces the reader to the basic concepts of L-PRF and sequentially elaborates on the scientific rationale and laboratory evidence for its justification as a viable biomaterial. The last article presented culminates in clinical case reports that support the advantages of using the material in clinical treatment.

All of the publications have been submitted to peer reviewed journals that are indexed on PubMed®, with six of the publications having been submitted to a journal accredited by The Department of Higher Education and Training of The Republic of South Africa. The South African Dental Journal was selected as the main target for publication, since this journal is disseminated to the majority of dentists in private and public sectors in South Africa. Two of the articles (VI and VII) were published before registration of the PhD but were included due to their recognised significance within the scientific literature. This is evident from the fact that they have collectively been cited at least sixty times by various authors in various international publications since 2013 (Google Scholar). One of the presented articles (III) was based on research conducted as part of a previous master's program at The University of Western Cape but was included based on its relevance to the biological properties of the current tested material, as well as the fact that results from the research had never been submitted for publication in an academic journal.

Of the disadvantages of presenting this thesis by publication, is the fact that although the articles represent different aspects of a universal theme, that this often resulted in repetition of concepts. This was necessary, based on the fact that the papers themselves were published independently of each other, and not as a series. Nevertheless, the cumulative repetition was mostly limited to background information and sought to reinforce the foundation for the current hypothesis with regards the development of the biomaterial. Another disadvantage was that the reference formats for each paper was carried out

according to the publication guidelines, therefore presenting some inconstancies across the various articles.

A brief overview of how the publications section of this thesis is constructed and linked is presented below;

- Chapter 3

    This chapter introduces the reader to the factors affecting the preparation of L-PRF which is presented as a narrative review of the recent scientific literature (I). This forms the basis for understanding of how manipulating certain parameters can result in the formation of a viable leukocyte- and platelet- rich concentrate. It also introduces a proposed classification system for leukocyte- and platelet- rich concentrates, based on the method of production.

- Chapter 4

    In this chapter we analysed the morphological ultrastructure by describing its basic architecture and its fibrin fibre characteristics (II). When compared to the literature, the biomaterial displays features that are consistent with classical L-PRF, but has enough distinct properties so as to distinguish itself from the newer L-PRF variants.

- Chapter 5

    A further analysis of the ability to store the new biomaterial is presented (III). We show that the material could be stored for at least 60 minutes in its original blood collecting tube without disintegrating as was previously

put forth by other authors. This has clinical implications, since it allows for more clinical treatment time to elapse between L-PRF preparation and its transfer to the recipient site.

- Chapter 6

No previous studies indicated whether preparing L-PRF using the modified method, had any biological effect on living cells. We therefore sought to determine this by using an in-vitro model (IV). We showed that the biomaterial had the ability to stimulate cellular proliferation significantly for at least 24 hours and that no cytotoxic or adverse effects were evident. This was independent of other bio-stimulants such as the addition of hyaluronic acid.

- Chapter 7

Fibrin has been tested as a potential drug carrier for a number of years. Because of the dense fibrin network of the tested biomaterial, we sought to determine whether it had the ability to store and release a systemically administered antibiotic (V). We showed that based on an in-vitro study, that the material was capable of storing and releasing the tested antibiotic for at least 24 hours. This may have therapeutic implications for localised drug delivery.

- Chapter 8

In this chapter, we report the clinical results of using the biomaterial for various oral surgical procedures. Positive outcomes are presented for cases in which the material is used to accelerate wound healing in bone

and stimulate bone regeneration in a sinus augmentation procedure (VI and VII). It provides evidence for the fact that the material can be used safely in humans and that it has a beneficial therapeutic effect.

The materials, methods and ethical considerations are presented in detail in each of the above articles. An appendix is attached at the end of this thesis and contains all of the original articles.

Chapter 2

2.1   Literature Review

In order to better understand the rationale for the use of platelet-based concentrates in wound healing therapy, we provide an overview of the latest concepts of the phenomenon and presents its biological relationship to platelet concentrates. The history of the development of autologous platelet concentrates and the evidence for its current use in clinical practice is also documented.

2.2   Current concepts of wound healing

Wound healing is a highly complex process mediated by both local factors and systemic mediators (Etulain, 2018). An excellent review of wound healing is presented by Broughton et al (2006), as well as by Krafts (2010), both of whom describe the phenomenon as a coinciding process of inflammation, proliferation, and remodeling. Although there may be variations in the terminology used, in the context of wound healing, most authors use the term "repair" to define tissue that is restored after trauma (Krafts, 2010). This encompasses two separate, but often overlapping components i.e. regeneration, and replacement. Regeneration is characterized by complete reconstitution of lost tissue, both in terms of structure and function, whereas replacement is essentially repair in which the wound area is replaced with tissue that does not have the same structure or function as the original. This tissue is sometimes referred to as "scar" tissue. Differences in wound healing are also evident with regards to the

type of tissue involved, the age of the patient, the systemic status of the individual, and the degree of wound damage (Broughton et al, 2006; Krafts, 2010). Because of the variation of tissue types, we limit this review to the wound healing associated with epithelial covered tissue (such as cutaneous tissue and the oral mucosa), and bone. Both of which are significant components of the oral and maxillofacial anatomy.

Immediately after a wound is created (traumatically or surgically), exposed collagen activates the coagulation cascade via both the intrinsic and extrinsic pathways (Etulain, 2018). This induces hemostasis which not only reduces the blood flow from the area, but also provides a fibrin-based scaffold that allows incoming cells to migrate to the area. In addition, this scaffold acts as a reservoir for cytokines and growth factors that are associated with wound healing (Krafts, 2010). Concurrently, an inflammatory response is initiated by cell signaling molecules that are released from the damaged tissue and the incoming platelets. These molecules include IL-1, TNF-α, TGF-β and PDGF, the result of which, is an influx of neutrophils that are concentrated at the edges of the wound margin after 24 hours (Broughton et al, 2006). These neutrophils then use the fibrin scaffolding of the blood clot to invade the area and break down local debris and bacteria. Monocytes are also attracted to the area and are transformed to macrophages within 48-96 hours after the wound was created. These macrophages serve various functions, including the release of cytokines and proteolytic enzymes designed to clear the wound from debris and foreign material. However, macrophages do not only play a role in inflammation, but also contribute to the tissue proliferation phase of wound healing by

releasing mediators that promote angiogenesis and increased collagen production (Behm et al, 2012).

With regards to cutaneous tissue and the oral mucosa, epithelialization occurs early in wound repair (Krafts, 2010). If the basement membrane remains intact, the epithelial cells migrate in its normal pattern i.e. from the basement membrane upwards. In this scenario, epithelial progenitor cells remain intact below the wound which allows for the normal layers of epidermis/mucosa to be restored within 2 to 3 days. If the basement membrane has been damaged, then epithelial cells located on the wound edge proliferate and send out projections to re-establish a protective barrier (Broughton et al, 2006). The underlying tissue in the meantime, undergoes increased angiogenesis, resulting from endothelial cell migration and capillary formation. This allows nutrients to be directly delivered to the sight, thereby stimulation tissue proliferation and the formation of "granulation tissue" (Krafts, 2010; Broughton et al, 2006). Granulation tissue is essentially formed due to an increase in collagen production by activated fibroblasts at the wound site and is characterised by a highly vascular immature tissue composed of mostly type III collagen (Gonzalez et al, 2016). This is followed by the maturation and remodeling phase where the collagen is laid down in an organized network which over the following months, remodels to a point where the healed site has 80% of the strength as compared to the lost tissue (Broughton et al, 2006).

Bone on the other hand, follows a different pathway to healing. Bone healing can be divided into two large categories, i.e. direct and indirect fracture healing (Marsell and Einhorn, 2011). This refers to whether the bone portions are

surgically approximated (direct healing) or not (indirect healing). Common to both, is that after the initial inflammatory phase (which is stereotypical and resembles that seen in soft tissue), that specific mesenchymal stem cells (MSCs) are recruited to the area in order to differentiate into osteogenic cells and initiate the process of new bone formation (Marsell and Einhorn, 2011). These stem cells are derived from the surrounding injured bone as well as from the peripheral blood. In indirect bone healing, and different to the healing process of soft tissue, the fibrin clot formed in the initial healing phase undergoes endochondral transformation, which brings about more rigidity to the traumatized tissue. At the same time, intramembranous ossification develops subperiostally at the distal and proximal portions of the fracture. A bridging process then occurs between the area of endochondral and intramembranous ossification, thereby improving mechanical stability and weight bearing ability of the entire wound site (Marsell and Einhorn, 2011).

For direct healing, the process is somewhat different, in that the fractured bone portions are directly in contact with each other, with a gap of less than 0.01mm. In this situation, cutting cones are formed at the ends of the osteons closest to the fracture site. The tips of the cones are formed by osteoclasts which resorb the region ahead of them. At the same time osteoblasts are stimulated distally, resulting in new bone formation and reconstitution of the lost tissue. No callus is formed and the process in independent of endochondral and intramembranous ossification (Marsell and Einhorn, 2011). However, common to both mechanisms of bone healing, is remodeling and that the area is healed without scar formation.

2.3   The significance of cytokines and growth factors in wound healing

Although wound healing is driven by the interaction and proliferation of various cell types, the underlying process is highly dependent on the participation of key cytokines and growth factors, all of which play a significant role in determining the final outcome. Growth factors (GFs) are specialized polypeptide molecules that bind to receptors on target cells, thereby stimulating cellular migration, proliferation, differentiation, survival, and secretion (Behm et al, 2012). These include;

2.4   Transforming growth factor beta (TGF-β)

TGF-β also sometimes referred to as TGFβ, belongs to a superfamily of GFs that has a wide range of functions such as BMPs, activins, inhibins, and Mullerian inhibiting substance (Gilbert et al, 2016). Members of the TGF-β family are widely recognized as key signal transducers and normally occur is three isoforms i.e. TGF-β1, TGF-β2 and TGF-β3. They are produced by platelets, endothelial cells, lymphocytes and macrophages, and effect their function by binding to cell-surface receptors that have serine/threonine kinase activity. This triggers phosphorylation of cytoplasmic transcription factors, referred to as Smads (small mother against decapentaplegic). These then enter the nucleus and affect gene transcription (Gilbert et al, 2016).

TGF-β has a wide range of functions affecting most of the processes governing tissue repair. It participates as a potent chemo-attractant and inflammatory mediator for various types of immune cells, including neutrophils, basophils, eosinophils, mast cells, and circulating monocytes. It also plays an important

role in angiogenesis and granulation tissue formation (Broughton et al, 2006; Krafts, 2010; Gilbert et al, 2016).

The proliferative phase of cutaneous wound healing involves three major TGF-β-mediated events such as re-epithelialization, angiogenesis, and extracellular matrix synthesis. In response to injury, epithelial cells located at the wound margins become activated and undergo alteration of their cytoskeleton. This allows them to lose cell to cell adhesion and increasing cellular motility. This is directly related to TGF-β and its ability to affect epithelial to mesenchymal transition of the epithelial cells (Gilbert et al, 2016).

All of the isoforms of TGF-β are also involved in the angiogenic phase of wound healing. This phase is dominated by the development of *de novo* blood vessels in the area which allows nutrients to be delivered to the rapidly growing tissue. It is thought that TGF-β stimulates the release of VEGF which directly encourages hematopoietic stem cells to promote angiogenesis. Another effect of TGF-β on angiogenesis has also been suggested, with the proposed mechanism being the same as that seen in its role of re-epithelialization i.e. its ability to stimulate epithelial to mesenchyme transition thereby allowing cells from existing vessels to branch and form new capillaries into the newly growing tissue. During this phase, TGF-β also stimulates the synthesis of the extracellular matrix (ECM) and plays a role in recruiting fibroblasts from the surrounding tissue and vasculature. Once the fibroblasts have entered the wound area, they undergo proliferation and the formation of a provisional ECM, most of the process being regulated by TGF-β. It does this by promoting collagen production and inhibiting matrix metalloproteinases (MMPs) (Gilbert et al, 2016; Behm et al, 2012).

The final phase of wound healing is characterized by apoptosis of the residual cells, wound contracture, the replacement of fibronectin, and the substitution of type III collagen by type I collagen. Wound contracture is mediated by myofibroblasts, i.e. a special cell type of fibroblast that has contractile properties. This is evidenced by the presence of Alpha-smooth muscle actin (α-SMA). The expression of α-SMA is directly controlled by TFG-β1 through Smad dependent and Smad independent activity (Gilbert et al, 2016; Behm et al, 2012). This control is limited to areas of stiff ECM often found in the late stages of granulation tissue formation.

From the above, it is therefore clear that TGF-β is a crucial component of all of the phases of wound healing and is required for optimal tissue regeneration. The therapeutic indication for the exogenous introduction of TGF-β to the area, therefore has a valid biological basis.

2.5 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) also known as Vascular endothelium growth factor is another GF that is critical for normal wound healing. An excellent review of its role in wound healing has been described by Bao et al (2009).

VEGF is a homodimetric glycoprotein that shares 20% of its amino acid homology with that of platelet-derived growth factor (PDGF). Due to alternative splicing of its mRNA during its production, five isoforms of VEGF exist. These include VEGF-A, VEGF-B, VEGF- C, VEGF-D, and placental growth factor. The

most studied variant is VEGF-A, hereforth referred to as VEGF (Bao et al, 2009; Behm et al, 2012).

VEGF is produced by a number of different cell types that participate in wound healing. These include endothelial cells, fibroblasts, smooth muscle cells, platelets, neutrophils, and macrophages. One of the major roles of VEGF, is stimulation of angiogenesis. Wound-healing angiogenesis involves multiple steps including vasodilation, basement membrane degradation, endothelial cell migration, and endothelial cell proliferation. This is followed by capillary tube formation and the anastomosis of parallel capillary sprouts, and finally, new basement membrane formation. VEGF plays a role in most of these processes (Broughton et al, 2006; Krafts, 2010; Bao et al, 2009; Behm et al, 2012). It has the ability to stimulate vasodilation by increasing vascular permeability. With an increase in permeability, an increased stretch of the vessel occurs, resulting in further activation of VEGF, thereby setting up a positive feedback loop.

It further facilitates ingress of new vessels into the wound site by setting up a proteolytic environment that ultimately destroys the basement membrane of endothelial cells, thereby allowing these cells to migrate freely into the wound site via chemotaxis. Once these cells have entered into the affected area, VEGF promotes endothelial cell proliferation by selective mitogenic activity (Bao et al, 2009). Furthermore, it delays senescence and restores the proliferative capacity of endothelial cells. The increased replication and increased absolute lifespan of endothelial cells augments VEGF-induced proliferation thereby promoting vascular formation. In wound healing, this vasculature provides a

conduit for nutrients and other mediators of the healing response as well as removal of metabolites.

The evidence therefore implicates VEGF as a significant factor in wound healing. Produced by inflammatory cells and local wound conditions, VEGF alleviates tissue hypoxia and metabolic deficiencies by promoting early events in angiogenesis, as well as endothelial cell function (Broughton et al, 2006; Bao et al 2009; Krafts, 2010; Behm et al, 2012). Maximal activity occurs during a "window" period approximately 3 to 7 days after injury and once the wound is granulated, angiogenesis ceases and blood vessels decline as endothelial cells undergo apoptosis.

Because of the biological effect of VEGF, several clinical trials have been started to investigate its therapeutic potential in the management of nonspecific limb ischemia, Buerger's disease, and myocardial ischemia (Bao et al, 2009). Other conditions that may benefit from the therapeutic use of VEGF is chronic wound management, the treatment of pressure sores, and diabetic ulcers. The use of VEGF in the management of surgical wound sites therefore has a sound biological basis.

2.6   Insulin-like Growth Factor (IGF)

Insulin-like Growth Factor (IGF) is one of the key players in skeletal development and the maintenance of the skeletal structures during life (Behm et al, 2012). It therefore has an essential role in the wound healing of skeletal tissue. However, its physiological significance is not limited to skeletal tissue,

with it playing a significant role in the regeneration of both muscle and neural tissue (Laron, 2001; Reible et al, 2018).

Having first been identified by Salmon and Daughaday (1957), IGF is now recognized as part of the family of insulin related peptides and chemically characterized as small peptide consisting of 70 amino acids with a molecular weight of 7649 Da. Two distinct forms exist: IGF-1 with a molecular mass of 7.5 kDa and isoelectric point of 8.5, and IGF-2 with a molecular mass of 7.4 kDa and isoelectric point of 7.02 (Laron, 2001; Reible et al, 2018).

IGF-1 is the most widely studied form with respect to cartilage injury and repair. Similar to insulin, IGF-1 has an A and B chain connected by disulphide bonds. The C peptide region has 12 amino acids. The structural similarity to insulin explains the ability of IGF-1 to bind to the insulin receptor. IGF-1 is secreted by many tissues, most commonly the liver and is transported to other tissues, acting as an endocrine hormone. IGF-1 is also secreted by other tissues, including platelets and cartilaginous cells, and acts locally as a paracrine hormone (Laron, 2001; Behm et al, 2012; Reible et al, 2018). Evidence indicates that the molecule acts as an anabolic hormone, as is seen in cases where there is underdevelopment of skeletal structures due to IGF-1 deficiency (Laron, 2001). This is most often seen in cartilaginous tissue where the addition of exogenous IGF-1 is found to stimulate chondrocytes and accelerate tissue repair. In addition, exogenous administration of IGF1 induces an increase in muscle oxidative enzymes and fatigue resistance. IGF-1 is therefore plays a significant role in the anabolic component of wound healing and is required for normal growth.

2.7   Fibroblast growth factor (FGF)

Fibroblast growth factor or FGF is a key player in tissue regeneration and repair (Belov and Mohammadi, 2013; Maddaluno et al, 2017). The FGF family includes 22 polypeptides that regulate migration, proliferation, differentiation, survival, metabolic activity and/or neural function in a variety of cells. FGFs range in size from 150 –300 amino acids and can historically be divided into seven subfamilies, however, other studies have proposed the existence of eight (Werner et al, 2011; El Agha et al, 2016; Maddaluno et al, 2017). According to their mode of action, FGFs can be divided into the following groups, i.e. paracrine, endocrine and intracrine. Paracrine FGFs are involved in the development of multiple organs such as the heart, lung, brain, muscle, kidney, hair, ear and limbs. Endocrine FGFs (FGF15/19, 21 and 23) are involved in multiple metabolic pathways such as glucose, lipid and phosphate metabolism (Belov and Mohammadi, 2013). They have low affinities which enables them to function in a hormone-like fashion. Intracrine FGFs (FGF11–14) are retained inside the cells where they control neuronal excitability. Most of these, act by binding to and activating transmembrane tyrosine kinase receptors, designated FGFR1-4 (Belov and Mohammadi, 2013; Maddaluno et al, 2017). Through highly complex molecular pathways, FGF is known to play a role in embryogenesis, gastrulation, somatogenesis, organogenesis, Vitamin D homeostasis and glucose metabolism (Belov and Mohammadi, 2013). During cutaneous wound healing, several types of FGFs are expressed at the wound site, including FGF1, FGF2, FGF7, FGF10, and FGF22. The expression of these are greatly increased at different phases of the repair process. FGF2

seems to be particularly important for wound angiogenesis, whereas FGF7, FGF10, and FGF22, are important regulators of wound re-epithelialization (Werner et al, 2011; El Agha et al, 2016; Maddaluno et al, 2017).

2.8 Platelet-derived growth factor (PDGF)

Patelet-derived growth factor (PDGF) is a two-chain polypeptide, which belongs to the growth factor family (Shah et al, 2014). The original source of PDGF was platelets, but PDGF has been isolated from a variety of normal and neoplastic tissues, including bone matrix and osteosarcoma cells (Shah et al, 2014). PDGF is contained in alpha granules of platelets and is released only during blood clotting or when platelets adhere at sites of blood vessel injury. PDGF may serve to promote wound healing since it is the most potent mitogen in serum for cells of mesenchymal origin including fibroblasts, glial cells, and smooth muscle cells. PDGF stimulates bone DNA and protein synthesis and may be a systemic or local regulator of skeletal growth. As a systemic growth factor, it could be released during platelet aggregation and have important effects in the early stages of fracture healing. PDGF also has a significant influence on wound healing since it is the most potent mitogen in serum for cells of mesenchymal origin including fibroblasts, glial cells, and smooth muscle cells (Shah et al, 2014).

Four different chains (A, B, C, and D) are identified in the structure of PDGF. PDGF is now considered as a family of five heterodimeric and homodimeric proteins (PDGF-AB, PDGF-AA, PDGFBB, PDGF-CC, and PDGF-DD) (Shah et al, 2014). The mature parts of the A- and B-chains of PDGF are 100 amino acid

residues long and show 60% amino acid sequence identity. Each chain has 8 cysteine residues, which are conserved between the 2 chains. PDGF is a highly basic glycoprotein with a pH 10.2 with both PDGF A and B having essentially equal amino acid composition and immunological reactivity (Shah et al, 2014).

PDGF has a direct role in wound healing by increasing the numbers of mesenchymal cells in the wound (Bhem et al, 2012; Shah et al, 2014). This is accomplished in two ways i.e. as platelets aggregate in the wound, they release PDGF which diffuses into the surrounding tissue and acts as a chemo attractant, recruiting cells into the wound, and secondly, at the higher concentrations found in the wound, PDGF increases the proliferation of the cells. In this way, it regulates the number of cells in the wound and the deposition of matrix. PDGF also activates cell membrane receptors on target cells, which in turn are thought to develop high-energy phosphate bonds on internal cytoplasmic signal proteins; the bonds then activate the signal proteins to initiate specific activities within the target cell (Shah et al, 2014). The most important specific activities of PDGF include mitogenesis, angiogenesis, and macrophage activation. It also stimulates mitogenicity and chemotaxis of fibroblasts, smooth muscle cells and neutrophils. Moreover, PDGF has been shown to stimulate the production of several matrix molecules like fibronectin, collagen, proteoglycan, and hyaluronic acid. PDGF may also be of importance at later stages of wound healing as it stimulates contraction of collagen matrices in vitro implicating a role in wound contraction in vivo (Shah et al, 2014). Furthermore, PDGF stimulates the production and secretion of collagenase by fibroblasts, suggesting a role in the remodeling phase of wound healing. From

the above, it can therefore be seen that PDGF is an essential component of wound healing.

2.9 The role of platelets and platelet concentrates used in wound healing

A portion of this review has previously been published as part of another original thesis by the same author (Peck, 2011). Due to the fact that most of physiology and literature with regards to history of platelet concentrates have not changed since then, we have used excerpts from that thesis and updated the information based on current information.

2.10 Platelets

Platelets are anucleate, discoid shaped blood cells that are derived from megakaryocytes in the bone marrow. They enter the peripheral blood, where they circulate for 7-10 days before 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 × 10^9/L of circulating blood (Ezeilo et al, 2002).

Structurally, platelets consist of the following three identifiable zones i.e.:

- A peripheral zone containing the plasma membrane, receptors and the open canalicular system (OCS). The OCS is a final common pathway for uptake of particulates and discharge of secretory products in thrombin-activated human platelets (Escolar and White, 1991).
- 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 which include α–granules, dense granules and lysosomes, with the α–granule being 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 µm^2 which is eight times more than the dense granules and approximately equal to that of the OCS. The α–granules contain a large number of mediators that include, amongst others, platelet factor 4, IL-8, PDGF, TGF-β and VEGF (Heijnen and van der Sluijs, 2015). Platelet dense granules (three to eight per platelet) form the second major secretory compartment. They contain mainly small molecules such as ADP, ATP, serotonin, calcium, pyrophosphate and polyphosphate. The remaining granules, the lysosomal granules, contain hydrolytic enzymes that dissolve phagocytosed debris. The functions of platelets are therefore directly related to the properties of their secretory granules (Blair and Flaumenhaft, 2009). This allows platelets to be participate in coagulation, wound repair, inflammation and angiogenesis (Heijnen and van der Sluijs, 2015).

2.11 The role of platelets in haemostasis and wound healing

When a blood vessel is injured, it immediately contracts at the site of injury. At the same time, platelets adhere to the underlying exposed collagen and form a temporary plug. This plug formation is divided into 3 interrelated stages that

includes platelet adhesion, a release reaction, and platelet aggregation. Platelet adhesion is trigged 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, which results in better adhesion (Ezelio, 2002). 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 polypepides 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 i.e. 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 α2-antiplasmin, which limit plasmin-mediated fibrinolysis, thereby promoting clot formation (Ezelio, 2002).

Evidence also suggests that platelets may form an integral part of the inflammatory response (Blair and Flaumenhaft, 2009). Platelet α–granules influence inflammation by secreting high concentrations of pro-inflammatory and immune-modulating factors. 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 known to recruit neutrophils in certain circumstances (Blair and Flaumenhaft, 2009; Tözüm and Demiralp, 2003).

The chemical mediators released by platelets also play an essential role in wound healing. The joint action of these mediators' results in revascularization of damaged tissue through;

- the induction of migration, proliferation, differentiation, and stabilization of endothelial cells for new blood vessels;
- restoration of damaged connective tissue through migration, proliferation, and activation of fibroblasts;
- and proliferation and differentiation of mesenchymal stem cells into tissue-specific cell types.

Based on the above physiological characteristics, the use of platelets in the form of autologous concentrates has been advocated for tissue regeneration.

2.12 Platelet concentrates and tissue regeneration

The exact history for the use of autologous platelet concentrates in wound healing is unclear and inconsistent in the literature. Nevertheless, in 1986 Knighton et al described the use of autogenous platelets to accelerate wound healing in chronic nonhealing wounds. According to our knowledge, this was one of the earliest accounts of autologous platelet concentrates being used as

an adjunct to wound healing in humans. In 1993 Hood et al and later Hill et al (1993), described a "newly developed autologous platelet-concentrated fibrin glue" that had wound healing properties. Four years later, Whitman et al (1997) introduced the use of autologous platelet concentrates to enhance wound healing for various maxillofacial procedures. These included mandibular reconstruction, sinus augmentation, reparation of Schneiderian membrane tears, the reconstruction of oral-antral fistulas and the control of haemorrhage. The platelet concentrate used in this case was similar to that previously described by Matras (1982) and 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 this platelet concentrate. 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 (2012b) proposing a standardised nomenclature to denote various platelet concentrates.

By the year 2000, PRP had gained widespread acceptance as a viable treatment option for various clinical procedures. Although research has focused on the ability of PRP to accelerate 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 enhanced wound healing were proposed. Central to all of them, was the ability of a high concentration of platelets to release growth factors and other mediators to stimulate the preferential migration of undifferentiated stem cells to the wound site.

2.13 PRP preparation methods

Since the original preparation method of PRP had been described, several modifications of the 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, 2012b).

This variation in preparation protocol has led to the non-standardisation of PRP with the result that potential biological differences may exist between them (Dohan Ehrenfest et al, 2010c; Dohan Ehrenfest et al, 2018). However, whether this translates into any clinical significance, remains a point of contention.

2.14 Safety and efficacy

The preparation of PRP is not purely autologous and the inclusion of various additives, some of which are animal-derived, are common to most of the preparation techniques. These are added to stimulate platelet activation include bovine thrombin and calcium chloride. 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 anti-coagulated blood (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 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 (Wai 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 thombin 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.

2.15 Joseph Choukroun and platelet-rich-fibrin (PRF)

Joseph Choukroun (cited by Dohan et al, 2006a) successfully introduced a new technique of concentrating autologous platelets for surgical use. 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. Different from traditional methods of producing PRP, Dohan et al (2006a) described the PRF preparation method 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).

2. The collected blood is immediately centrifuged in a tabletop centrifuge at a relative centrifugal force of 400 x g for 10-12 minutes (Dohan et al, 2006a; Dohan et al, 2006b). 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. Platelets and leukocytes are trapped in large numbers within the fibrin mesh, and this portion of the when isolated, is termed PRF (Gassling et al, 2010).

3. PRF is then separated from the red blood cells by using various instruments such as surgical tissue forceps or scissors (Dohan et al, 2006a; Dohan et al, 2006b).

4. The resultant biomaterial can then be used as is, or it be compressed to form an "autogenous membrane" or a dense "plug" used that is used for post dental-extraction treatment (Dohan et al, 2006a; Dohan et al, 2006b)

The above procedure is often denoted as the "classical" method of PRF production. This has however changed recently, with modifications made to the tubes and the centrifuge used to produce PRF (Miron et al, 2018). Changes have also been made to the time and centrifugal force used, with the resultant preparations termed A-PRF, APRF+ and IPRF respectively. Interestingly, PRF prepared using the classical protocol uses different preparation guidelines to that currently advocated by the original proponents of the technique (Miron et al, 2018). Nevertheless, and irrespective of the protocol used to prepare PRF, it distinguishes itself from PRP in that it is easier to prepare, requires little

specialized equipment, uses no additives, is less expensive, and requires less time (Dohan et al, 2006b; Dohan Ehrenfest et al, 2010a). The term PRF and L-PRF is often used interchangeably in the literature and this trend has been continued in this review.

2.16 PRF: Structure, biological properties and clinical applications

Morphologically, PRF appears as a gel like clot that has two main areas: a yellow portion constituting the main body, and a red portion constituting the upper most part of the red blood cell layer (Paper III). 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, 2010b, 2010b; Gassling et al, 2010; Kang et al, 2010).

An analysis of the concentration of platelets found in PRF showed that it trapped $\pm$ 97% of the total platelets available (Dohan Ehrenfest al., 2010a). Different to other platelet concentrates, PRF also contains a high concentration of leukocytes. Up to 50% of available leukocytes can be trapped in a platelet concentrate using the Choukroun PRF preparation method (Dohan Ehrenfest et al, 2010a).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, 2006a; Dohan et al, 2006b). One study compared PDGF-AB, PDGF-BB, and TGF-β levels in PRP samples that were either leukocyte-rich or leukocyte-poor. The presence of leukocytes resulted in significantly

higher mean absolute levels of PDGF-AB and PDGF-BB compared to leukocyte-poor PRP (Zimmerman et al, 2003). For TGF-β, higher concentrations were detected for the leukocyte containing PRP when its levels were related to platelet concentration.

Dohan Ehrenfest et al (2010a) 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, 2010a). This architecture has been confirmed by recent independent animal studies (Bai et al, 2017). Newer generation platelet and leukocyte concentrates such A-PRF, A-PRF+ and IPRF have a different ultrastructure and biological profile compared to PRF and as such are not considered equivalent to the original biomaterial (Miron et al, 2017a; Miron et al, 2018, Fujioka-Kobayashi et al, 2017).

When compared to traditional PRP, PRF shows a higher concentration of growth factors, including PDGF and TGF-β (Gassling et al, 2010; He et al, 2009; Kang et al, 2011). Because no additional activators are used during its preparation, the material undergoes slow polymerization, thereby allowing it to trap a number of key cytokines. These cytokines are released over a longer period when compared to PRP. This characteristic has been proposed as one of the major reasons that the biomaterial displays positive clinical outcomes.

Several researchers have studied the clinical and biological effects of PRF on wound healing. Shen et al (2008) showed that PRF had the ability to inhibit epithelial cell growth, enhance osteoblast proliferation, and increase gingival fibroblast proliferation. He et al (2009) analyzed the effect of PRF and PRP 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. Jang et al (2010) showed that when PRF was combined with silk powder, it could successfully be used to treat peri-implant bone defects. Choukroun et al (2006) evaluated the potential of PRF in combination with freeze-dried bone allograft to enhance bone regeneration when used in lateral sinus floor augmentation. At 4 months, the histologic maturation of the test group appeared identical to that of the control group at 8 months i.e. the quantities of newly formed bone were equivalent. The authors therefore concluded that PRF accelerated bone regeneration when was used in combination with freeze-dried bone to perform sinus floor augmentation.

2.17 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 tooth. The patient initially presented with gingival recession of 5 mm. After the surgical site was prepared, an autologous PRF membrane 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, the author 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 for sinus augmentation. 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 bone height of 4.5 to 8 mm, a healing period of 2-3 months was found to be sufficient to resist a torque of 25 Ncm 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 (Simonpieri et al, 2011, Simonpieri et al, 2009, 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 guided bone regeneration (GBR) procedures. Simonpieri et al (2009), reported on a new technique for maxillary reconstruction using a combination of freeze-dried bone allograft, 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, 2009).

Toffler et al (2014) 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 and mixed with the bone graft material. Further PRF membranes were then placed over the graft material to isolate the wound from the overlying tissue.

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 (2010), a PRF membrane can be compressed into a "plug" using 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.

PRF has been used to repair perforations of sinus membranes after injury during sinus augmentation procedures. Choukroun et al (2006) 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 is a viable treatment option in the management of iatrogenic sinus perforations (Mazor et al, 2009).

2.18 Current controversies regarding PRP and PRF

Although widely used in a variety of clinical fields, the ability of PRP to enhance wound healing has been questioned (Wang et al, 2007; Trombelli et al, 2008; Plachokova 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 (Plachokova 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 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 results in a number of clinical case reports, it however suffers from the same scientific weaknesses as PRP in that the biomaterial currently lacks long-term controlled trials that would endorse its routine use in surgical procedures. Recently, a 5-year follow up report has been published which demonstrates the clinical safety and efficacy of using this biomaterial (Simonpieri et al, 2011).

Because of the growth in popularity of the biomaterial, a number of systematic reviews with regards to its clinical efficacy has recently been published (Najeeb et al, 2017; Miron et al, 2017b; Miron et al, 2017c; Ali et al, 2015). In most cases these reviews are not conclusive nor show any major clinical advantage for the use of PRF in routine dental surgery. Most studies conclude that heterogeneity in the literature limits the evidence that is available for further analysis. Moreover, the term PRF is used in the literature to denote a range of biomaterials that may all not have been prepared using the same protocol (Miron et al, 2018). As such, most authors suggest that larger and better controlled clinical trials are needed to study the potential of this biomaterial.

2.19 Summary

The use of autologous platelet concentrates has become common for the management of a wide range of clinical scenarios (Creslik-Bielecka et al 2012; Harrison, 2018). However controversy exists as to how these biomaterials should be prepared and applied (Zimmerman et al, 2003; Bielecki et al, 2012; Kobayashi et al, 2016; Dohan Ehrenfest et al, 2012a; Ghanaati et al, 2018; Kubesch et al, 2018) . We therefore sought to investigate whether a biologically viable and clinically effective platelet concentrate could be produced using standard locally available laboratory equipment. The findings are presented in a series of articles that have been published in peer-reviewed journals.

Chapter 3

3.1 Factors affecting the preparation, constituents, and clinical efficacy of leukocyte- and platelet- rich fibrin (L-PRF)

The use of leukocyte and platelet- rich fibrin (L-PRF) in various medical procedures has enjoyed exponential growth recently (Miron et al, 2017a; Miron et al, 2017c). An electronic search of the PubMed ® database, showed that 756 publications having being reported during the past 5 years. Of these, 56 or 7.5% have been classified as clinical trials whereas case reports constitute a much higher percentage (13.5%). In almost all of the reports, the method of L-PRF preparation has not been standardised (Miron et al, 2017c; Miron et al 2018). The heterogeneity of the methods used, makes direct comparisons between the studies challenging, since one cannot assume that the platelet concentrate produced by these varying methods are the same. Based on the above, and the fact that the author has been approached by several clinicians, with regards to standardised settings for the production of L-PRF, it was decided to elaborate on the factors affecting the preparation of L-PRF by publishing a narrative review, using relevant data derived from the literature. In our report, we propose a new classification of L-PRF type platelet concentrates, so as to distinguish L-PRF (produced by the original protocol), from other types of platelet concentrates. We also highlight the most common factors that influence its preparation. The report draws attention to the diversity of methods used to produce L-PRF and the fact that these different preparation methods may result in differences in the biomaterial formed. Evidence is presented to substantiate

these claims. Emphasis is placed on the centrifuge force and its role in optimising the production of L-PRF. Biological factors such as pH, growth factor release, the influence of bone morphogenic proteins and the presence of stem cells are addressed. Mention is also made of the clinical success of L-PRF produced by non-standardised methods.   The resultant article was published in The South African Dental Journal of August of 2016.

3.2 Published article

Title: Factors affecting the preparation, constituents, and clinical efficacy of leukocyte- and platelet- rich fibrin (L-PRF)

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Abstract

Platelet- rich fibrin (PRF) was first introduced by Choukroun *et al* in 2001 as a method of concentrating autologous human leukocytes, platelets and fibrin for autotransplantation into surgical wound sites to accelerate healing. Even though several clinical reports have documented the use of L-PRF, controversy still exists with regards to several aspects of this biomaterial. Several publications report the use non-standardised methods to prepare L-PRF, resulting in variable clinical results. The impact of the type of centrifuge, as well as the growth factor release kinetics have recently been studied and have yielded new insights into the structure and function of L-PRF. The presence of bone morphogenic proteins as well as stem cells has also been documented. In this report we analyse various factors affecting L-PRF preparation and its constituents and highlight some of the controversies surrounding the biomaterial.

Introduction

Platelet- rich fibrin (PRF) was first introduced by Choukroun *et al* in 2001 as a method of concentrating autologous human leukocytes, platelets and fibrin for autotransplantation into surgical wound sites to accelerate healing [1]. This method of concentrating blood platelets was different to previous techniques in that it only centrifuged the collected blood once, no anticoagulant agents were added, and leukocytes and fibrin were deliberately included in the final product. Previous similar techniques had only sought to concentrate platelets, with little consideration for the other constituents [2,3]. Choukroun's protocol (Process protocol, Nice, France) was simple and essentially consisted of collecting venous blood into dry glass tubes, afterwhich the tubes would be spun at a low centrifuge speed to allow the blood to separate into the constituents [4]. This resulted in 3 distinct layers forming in the blood collecting tube, i.e. a red blood cell layer at the bottom of the tube, an accellular layer at the uppermost part of the tube, and a leukocyte- and platelet- rich fibrin (L-PRF) layer formed in the middle of the tube [4]. The L-PRF layer was considered as the active biomaterial and has since its development, been promoted as an agent that accelerates wound healing and tissue regeneration [5]. Even though several clinical reports have documented the use of L-PRF in oral and extra-oral surgical procedures, controversy still exists with regards to several aspects of this biomaterial. In this report we attempt to highlight some of these.

The terminology and classification of L-PRF

In an attempt to distinguish various platelet concentrates from each other, Dohan Ehrenfest *et al* used three key parameters i.e; the preparation process, the pharmacological properties, and the characteristics of the final material to establish a functional classification of platelet concentrates [5]. Using these parameters with specific criterion, the authors were able to classify platelet concentrates into four distinct categories (Table 1) [5].

Table 1: Categories of platelet concentrates as proposed by Dohan Ehrenfest *et al* [5]
1. **Pure platelet-rich plasma (P-PRP)**
2. **Leucocyte- and platelet-rich plasma (L-PRP)**
3. **Pure platelet-rich fibrin (P-PRF)**
4. **Leukocyte- and platelet-rich fibrin (L-PRF)**

Although this classification elucidates the distinction of various platelet concentrates simpler, it is not the only system to classify platelet concentrates that exists, and other systems have since been described [7]. Further, Dohan Ehrenfest's proposed classification is widely quoted in the literature and at the time of its publication, Choukroun's PRF was the only platelet concentrate included in the category of L-PRF.

As the popularity of Choukroun's protocol for the production of L-PRF grew, publications emerged that purported to produce L-PRF, but had not used the exact criterion as described by Choukroun (Process protocol, Nice, France) [8-11]. It is unclear whether L-PRF not produced by this method can be classified as a true L-PRF [4]. Publications incorrectly use the term L-PRF and Choukroun's PRF interchangeably, even though the exact method as described by Choukroun is not used to produce the resultant platelet concentrate [12]. This has lead to incorrect assumptions and a clear classification of platelet concentrates that is universally accepted is therefore sought. For the purposes of clarity, the following proposed terminology will be used throughout this article;

- L-PRF – Leukocyte and platelet-rich fibrin. Defined as a broad and all-inclusive category that is used to describe a mixed platelet, leukocyte and fibrin concentrate prepared using no-anticoagulants and a single spin centrifuge technique.

- L-PRF (C) – Leukocyte and platelet-rich fibrin (Choukroun type). Defined as a specific leukocyte and platelet-rich fibrin prepared using Choukroun's protocol i.e. *(the equipment and the preparation method follows the exact recommended protocol as outlined by Choukroun)*.

- L- PRF (I/E) – Leukocyte and platelet-rich fibrin (Intraspin/ EBA 20 type). Defined as a specific leukocyte and platelet-rich fibrin prepared using either an Intra-spin (Intra-Lock International, Boca-Raton, FL, USA), or EBA 20 (Andreas Hettich GmbH & Co KG, Tuttlingen, Germany) centrifuge and following the recommended protocol as outlined by the Dohan Ehrenfest *et al* [4].

- L-PRF (O) – Leukocyte and platelet-rich fibrin (Other). Defined as a leukocyte and platelet-rich fibrin prepared similar to L-PRF (I/E) and L-PRF(C), but using a non-purpose built centrifuge.

Techniques and methods of producing L-PRF

Choukroun's initial method of producing L-PRF was intended to be a simple technique that would allow for the production of high quality platelet and leukocyte concentrates, that could easily be prepared and used in everyday healthcare facilities [4]. This method specifically used a PC-02 table centrifuge and a collection kit from Process (Nice, France) [4].Further a blood sample is to be taken without anticoagulant in 10-mL blood collecting tubes which were then immediately centrifuged at 3000 rpm (approximately 400g of relative centrifugal force) for 10 minutes. The formed L-PRF clot was then removed from the blood collecting tube and used as required.

The influence of centrifuge type and relative centrifugal force on L-PRF

Even though Choukroun's protocol was clearly outlined, several publications were subsequently produced that did not follow the intended protocol [13]. Key to the difference between these publications and the proposed protocol, was the lack of use of a specific centrifuge (PC-O2, Process, Nice, France) and a specific relative centrifugal force (400g). In many publications, the relative centrifugal force was not reported, however the centrifuge speed and time was rather quoted [13-15]. This is a significant deviation from the protocol since the influence of the relative centrifugal force is underestimated.

Relative centrifugal force (RCF) can be defined as the amount of accelerative force applied to a sample in a centrifuge [16]. It is not equivalent to revolutions per minute (RPM) and the terms cannot be used interchangeably. Centrifuges work by putting samples in rotation around a fixed axis, thereby resulting in an accelerative force being applied perpendicular to the axis [16]. This resultant force causes the separation of various elements in the sample based on the individual weight of the sample elements and is the basis for blood separation techniques carried out by laboratory centrifuges. RCF is measured in multiples of the standard acceleration due to gravity at the Earth's surface (x g) and is based on two specific variables i.e. how wide the rotor is and how fast it is moving [16]. The radius of the centrifuge or rotor is as critical to the process as the RPM is. Processes where the RPM and the rotor radius are identical, are comparable and any deviation from reporting, may be inaccurate. Consequently, RCF will only be constant for centrifuges with the same rotor radii. If an RPM setting from a protocol where the operator used a centrifuge with a different radius from yours, you will get a different RCF [16]. Therefore one cannot assume that all centrifuges used for producing L-PRF and spinning at 3000RPM will produce a RCF of 400g. This is a significant parameter that is often misunderstood.

The effect of varying RCF during platelet concentrate preparation was recently reported [17]. In their analysis of various factors affecting the preparation of Platelet-rich plasma (PRP), showed that changes in RCF significantly influenced the platelet yield even though other parameters such as period of centrifuge time as well as temperature remained constant. Dhurat and Sukesh

reviewed several PRP preparation methods [18]. Based on their analysis, it was shown that authors using different RCF parameters resulted in variations in the platelet yields of the PRP produced. More significantly, scrutiny of the literature reveals that although PRP was clinically used for several years, that no standardised protocol has been documented. With regards to the preparation methods of L-PRF that are published in the literature, similar inconstancies exist.

Other centrifuge parameters that may influence L-PRF preparation

In a recent series of articles recently published, the effect of various parameters on the quality of L-PRF was investigated [19-21]. Using the same centrifugal force (400g) as well as the same type of blood collecting tubes, the authors tested 4 different commercially available L-PRF centrifuges. These results indicated that centrifuge vibration as well as centrifuge type, significantly affected the quality and quantity of the L-PRF clot produced. Under scanning electron microscope (SEM) analysis, the L-PRF clots produced from the different centrifuges showed variations in cell morphology and fibrin architecture, with some cells showing signs of significant damage. These differences were attributed to the type of centrifuge used. The Intra-Spin L-PRF centrifuge (Intra-Lock International, Boca-Raton, FL, USA), displaying cells with the most stable and normal shape [20]. It is therefore critical that all the factors are reproduced to be able to standardise the biomaterial. Researchers cannot simply recreate the biomaterial by using any centrifuge with a setting of 400g RCF at the appropriate spin time.

Growth factors and their release kinetics

The preferred use of L-PRF in clinical practice is due largely to its reported release of autogenous growth factors. It is assumed that the high concentration of these growth factors result in reduced healing time as well as the stimulation of tissue regeneration [8]. These growth factors have been well documented in the literature [22]. Recently however, the release kinetics of these growth factors has been questioned [23]. Schär *et al* prepared L-PRF (I/E) with a single spin protocol at 400g for 12 minutes using an EBA 20 (Andreas Hettich GmbH & Co KG, Tuttlingen, Germany) centrifuge [23]. This is the same centrifuge as the Intra-Spin L-PRF centrifuge (Intra-Lock International, Boca-Raton, FL, USA) [24]. Both these centrifuges have recently been upgraded [25,26]. The authors compared the release of various growth factors from L-PRF, L-PRP and a coagulated blood clot. Based on these results, it was demonstrated that the total growth factor release of vascular endothelial growth factor (VEGF) as well as interleukin-1β (IL-1β) was higher for the blood clot than any of the platelet concentrates. Furthermore, no statistically significant difference in the amounts of insulin-like growth factor-1 (IGF-1) as well as platelet-derived growth factor AB (PDGF-AB) that were released, could be established between the blood clot and the various platelet concentrates. The highest concentration of transforming growth factor β1 (TGF-β1) released, was that from the L-PRF (I/E) clot. When investigating the release kinetics of L-PRF (I/E), L-PRP and the blood clot, the researchers reported that the various growth factors were released at different times as well as for different lengths of time. Upon examining this effect on the migration on human bone marrow-derived mesenchymal stem cells (MSC) and Human

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umbilical vein endothelial cells (HUVEC), it was found that no difference in the overall patterns of migration were seen for any of the groups tested. However it was reported that IGF-1 had a positive correlation on the migration of both cell types whereas PDGF-AB had a negative correlation to both cell types i.e. MSC and HUVEC. Interestingly, IGF-1 had the highest concentration in the blood clot and there was no difference in release kinetics of this growth factor when compared to L-PRF and L-PRP [23].

In a similar study, where the release of growth factors as well as the effect of platelet concentrates on tendon cells was compared to that of a whole blood clot, it was shown that the platelet concentrates had the ability to significantly increase cell proliferation as compared to that of the blood clot [27]. However, it must be pointed out that the technique of preparing these platelet concentrates was completely different between the two studies, thereby influencing the final architecture and possible biological properties of the various platelet concentrates used.

Bone morphogenic proteins

Bone morphogenic proteins (BMPs) are low molecular weight glycoproteins that are responsible for ectopic bone formation [28]. First described in the 1960's, these proteins play a critical role in various aspects of cell function, differentiation and tissue repair. More significantly, they are crucial in the maintenance of skeletal integrity and bone fracture healing. BMPs are released and synthesised by a number of cells including osteoblasts, osteoprogenator

cells, chondrocytes, platelets and macrophages [28,29]. It is therefore clear that the synthesis of these key proteins is not restricted to bone forming cells.

It has recently been shown that L-PRF (I/E) releases BMP-2 over a period of 7 days, but that the amount of BMPs that are released, is relatively small [21]. Dohan Ehrenfest *et al* found it difficult to explain the exact origin of these BMPs, but attributed this to the presence of leukocytes in the platelet concentrate [21]. However it has been shown previously that platelets themselves contain significant amounts of BMP-2 and that the release of these proteins is pH dependent [30]. As a result, it has been suggested that platelet's release of BMP-2 may play a significant role in the initial stages of bone fracture healing, since the pH in this environment is optimal for platelet activation [30]. Other researchers have found that other BMPs such as BMP-6, BMP-7 and BMP-4 are also released by platelets, and that the concentration of BMPs contained in platelets, is patient dependent [31,32]. It has further been shown by genome-wide micro analysis that lysed platelets have the ability to upregulate proliferative pathways of osteoblast like cells *in-vitro* [33].

The findings from various studies investigating the BMP potential of L-PRF as well as its variants is potentially groundbreaking, however in light of the above, future studies need to take into account patient variation as well as the pH of the test environment [21,28,30,32]. This may have clinical implications and explain the reasons for inconsistent clinical outcomes of platelet-rich concentrates when used for bone grafting or regeneration. By implication then, it would currently be difficult to control the amount of BMP released from platelets when used in a clinical setting.

Stem cells

Stem cells are undifferentiated cells that can differentiate into specialized cells, including more stem cells or other cells types during development [34]. Recently, a variant of L-PRF(C) has been analysed, and thought to contain hematopoetic stem cells (HSC) [35].The presence of these HSC cells are mostly determined using immunohistochemical analysis for the detection of specific cell markers, in this case, CD34. This is a transmembrane phosphoglycoprotein that is predominantly used as a marker for HSC as well as hematopoetic progenitor cells [36]. Although traditionally linked to cells of hematopoetic cell origin, CD34 has recently been linked to other nonhematopoetic cells types such as mesenchymal stem cells (MSC), endothelial progenitor cells and interstitial dendritic cells [36]. Therefore, the mere presence of CD34 positive cells, does not assume that a specific cell type such as HSC, exists. In order to verify the existence of HSC, the cells should in addition to the presence of CD34, display other traits such as a low expression of CD90, a lack of expression of CD38 and human leukocyte antigen-DR (HLA-DR), as well as a panel of mature lineage markers (lin⁻) [36].

The potential of CD34 positive cell types in L-PRF(C) and its variants appears promising, but requires further investigation due to the variation in CD34 detection methods. Almost all CD34 detection methods use antigen-antibody interactions. These interactions are noncovalent and are reversible, therefore potentially affecting the detection of the CD34 marker. Because of this, it is suggested that multiple methods be used to verify the presence of CD34 [36].

Although peripheral blood has been used as a source for CD34 positive cells in many forms of therapy, the baseline concentration of these cells in peripheral blood is relatively low [37,38]. As such, most therapies that require the use of CD34 positive cells enrich the presence of these cells in the vascular by using granulocyte colony-stimulating factor (G-CSF) [39]. This allows for an adequate amount of cells to be harvested for local or systemic transplantation. Whether the levels of CD34 positive cells of L-PRF and its variants are at therapeutic concentrations, requires further analysis.

Clinical results from studies using L-PRF

Several randomised controlled trials have been published that involve the use of L-PRF in the clinical management of a variety of disorders [40-43]. These trials have contradictory results, which may be related to the variation of techniques and equipment used to prepare L-PRF [41-45]. Nevertheless, several articles present positive clinical outcomes even when not using standardised preparation techniques [46-49]. Most of these publications are case reports, however, even randomised controlled trials where the RCF is not verified, have shown positive clinical outcomes [43]. This is similar to reports about the use of PRP, which show a variety of clinical results based on the various methods of producing PRP [18]. Further research is therefore required to verify clinical differences for the various methods of preparing L-PRF.

Does generic L-PRF exist?

L-PRF (O) is prepared using the RCF of 400g as well as the centrifuge time of 12 minutes, using a non-purpose built table top centrifuge and standard blood

collecting tubes. This method allows for the preparation of a platelet and leukocyte concentrate without the need for specialised equipment. Several case reports have demonstrated positive clinical results using this preparation method [50,51]. However one cannot assume that this generic type of L-PRF has similar properties to that of L-PRF(C) or to L-PRF(I/E) since it has previously been showed that the centrifuge type may a play a significant role in the final morphological features of the end product [19,20]. Further analysis of this biomaterial is therefore required to determine equivalence to the established protocols for L-PRF preparation.

Conclusions

The introduction of L-PRF as an bioactive material with possible regenerative properties has resulted in it being adopted for use in various clinical procedures. However, the widespread use of this material has resulted in several variants of it being produced. Whether the biological properties of all these variants are similar, is unknown. Contradictory clinical results are reported in the literature with several generic types of L-PRF showing diverse results. In order to minimise the controversies associated with L-PRF, further research is required to determine which factors affect the biological properties of the material and whether these factors are clinically beneficial and relevant.

Disclosure policy

The authors declare no conflict of interest regarding the publication of this paper.

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3.3 Conclusion

The current report highlights various factors that affect the preparation of L-PRF and concludes that these factors may play a role in the structure, biological behaviour, and clinical characteristics of the resultant biomaterial. To the author's knowledge, it is the first such publication that addresses these factors as a collective, in a single manuscript. It also critiques the claimed biological properties of the biomaterial, specifically the concentration of its BMP release, as well as the significance of the detected stem cells. Patient variation is mentioned as an important contributing factor that influences its biological properties. This implies that it may be difficult to reproduce the exact biomaterial from different subjects. It is also shown that even though L-PRF is prepared using different protocols, that this variation does not necessarily imply negative clinical outcomes. Studies showing positive clinical outcomes, where a non-standard L-PRF preparation method was used, are highlighted. However, even though the clinical reports of the use of L-PRF are largely positive, inconsistencies are eluded to in the literature. The influencing factors highlighted in this paper may explain these inconsistencies. Furthermore, the authors question whether, based on the literature reports where positive clinical outcomes were observed when non-standard L-PRF preparation protocols were used, if a generic, biologically active, and clinically beneficial L-PRF, could be prepared using non-specialised equipment and materials. Based on the author's previous clinical reports (VI), as well as considering the factors affecting the preparation of the biomaterial, we further tested this hypothesis, the results of which are further expounded upon in subsequent chapters.

Chapter 4

4.1 The effect of preparation method on the fibrin diameter of leukocyte- and platelet-rich fibrin (L-PRF).

The structural properties of L-PRF are well known and have previously been reported upon in the literature (Dohan Ehrenfest et al, 2010a). It has also been shown that differences in preparation method could influence this structure, resulting in biomaterials that may not behave the same biologically (Dohan Ehrenfest et al, 2018). This may have clinical consequences. A significant feature of L-PRF is its fibrin fibre architecture which consists of an interwoven mesh of fibrin fibres interspersed with platelets and leukocytes.   It is this structure that is assumed to impart reliance to the biomaterial thereby allowing for the release of growth factors over an extended period (Dohan Ehrenfest et al, 2006a). This is a significant distinguishing factor that differentiates L-PRF from other platelet concentrates. Since the structural properties of L-PRF were originally described, subsequent publications have further investigated these characteristics, with the most comprehensive of those being that of Bai et al (2017).   The study however had several limitations, including the use of animal blood, and a centrifuge that did not conform to that of the original protocol. A similar study was carried out by Sam et al (2015) in which they examined the mechanical and surface characteristics of L-PRF using a scanning electron microscope. Their study had similar limitations that plagued most L-PRF investigations i.e. the method used to prepare L-PRF did not follow the original protocol, making comparisons, difficult. In the present study we determined

whether modification of the original protocol used to prepare L-PRF (by using non-standardised equipment), influenced the fibrin fibre diameter and architecture of the resultant biomaterial. This is essential, since fibre diameter is known to be directly related to the stiffness of the clot and hence susceptibility to dissolution (Wei Li et al 2016). To the author's knowledge, no similar investigation had previously been published with regards to this specific platelet concentrate. It allowed for the first time, a description of the morphological characteristics of this modified version of L-PRF, a biomaterial which had previously been proven to have positive clinical outcomes (VI). For this investigation we chose to examine L-PRF that was prepared using two different methods, and examined their gross and ultrastructural morphology using SEM. The resultant manuscript was accepted for publication in the South African Dental Journal of May 2018.

4.2   Published article

Title: The effect of preparation method on the fibrin diameter of leukocyte- and platelet-rich fibrin (L-PRF).

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The authors declare no conflict of interest regarding the publication of this paper. This paper forms part of the requirements of the partial fulfilment towards the degree PhD.

Abstract

Introduction: The use of leukocyte and platelet- rich fibrin (L-PRF) in regenerative surgery has increased exponentially in the last decade. Because of it's success, various centres have introduced the biomaterial as a routine inclusion in oral grafting procedures. Evidence suggests that the physical properties of fibrin fibers play an essential role in homeostasis of blood clots. Only a limited number of studies have investigated the features of fibrin fiber diameter of L-PRF concentrates. **Aims and objectives:** To investigate and compare the fibrin network and fiber diameter of L-PRF prepared by two different methods. **Methods:** Blood was collected from a single volunteer using established protocols. The resultant L-PRF clots were then prepared and examined using scanning electron microscopy. The results were subjected to statistical analysis. **Results:** L-PRF prepared using the modified method had larger diameter fibers than that prepared using the official protocol. The difference was statistically significant (P=0.001). There was also a larger amount of thicker fibers observed in the modified L-PRF group. **Conclusions:** Preparation methods affect the fiber diameter of L-PRF. This may have consequences that influence the biological properties of the biomaterial.

Introduction

The use of leukocyte and platelet- rich fibrin (L-PRF) in regenerative medicine has increased exponentially in the last decade [1]. First introduced by Choukroun *et al* in 2001, this autologous, blood derived platelet concentrate that has been extensively used in oral surgical procedures, with clinical success in the fields of surgical implantology and regenerative periodontology [1, 2]. Because of its popularity, various centers have introduced the biomaterial as a routine inclusion in oral grafting procedures [1]. Although Choukroun had specified the original method of L-PRF preparation, this protocol had been modified over time, with several variants of L-PRF now being made available [3, 4]. The modification of the original protocol included updating the equipment and materials used, as well as varying the centrifuge time and force [4]. This resulted in two further variants of L-PRF being introduced i.e. advanced PRF (A-PRF) and advanced PRF+ (A-PRF+) [4]. Both of these variants use a lower centrifugal force when compared to the original protocol and this is thought to have distinct advantages over the original preparation method [3, 4].

An important component of the biological advantages of L-PRF concentrates, is the specific structure which comprises a dense fibrin network, with high concentrations of platelets and leukocytes interspersed within the mesh of fibrin fibers [5,6]. It is thought that this allows growth factors to be trapped, and released over a prolonged period thereby enhancing healing and regeneration [6, 7]. Recently, the role of fibrin structure has been researched for its role in blood clots [8-11]. Evidence suggests that the physical properties of fibrin fibers play an essential role in homeostasis of blood clots by affecting their mechanical and

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biological contribution properties [8, 12]. However, only a limited number of studies have investigated the features of fibrin fiber diameter of L-PRF concentrates [13, 14].

With the increase in popularity of L-PRF, several authors have reported positive clinical results using L-PRF that is prepared using non-standardised equipment or materials [15-17]. An example of this type of "modified" L-PRF has been clinically documented in previous publications by Peck *et al* [18, 19]. This deviation from the proposed protocol for L-PRF may result in structural or functional consequences to the biomaterial that is being produced [20-22]. In this report, using scanning electron microscopy (SEM), we analyse and compare the fibrin network and fiber diameter of two different methods of L-PRF preparation i.e. our modified protocol for the preparation of L-PRF as previously described by Peck *et al*, and A-PRF+, prepared via the recommended protocol as previously outlined in the literature [4, 18, 19].

Materials and methods

The study was conducted at the Dental Faculty, University of the Western Cape, Cape Town, South Africa in 2017. Ethical clearance was obtained from the research ethics committee of the above said university with the following reference number: BM 16/3/31

Preparation of the L-PRF samples

Thirty six milliliters of blood was obtained from a single 30 year old healthy male volunteer via venipuncture of the left antecubital vein. Two methods were used to prepare L-PRF, i.e.;

1. Modified L-PRF:

   Blood samples were collected in 9ml blood collecting tubes that contain clot activator i.e., Vacuette® 9ml serum tubes with Z Serum Clot Activator (Greiner BioOne International AG, Germany). The blood samples were then immediately centrifuged at 400g for 12 minutes in a standard benchtop centrifuge (PLC-03, Hicare International, Taiwan).

2. A-PRF+:

   Blood samples were collected in 10 ml A-PRF+™ tubes (Process for PRF, Nice, France), and centrifuged for 1300RPM for 8 minutes, using a dedicated tabletop centrifuge (PRF DUO™, Process For PRF, Nice, France).

After being centrifuged, the blood from both groups separated into 3 distinct layers (Fig 1). From previous reports, the layers could be distinguished as a topmost layer consisting of acellular platelet poor plasma, Modified L-PRF/A-PRF+ in the middle, and red blood cells at the bottom of the test tube [5, 6]. In order to standardise L-PRF thickness, the resultant L-PRF clots from both groups were removed from the tubes, and subjected to controlled compression, using a specifically designed tool i.e., the PRF Box ® (Process for PRF, Nice,

France) (Fig 2) [23]. All the samples were then transferred for preparation for scanning electron microscopy analysis.

Preparation for scanning electron microscopy (SEM)

All the samples were fixed using 2.5% gluteraldehyde in phosphate buffered saline (PBS) for 1 hour. Each specimen was removed and washed in PBS for 5 minutes (twice) and thereafter distilled water for 5 minutes (twice). The specimens were then dehydrated serially with 50%, 70%, 90% and 100% ethanol, with each dehydration step taking 10 minutes. The samples were then transferred to the Electron Microscope Unit, Department of Physics, University of the Western Cape, for critical point drying, gold-palladium coating, and mounting for SEM analysis.

Scanning electron microscope observation

The surface microstructure of each specimen was analysed using the same scanning electron microscope (AURIGA Field Emission High resolution SEM, Carl Zeiss Microscopy GmbH, Jena, Germany). All measurements of the fibrin networks were carried out at 10 000 times magnification. At least 200 fibers were randomly selected from each group and measured for analysis using ImageJ version 1.51o software developed by Wayne Rasband (National Institutes of Health, USA). The data was captured using Microsoft Excel 2010 (Microsoft Corporation, Washington, USA) and statistically analysed using the Mann-Whitney U and Wilcoxon W tests.

Results

Descriptive analysis of Modified L-PRF and A-PRF+ fiber networks

Under low magnification (1000 times) both samples show similar morphological characteristics. A dense mesh of fibrin fibers was evident with scattered cells seen on the surface for each sample (Fig 3). For the Modified L-PRF sample, more irregular bodies were present, possibly signifying the presence of platelets (Fig 3) [24]. This may be characteristic of the region observed and may not be representative of the sample as a whole [6]. No distinction in fiber architecture for the 2 groups was seen at this level of magnification.

When observing the samples at higher magnification (10 000 times), the fiber mesh showed distinct characteristics (Fig 4 and 5). For both groups, the fibers were densely arranged in a netlike structure with clear spacing and crosslinking seen. Distinct differences could be noted with regards to fiber diameter, with both groups showing non-uniform fiber thickness. Thicker fibers were interweaved with thinner fibers and many of the fibers showed irregular surface characteristics indicative of platelet-fiber interaction [24]. The presence of cellular bodies could be noted for both groups. Distinguishing between both groups solely on the basis of SEM analysis, was difficult.

Analysis of Modified L-PRF and A-PRF+ fibrin fiber thickness

A total of 437 fiber samples were analysed from both groups. The fibers were measured in nanometres (nm) with a range of 100-808nm and a mean of 281nm being observed for the Modified L-PRF group. For the A-PRF+ group, fiber diameters ranged from 43-742nm with a mean diameter of 256nm. For

both the groups, statistical analysis revealed that the mean fiber diameter was not a clear reflection of the various fiber groups present. It also showed that that the fibrin fiber diameters were not equally distributed in each of the groups and that a wide range of fiber diameters were present (Fig 6). These fiber groups were present in both Modified L-PRF and A-PRF+, but were significantly different in their distribution for the two versions of L-PRF. Of the two groups tested, Modified L-PRF was characterized by larger diameter fibers and had a higher amount of thicker fibers when compared to A-PRF+ (Fig 6).

Because of the wide range of fiber diameters noted in each of the sample groups tested, arbitrary size classes were selected to better illustrate the range of fiber diameters seen. This size selection was based on a previously published model which analysed human fibrin networks [25]. Consequently the fiber diameters were categorised into 3 groups based on "thickness" i.e. thin (lowest thickness to 85nm), intermediate (86 - 202nm), and thick (203nm to highest). This is represented by the histogram A.

Discussion

L-PRF in its various forms has shown promising results in both *in-vitro* and *in-vivo* studies [1, 15-19]. This is attributed to the structural integrity of the biomaterial which results in a matrix that contributes to the prolonged release of various growth factors as well as provides a network for the migration of cellular components [2-6]. The process of preparing L-PRF was initially documented by Choukroun in 2001 [2]. However since the original protocol was introduced, several authors have attempted to replicate the platelet concentrate using non-

standardized methods [15-19]. Although clinically successful, the ultrastructure of these L-PRF variants has not been reported upon extensively.

The data from this study indicates that using both the A-PRF+ protocol as well as the modified protocol for the preparation of L-PRF that we used, may yield L-PRF clots that appear similar macroscopically. Both preparation methods resulted in separation of the centrifuged blood into 3 distinct layers as previously described. When examining L-PRF clot size, the resultant L-PRF clots appeared smaller for the Modified L-PRF group, however this was not quantified statistically. We speculate this may be due to using a 9ml blood collecting tube as opposed to 10ml in the A-PRF+ group. However the size of the collecting tube may not be the only factor affecting L-PRF size, since recent research indicates that using different centrifuges may affect L-PRF clot size [20-22].

Compressed L-PRF clots from both groups appeared similar under low magnification (x1000), with a dense fiber network and large range of fiber diameters being seen. The density of the fiber networks seen in this study has previously been reported upon and is thought to be related to the compression of the clots in the PRF-Box™ [23]. When the fiber diameters were analysed as thin, intermediate, and thick, the majority of fibers in both groups were classified as thick. This differs from the findings of Vieira et al 2009, who reported that the predominant fiber patterns were thin.

Previous studies that have examined the features of fibrin associated with L-PRF, have often used the mean diameter of the fibers to describe the

characteristics of the L-PRF clot or are limited to a subjective description of the fiber diameter [13, 20]. In the present study, statistical analysis revealed that using the mean diameter, did not reflect the range and frequency of the fiber types observed. Therefore when analyzing fiber diameters for Modified L-PRF and A-PRF+, we identified clear groupings (Fig 6). These were statistically significantly different for the two L-PRF groups, with the Modified L-PRF showing a higher number of larger diameter fibers as compared to A-PRF+ (Table 1). The exact reasons for this difference in not known, but is probably due to differences in the centrifugal force used, centrifuge time, centrifuge make, agents present in the blood collecting tubes and shape of the blood collecting tubes [20, 26]. Because all the samples were taken from the same individual in a 15 minute period, the above may serve as an adequate explanation for the differences in fiber diameters seen. However, one must consider that for different individuals or in instances where blood is collected from the same individual at different times, that other factors such as pH, the presence of zinc, ionic strength, concentrations of calcium, polyphosphate, fibrinogen, fibrinogen binding proteins and thrombin, may also influence fiber thickness and network density [9,12].

The tubes used to collect blood to prepare Modified L-PRF, are coated with silica. Silica is a known procoagulant. It binds to plasma proteins and has the ability to cause reversible structural changes to these molecules [27,28]. This is dependent on the size of the silica particle, with smaller silica particles demonstrating the ability to shorten coagulation time and increase the activation of factor X, whilst at the same time activate platelets [28]. The fact that silica

coated tubes were used in the preparation of the Modified L-PRF, may further contribute to the morphological differences seen between the 2 groups.

The individual fiber diameters seen in the samples tested may have implications for the mechanical stability of each L-PRF clot [29]. Clots with thicker fibers tend to be less elastic than those with thinner fibers and may be more readily degraded by the fibrinolytic system [8, 29]. This may have consequences for the biological behaviour of L-PRF since structural integrity and the controlled release of growth factors is thought to be a major contributing factor to its clinical success. Therefore, one might assume that because A-PRF+ has a lower amount of thick fibers than Modified L-PRF, that its rate of dissolution may be slower than that of the Modified L-PRF. As such, its ability to remain intact may be prolonged as compared to other platelet concentrates and may explain the extended release of growth factors as recently reported [3,4].

Conclusions

To the author's knowledge, this is the first presentation of the morphological fiber characteristics of an L-PRF clot prepared using a specific modified protocol as previously described by the authors. When compared to an established protocol, the resultant L-PRF clot appears morphologically similar to it's A-PRF+ counterpart with a dense fibrin network interspersed with platelets and other blood cells. However, clear differences are noted for the fibrin fiber diameter with Modified L-PRF showing a higher proportion of larger diameter fibers. The reason for these differences is speculated to be associated with the different protocols used to prepare the platelet concentrates. One might therefore

assume that changes in the protocol of preparation of L-PRF may directly affect it morphological structure. Whether these differences affect the clinical efficacy of these biomaterials is unclear, and warrants further research.

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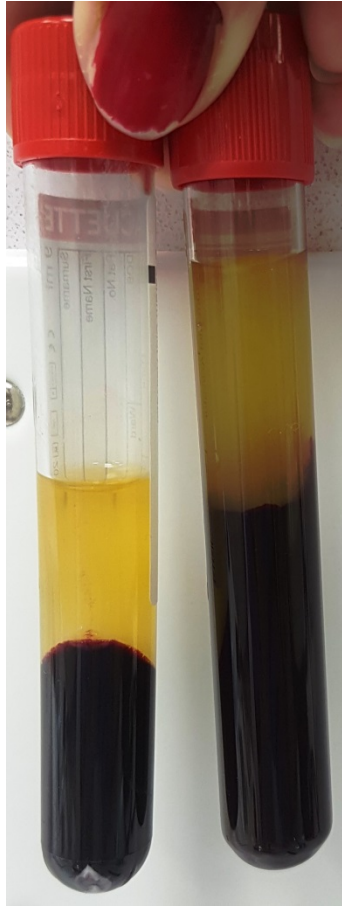
Figures

Figure 1. Modified L-PRF (left) and A-PRF (right)
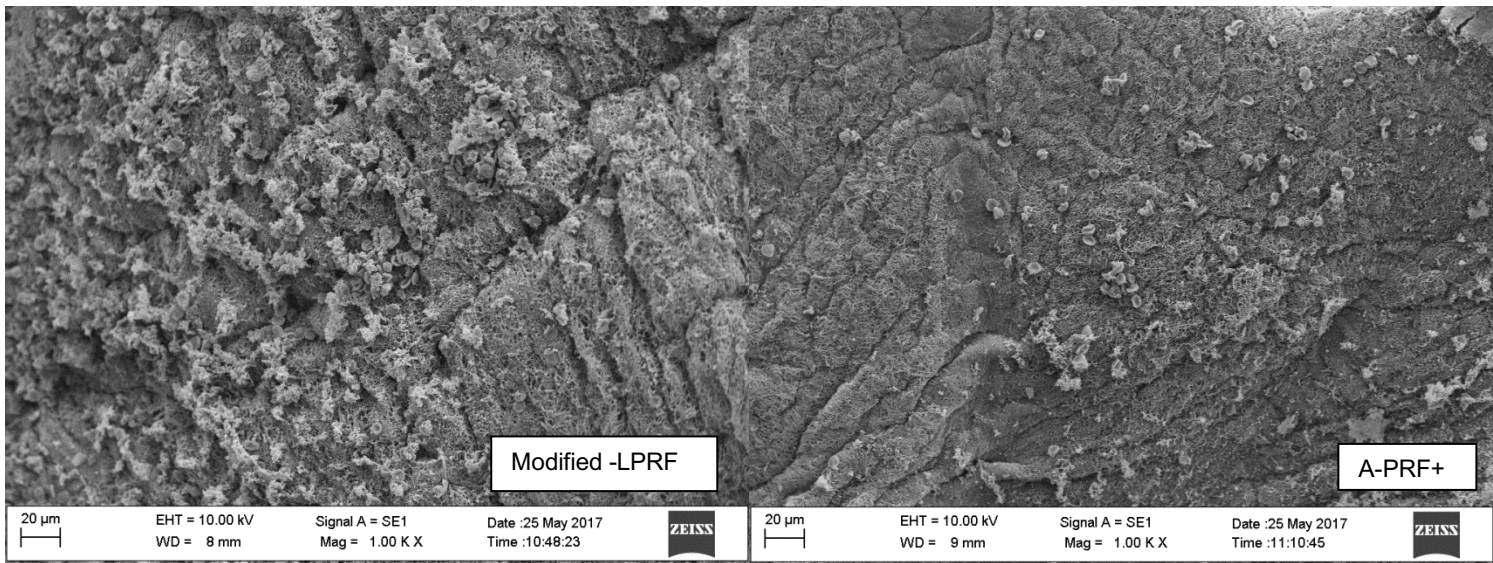
Figure 2. PRF Box with prepared L-PRF before compression

| 20 µm | EHT = 10.00 kV | Signal A = SE1 | Date :25 May 2017 | ZEISS |
| | WD = 8 mm | Mag = 1.00 K X | Time :10:48:23 | |

Modified -LPRF

| 20 µm | EHT = 10.00 kV | Signal A = SE1 | Date :25 May 2017 | ZEISS |
| | WD = 9 mm | Mag = 1.00 K X | Time :11:10:45 | |

A-PRF+

Figure 3. Modified L-PRF and A-PRF+ (Low magnification)
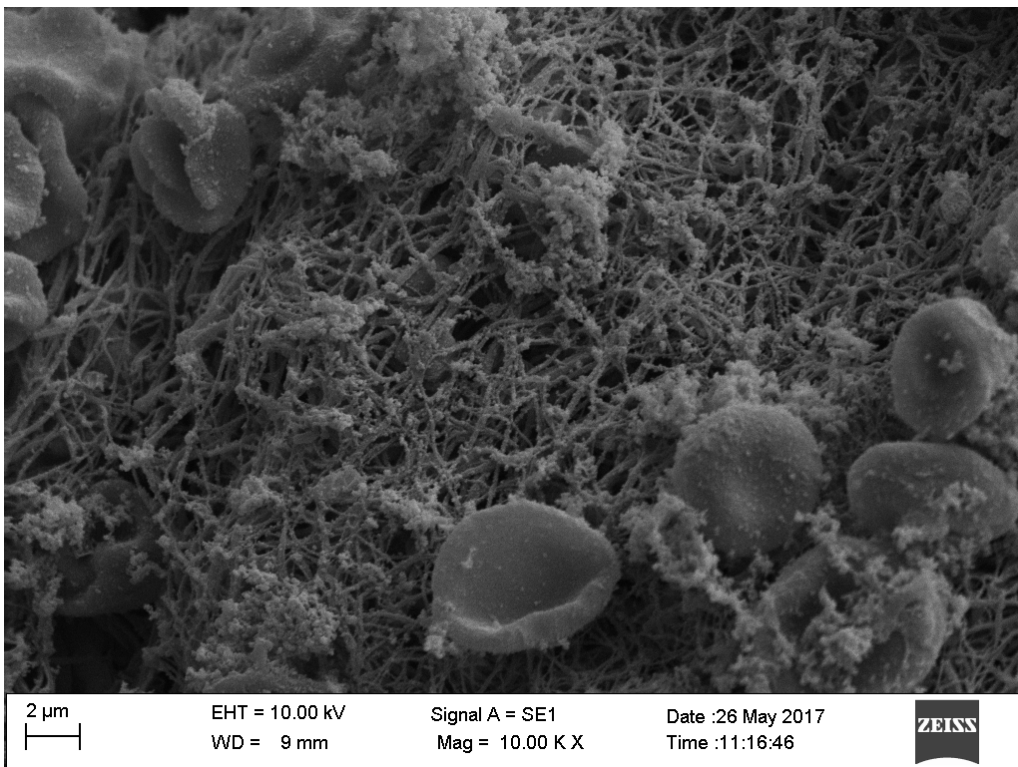
Figure 4. Modified L-PRF (High magnification)
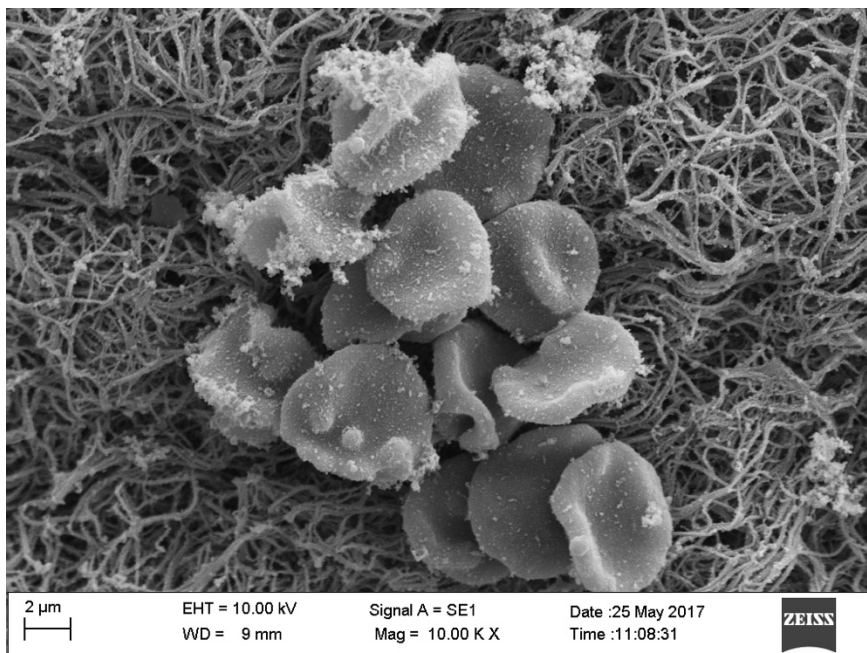
Figure 5. A-PRF + (High magnification)
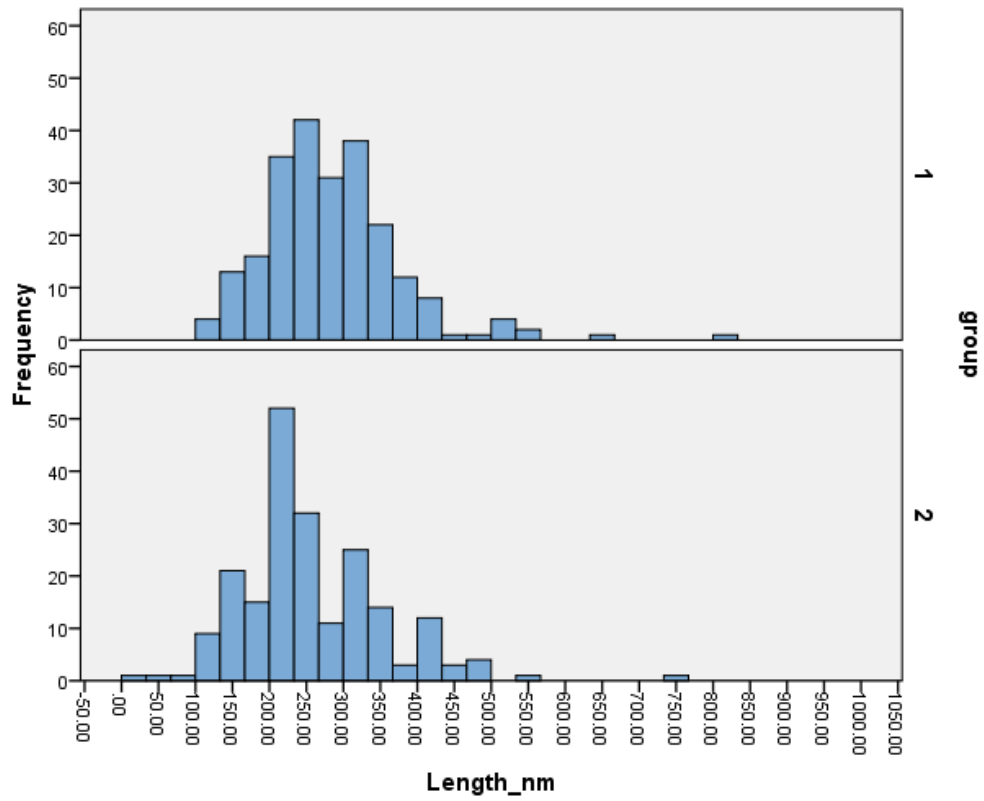
Figure 6. The distribution of fiber diameters for Modified L PRF (group 1) and

A-PRF+ (group 2) measured as length in nm

Ranks				
	Group	N	Mean Rank	Sum of Ranks
Length_nm	M-PRF	231	238.06	54992.00
	A-PRF+	206	197.63	40711.00
	Total	437		

Test Statistics[a]	
	Length_nm
Mann-Whitney U	19390.000
Wilcoxon W	40711.000
Z	-3.343
Asymp. Sig. (2-tailed)	.001
a. Grouping Variable: Group	

Table 1 a,b. Results of testing the difference in fiber diameter (Length in nm)

between both groups with the Mann Whitney test:
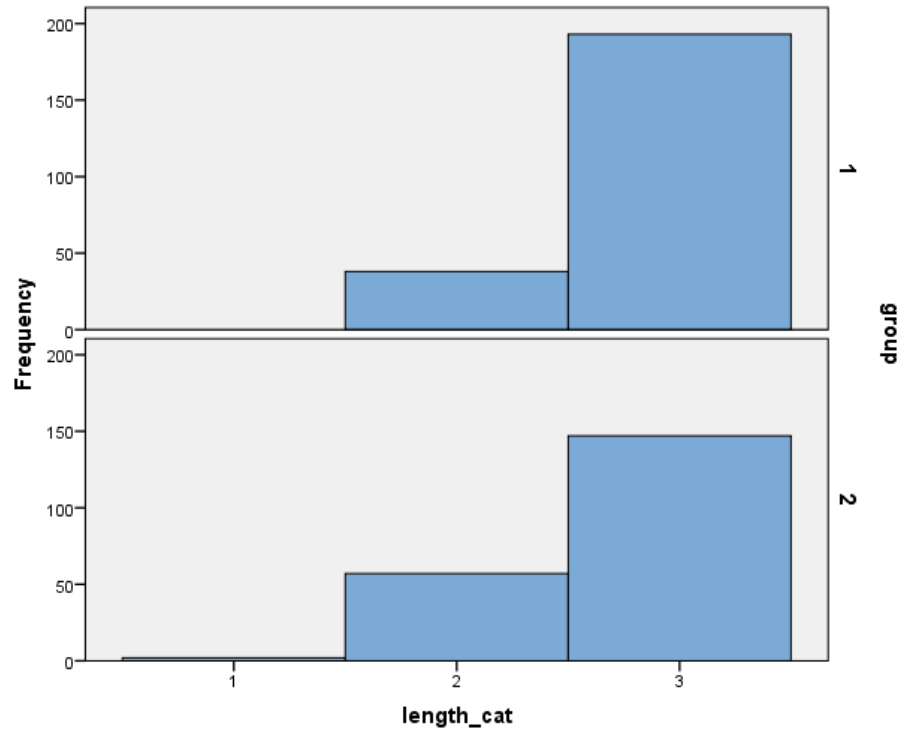
Histogram A. The distribution of "thin, intermediate and thick" fibers of

Modified L-PRF (group1) and A-PRF+ (group 2)

4.3 Conclusion

The current investigation, to the best of our knowledge, is the first to describe the gross and ultrastructural features of L-PRF prepared using a protocol that was previously introduced by the author. The findings showed that L-PRF produced in this way, had specific characteristics that include; a similar gross and microscopic surface morphology to that of L-PRF prepared using a lower centrifugal force. This included a dense mesh-like network of fibrin fibres interspersed with cellular components that may be indicative of platelets and leukocytes. The fibrin fibres had a non-uniform diameter with a mean of 281nm, and a distinct predominance of fibres that could be regarded as thick (> 203nm). These "thick" fibres were found in higher amounts as compared to that of the L-PRF produced by a lower centrifugal force. Except for the differences in the amount of "thick" fibres, both groups showed very similar morphological characteristics. This may partly explain similarities seen in the clinical performance of these biomaterials, both which show the potential to accelerate wound healing. The effect of this biomaterial on cellular growth is further expounded upon in subsequent chapters.

Chapter 5

5.1 Platelet- Rich Fibrin (PRF) - The effect of storage time on platelet concentration

The development of autologous platelet concentrates has resulted in various forms of these biomaterials being used to stimulate faster wound healing over the past two decades (Dragonas et al, 2018; Najeeb et al, 2017; Ali et al, 2015). Most of these materials are prepared during or slightly prior to the clinical procedure. This is thought to result in an optimum biomaterial, which has maximum clinical efficacy. With the development of Choukroun's method of producing L-PRF, this philosophy had been perpetuated, with the initial literature indicating that the biomaterial can only be stored for up to 15 minutes in the blood collecting tube after formation. It was assumed that this would preserve its mechanical properties (Dohan Ehrenfest, 2010c). This in turn, is thought to affect its biological behaviour (Dohan Ehrenfest et al, 2012a). This has clinical implications in that the biomaterial must be prepared during the procedure and be transferred to the wound as soon as possible or if stored, requires the need for specialised equipment (Dohan Ehrenfest et al, 2010c). With increased complexity of different procedures, this often meant that phlebotomy had to be carried out whilst the patient was sedated and dressed with sterile drapes. This increased the likelihood of contamination of the surgical area, especially if the phlebotomy was carried out by someone not directly involved in the surgical procedure. We therefore sought to examine whether storage or L-PRF (using a modified preparation method), for a period of longer

than 15 minutes, in its blood collecting tube,had a negative effect on the biomaterial, with specific reference to its platelet concentration. The measurement of platelet concentration was used as an indirect measurement of L-PRF breakdown, since platelets adhere to the fibrin network of the biomaterial and are released upon its dissolution. This investigation formed part of a Masters Degree, with parts of the results obtained being submitted as a thesis (Peck, 2011). The resultant data was gathered and formulated into a scientific paper that was subsequently published in the South African Dental Journal of November of 2015.

5.2   Published article

The effect of storage time on the platelet concentration of platelet- rich fibrin (PRF)

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Running Title: Storage time and the platelet concentration of platelet- rich fibrin

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Title: Platelet- Rich Fibrin (PRF) - The effect of storage time on platelet concentration

Abstract

The aim of this study was to determine whether storage time had a significant effect on the platelet concentration of platelet-rich fibrin (PRF). Three blood samples were drawn from each participant into a sterile (non-anticoagulant containing) blood sampling tube. Two of the blood samples were centrifuged to form PRF. The third non-centrifuged sample was used to measure the baseline blood platelet concentration. After PRF had formed, it was removed from the respective test tubes at different time intervals i.e. immediately after centrifugation (Group A) and after 60 min of storage in the blood collecting tube (Group B). The residual blood from each group was tested for platelet concentration and compared to the baseline reading *(as an indirect measure of the platelet concentrate of PRF).* The PRF produced in Group A (PRF A) had a mean platelet concentration of $274 \pm 57.8 \times 10^9$/L, whereas the PRF of Group B (PRF B) was $278 \pm 58.2 \times 10^9$/L. A statistically significant difference was seen between the groups ($p < 0.001$). **Conclusions:** Storage time has a significant effect on the platelet concentration of PRF. Further research is required to determine whether this has any clinical relevance.

Introduction

Wound healing is a complex process characterised by the repair and reconstitution of lost or damaged tissue. Identification of the pathological and biochemical mechanisms that regulate tissue repair and homeostasis has long been regarded as central to their therapeutic exploitation in the clinical setting. By the mid-1990s, several methods were proposed to enhance wound healing, including the administration of high concentrations of human platelets to affected areas [1-6]. It was assumed that platelets optimised wound healing by promoting the secretion of growth factors (GFs) necessary for tissue repair [7-9]. The most common platelet concentrate used in these procedures is platelet-rich-plasma (PRP) and by the early part of the 21st century, its use in various surgical procedures was commonplace [1, 4, 10, 11]. However, the preparation of PRP often requires the use of specialised equipment, chemicals and animal-derived additives. This increases the risk for complications secondary to allergic reactions to certain animal-derived additives [12-14]. As a result, researchers have sought more efficient and safer methods of concentrating platelets for surgical use [1]. This led to the production of platelet-rich-fibrin (PRF), a platelet concentrate that neither contained additives nor required the use of specialised equipment during its preparation. First introduced by Choukroun *et al* (2001), PRF has been studied extensively and is now regarded as a biological scaffold different to PRP [15]. However, even though the use of PRF is gaining widespread clinical acceptance, several questions regarding its biological stability remain unanswered [16, 17]. The aim of this this study was to analyse the effect of storage time on the platelet concentration of this unique biomaterial.

Materials and Methods

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 participants were fully informed of the research protocol and had to sign a declaration of informed consent before being allowed to participate in this cohort analytical study. A total of 30 healthy participants (16 females and 14 males) were enrolled into the study. Participants were drawn from the current patient pool as well as staff members based at the Dental Faculty, University of the Western Cape. All participants had 3 separate blood samples collected by venipuncture. Two of the samples were acquired using tubes containing clot activators, i.e., Vacuette® 10 ml serum tubes with Z Serum Clot Activator (Greiner BioOne International AG, Germany), whereas the remaining blood sample was drawn into a 10 ml BD Vacutainer® tube that contained dipotassium-EDTA, an anticoagulant (BD Diagnostics, New Jersey USA).

All the blood samples collected in the anticoagulant containing tubes were used to measure baseline platelet concentrations, whereas the remaining blood samples (collected in the Clot Activator containing tubes) were used to prepare PRF by centrifugation (400g for 12 minutes) in a standard benchtop centrifuge (PLC-03, Hicare International, Taiwan) (Figure 1). Therefore, from each study participant, 2 samples of PRF were obtained (Figure 2). The 2 PRF samples were then randomly allocated into either group (Groups A or B) using a simple coin toss.

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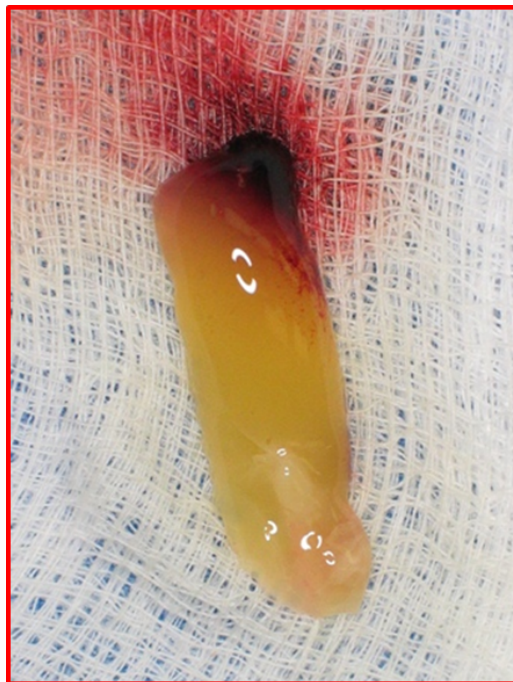**Figure 1**: Benchtop centrifuge

Figure 2: Prepared PRF

For Group A, the PRF was removed from the tube immediately after preparation (0 min), whereas for Group B, the PRF remained in the tube and was only

removed after 60 minutes. The PRF produced from each group was designated PRF A and PRF B respectively. Because a direct measurement of platelet concentration of PRF is not yet possible, we calculated the PRF concentration for each group indirectly, by determining the numerical difference between the residual platelet concentration (of the remaining serum after removal of PRF) and the baseline platelet concentration for each specific study participant. In this study, platelet concentration analysis was carried out using an electronic automated cell counter (Advia 2120, Siemens AG, Erglangen, Germany).

Data was collected and entered into a spreadsheet (Microsoft Office 2010 Excel, Microsoft Corporation, Washington). The results were compared and analysed statistically using SPSS® Version 13 for Windows.

Results and Discussion

A total of 30 participants (16 females and 14 males) were entered 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.

Platelet concentrations obtained from analysis of all 30 participants were within the normal laboratory reference range of 170-400 × 10^9/L of circulating blood. None of the participants displayed any significant haematological disease. The mean blood platelet concentration was 282.8 × 10^9/L **(Table 1).** There was no significant difference between the genders.

Table 1: Mean baseline platelet concentration in cells x 10^9/l of blood		
	Baseline	
	Mean	SD
Males	275.14	43.13
Females	289.50	69.64

After removal of formed PRF from Group A, the residual serum yielded minimal concentrations of platelets. The mean concentration of remaining platelets was 7.9 × 10^9/L **(Table 2).** For Group B, serum platelet concentrations were also minimal after removal of the prepared PRF with the mean concentration of the platelets being 4.0 × 10^9/L **(Table 2)**.

The residual mean platelet concentration of Group A was higher than that determined for Group B with the mean difference in platelet concentrates between the 2 groups being 3.90 × 10^9/L. Using a non-parametric Signed Rank Test, the statistical significance of the differences in platelet concentrates between the Groups A and B was analysed. The difference between the 2 groups was statistically significant ($p < 0.001$).

Table 2: Mean baseline platelet concentration of residual blood in cells x 10^9/L				
	Mean	N	Std. Deviation	Std. Error Mean
Group A	7.9	30	3.03	0.55
Group B	4	30	1.93	0.35

The platelet concentration of PRF for both groups was calculated using the difference between baseline and residual platelet concentrations **(Table 3)**. A paired t-test showed a statistically significant difference between the 2 groups ($p < 0.001$) **(Table 4)**.

Table 3: Mean calculated PRF platelet concentration in cells x 10^9/L of blood				
	Mean	Std. Deviation	Minimum	Maximum
PRF A	274.9	57.8	169	387
PRF B	278.8	58.2	171	390

Table 4: Statistical analysis of PRF A and PRF B							
Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Significance (2-tailed)
			Lower	Upper			
-3.9	3.133	0.572	-5.07	-2.73	-6.818	29	0.001

* Paired t-test performed, P value is significant

Discussion

The purpose of this study was to determine whether storage time had any significant effect on the platelet concentration of 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. This was in accordance with a previously published method [18]. 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. The results from this study indicate that there is a significant difference in the mean platelet concentration of PRF when stored for 0 and 60 minutes.

From the platelet counts obtained in both test groups it was clear that a significant proportion 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 97-98% of the platelets were concentrated in the PRF clot (Table 3). This observation is similar to results published for previous studies (97%) [19]. 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 acts as a reservoir for a concentration of growth factors (GFs) required in the initial stages of wound healing [20].

Although the physiology of PRF has been studied extensively, very few reports adequately document the ability of this platelet concentrate to be stored [21]. Previous research has indicated that storing 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 [22]. Instead, several authors recommend storing the biomaterial in a metal dish or a proprietary designed storage box [17]. Data regarding the maximal storage time and ideal storage temperature of PRF are largely lacking.

In this study, we used standard blood collecting tubes with clot activators to store PRF for at least 60 minutes at room temperature. This particular duration of time was chosen based on the average time of typical periodontal surgical procedures at the Faculty of Dentistry, University of the Western Cape. The results of the study indicate that by using the presented protocol for platelet

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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 PRF. Indeed, storing the concentrate for 60 minutes resulted in a form of PRF that had significantly higher concentrations of platelets compared to non-stored PRF. The reason for this phenomenon is unclear, 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 [22]. As a result, a longer contact time between blood and these activators may enhance fibrin activation and clot formation, with subsequent platelet entrapment [18].

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 occur. 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 PRF to sustain its platelet concentrate over the tested time may have significant clinical implications [8, 9, 22-24]. Rather than drawing blood during the 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 [18,19]. 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 formed elements 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 [24]. Previous studies indicate that several of these factors play an essential role in osteogenesis and periodontal regeneration [20, 25]. 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 [23]. 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 [23]. 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 [18,19, 26]. 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 it has been suggested that storing it in near-freezing temperatures is not advisable. In this study, all the samples were stored at room temperature. It was clear that storage under these conditions had no detrimental effect on the platelet concentration of the PRF clot.

Although the present study showed statistically significant differences between the two groups tested, some 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 the above 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. Further investigations are warranted to determine whether this has any clinical implications.

Disclosure policy

The authors declare no conflict of interest regarding the publication of this paper. This paper forms part of the requirements of the partial fulfilment towards the degree PhD.

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5.3 Conclusion

The above investigation examined the effect of storage time on the platelet concentration of L-PRF. To the author's knowledge, this was the first paper published concerning this parameter. A significant finding was that 97% of platelets could be concentrated into a viable biomaterial using the method proposed by the author. It was also shown that storage of this specific L-PRF was possible for at least 60 min after preparation without any physical deterioration. Furthermore, the platelet concentration increased over that period of time, therefore rejecting the hypothesis that L-PRF would breakdown and release platelets if it was not used within 15 minutes of preparation. This implies that the biomaterial can be prepared before the surgical procedure, without increasing surgical treatment time. Because the investigation was limited to platelet concentration, further analysis was carried out on the efficacy of the biomaterial to stimulate cellular growth. The results are presented in a subsequent chapter of this thesis.

Chapter 6

6.1 The effect of L-PRF (prepared by a modified protocol) on cellular activity

The biological effects of L-PRF, has been extensively commented upon in the literature (Dohan et al, 2006a; Miron et al, 2017a; Miron et al, 2017c; Draganos et al, 2018). The biomaterial has been compared to other platelet concentrates and found to have a distinct morphology as well as have a stimulatory effect on various cells types (Dohan Ehrenfest et al, 2010a). Although the initial research was limited to cells of the oral region, L-PRF has proven to have similar effects on extra-oral tissues such as cartilage, ocular tissue, adipose tissue, meniscal tissue, periosteal cells, tympanic membrane tissue and salivary gland cells, amongst others (Verma et al, 2017; Arbildo et al, 2017; Miron et al, 2017c). A large proportion of the research investigating the effect of L-PRF, use the MTT assay as measurement of how L-PRF affects cell proliferation and viability. MTT is a standard laboratory assay that measures these parameters. It reflects the capacity of the mitochondria in living cells to convert MTT to purple formazan crystals that can be solubilized with dimethylsulphoxide (DMSO) or isopropanol. This effect is directly proportional to the cell number and is measured with a spectrophotometer at a wavelength of 540 nm. Rapidly dividing cells i.e. cells which show high rates of proliferation, reduce MTT at a faster rate than cells with low proliferative potential. The MTT assay therefore measures the ability of the cell to reduce MTT (viability) as well as the rate of cell division (proliferation). As a result, the MTT assay can also be used to measure

cytotoxicity (loss of viable cells) and cytostatic activity (shift from proliferation to quiescence).

Previous research indicates that L-PRF has a direct positive effect on cellular growth (Dohan et al, 2006). This is commonly seen during the early phase of L-PRF and cell interaction, with an initial proliferative burst often noted during the first few days of exposure (Dohan Ehrenfest et al, 2012a). The exact mechanism of how L-PRF interacts with these various cells is unknown but is speculated to involve the high concentration of GFs that the biomaterial brings to the site (Miron et al, 2017c, Miron et al, 2018). The L-PRF tested in this study is prepared using modified parameters that were initially developed by Choukroun et al (2001). These parameters may influence the biological properties of the material, as has been mentioned previously (I). The effects of diverting from the original protocol have increasingly become a point of contention, with many study results recently being called to questioned (Miron et al, 2018). It was therefore prudent that the L-PRF as prepared by the author be investigated for its biological effects on cells. The following published paper presents the results from an in-vitro investigation of the effect of L-PRF (prepared, using the authors method), either alone or in combination with another biomaterial, on living cells. Results from the study were published in the South African Dental Journal of 2018.

6.2   Published article

The *in vitro* effect of leukocyte- and platelet-rich fibrin (L-PRF) and cross-linked hyaluronic acid on fibroblast viability and proliferation.

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List of abbreviations

3T3 cells	mouse fibroblast cell line
bFGF	basic fibroblast growth factor
clHA	cross-linked Hyaluronic acid
DMEM	Dulbecco's Modified Eagles Medium
EDTA	ethylene-diamine-tetra-acetic acid
EGF	epidermal growth factor
FBS	foetal bovine serum
HA	hyaluronic acid
HGF	human gingival fibroblasts
L-PRF	leukocyte- and platelet-rich fibrin
PDGF-AB	platelet-derived growth factor
TGF-β1	transforming growth factor beta 1
VEGF	vascular endothelial growth factor

Abstract

Introduction. Leukocyte- and platelet-rich fibrin (L-PRF), an autologous derived platelet and leukocyte concentrate, was first introduced by Choukroun *et al*. in 2001 and is currently used for several oral and maxillofacial procedures. Hyaluronic acid (HA) is an anionic, glycosaminoglycan that shows a wide range of physiological actions, acting as a cellular scaffold, stimulating cell adhesion and migration, as well as having anti-inflammatory effects. **Aims and Objectives:** To investigate the effect of cross-linked HA and L-PRF on fibroblast viability and proliferation. **Methods:** An *in vitro* laboratory study was conducted at The University of the Western Cape. L-PRF was prepared from a single healthy volunteer and cross-linked HA was obtained from a commercially available product. Analysis was carried out on 3T3 cells using MTT assay. **Results:** Both L-PRF and cross-linked HA are associated with cell viability and increased cell proliferation. L-PRF had it strongest proliferative effect after 24 hours whereas HA had it strongest effect after eight days ($p < 0.05$). The combination of HA and L-PRF was not significantly better than the control ($p > 0.05$). **Conclusions:** L-PRF prepared in a specific manner as outlined, as well as commercially available cross-linked HA, is compatible with cell growth and proliferation.

Introduction

Leukocyte- and platelet-rich fibrin (L-PRF), an autologous derived platelet and leukocyte concentrate, was first introduced by Choukroun *et al.* in 2001 and is currently used for a number of oral and maxillofacial procedures.[1] The use of L-PRF has gained popularity in the field of surgical implantology, specifically for procedures involving soft and hard tissue augmentation.[2-5] The preparation of L-PRF differs from those of previous platelet concentrates in that it involves the use of a single spin protocol that results in an easily manipulated biomaterial that can be applied directly to the site of surgery.[1] It is assumed that the concentrated growth factors associated with L-PRF optimize wound healing and decrease surgical recovery time.[2,5] Consequently, L-PRF has been used in various fields, including dermatology and periodontal regeneration .[2-5]

Hyaluronic acid (HA) is an anionic, glycosaminoglycan.[6,7] It is a naturally occurring molecule that is found in high concentrations in the extracellular matrix of skin, cartilage, bone and periodontal ligament.[7] Even though the exact function of HA has not yet been established, the molecule shows a wide range of physiological actions, acting as a cellular scaffold, stimulating cell adhesion and migration, as well as having anti-inflammatory effects.[7] Commercially synthesized HA, in various forms, has been used in tissue engineering, dermatology, orthopaedics, and more recently, periodontal regeneration.[7-10] Traditionally, two forms of commercially synthesized HA exist, i.e., non-cross-linked HA, and cross-linked-HA, resulting in different properties and indications for each form of HA.

The aim of the study was to investigate the effect of cross-linked HA and L-PRF on fibroblast viability and proliferation.

Materials and methods

The study was conducted at The University of the Western Cape, Cape Town, South Africa, in 2017. Ethical clearance was obtained from the Research Ethics Committee of the above university (reference number: BM 16/3/31). Informed consent was obtained from the blood donor.

Preparation of the L-PRF

Thirty six milliliters of blood was obtained from a single 56 year old healthy female volunteer. The blood samples were collected in blood collecting tubes that contained clot activator i.e., Vacuette® 9-ml serum tubes with Z Serum Clot Activator (Greiner BioOne International AG, Germany). These were then immediately centrifuged at 400xg for 12 minutes in a standard benchtop centrifuge (PLC-03, Hicare International, Taiwan) as has been previously described.[11]   Centrifugation separated the blood into three distinct layers (Fig 1). The layers could be distinguished as a topmost layer consisting of platelet poor plasma, L-PRF in the middle, and red blood cells below. L-PRF was then removed with a sterile forceps, separated from the underlying red blood cells and shredded into smaller fragments using surgical scissors.

Hyaluronic Acid (HA)

A commercially available cross-linked HA (hyaDent BG, Bioscience GmbH) was used in the present study. It is sold as a clear gel contained in a dental

anaesthetic-type cartridge and is applied using a dental syringe and a specifically supplied needle. This specific formulation contains 2.0 mg/ml of sodium hyaluronate that is cross-linked with butanediol digylcidyl ether, resulting in a HA molecule with a unique chemical structure.

Cells and Cell Culture

In this investigation, the 3T3 fibroblast cell line was obtained from The National Repository for Biological Materials (Sandringham, South Africa). The 3T3 cells were incubated at 37°C in 5% carbon dioxide and 95% humidity in Dulbecco's Modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS) and 1% penicillin– streptomycin mix. Cells were grown to about 80% confluence and then trypsinized using trypsin-ethylene-diamine-tetra-acetic acid (EDTA). The cells were then seeded into 3 x 96-well plates and treated as follows;

- Plate 1 – 1 drop of HA *(HA group)*

- Plate 2 – 1 drop of HA added to a 1 mm x 1 mm fragment of L-PRF *(HA + L-PRF group)*

- Plate 3 – 1 mm x 1 mm fragment of L-PRF *(L-PRF group)*

All the plates had an equal number of controls containing only DMEM. After a culture period of 24 hours to 10 days, each group was removed and proliferation and viability evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[12] A minimum of 5 replicate experiments for each group was performed to ensure reproducibility.

Cell Viability and Proliferation Assay

Cell viability (the metabolic activity of the cell) and cell proliferation (rate of cell division) were measured using the standard MTT assay (MTT, Sigma Chemical Co., Baltimore, MD, USA). The MTT assay or test is a sensitive and quantitative colorimetric assay that measures these parameters.[12] It reflects the capacity of the mitochondria in living cells to convert MTT to purple formazan crystals that can be solubilized with dimethylsulphoxide (DMSO) or isopropanol.[12] This effect is directly proportional to the cell number and is measured with a spectrophotometer at a wavelength of 540 nm. Rapidly dividing cells i.e. cells which show high rates of proliferation, reduce MTT at a faster rate than cells with low proliferative potential. The MTT assay therefore measures the ability of the cell to reduce MTT (viability) as well as the rate of cell division (proliferation). As a result, the MTT assay can also be used to measure cytotoxicity (loss of viable cells) and cytostatic activity (shift from proliferation to quiescence). For the current study, readings were taken from all groups with the control group set at 100%.

Data Analysis

Data from the MTT assay were captured using Microsoft Excel 2010 (Microsoft Corporation, Washington, USA) and statistically analyzed using the one-way ANOVA and Tukey's test.

Results

With the control group set at 100%, the mean optical densities of each of the test groups were divided by that of the control group and expressed as a

percentage of the control value. The data from the MTT assay was correlated, and is presented in Figures 1-4. Statistical analysis is represented by Tables 1 and 2.

According to the results obtained, both L-PRF and cross-linked HA (clHA) as well as the combination of the two materials were compatible with cell viability and proliferation (i.e. none of the tested biomaterials tested displayed evidence of being cytotoxic or cytostatic). Cross-linked HA showed a statistically significant reduction in cell number when compared with the control group at 24 hours ($89.98\% \pm 9.83$, $p < 0.05$). Thereafter cell viability steadily increased, reaching $100.88\% \pm 6.46$ at day 10. Peak cell proliferation associated with HA was seen on day 8 and recorded as $112.20\% \pm 9.69$. This was significantly greater than the proliferation seen in both other test groups for the same time period ($p < 0.05$). The HA + L-PRF group showed a cell viability of $85.44\% \pm 12.35$ at 24 hours. This was statistically significant ($p < 0.05$) when compared with the control group, indicating a negative effect on cellular proliferation in the first 24 hours. This difference was not significant for days 2, 5 and 7, but was again recorded on days 8 and 10 ($p < 0.05$).

The L-PRF group showed the highest cell viability and proliferation after 24 hours ($108.99\% \pm 5.43$). This was significantly higher than the other groups tested as well as higher than the control group ($p < 0.05$). At day 10, no significant differences were seen between all the test groups, however the HA + L-PRF group showed a significantly reduced number of cells as compared with the control ($p < 0.05$).

A comparison of the proliferative effects of the test groups indicate that both groups containing clHA showed a pattern of cellular proliferation that steadily increased from day 1 to peak at day 8 (clHA group) and day 7 (HA+L-PRF group) respectively. This was different to that observed for the L-PRF group where peak cell proliferation was seen after 24 hours and steadily decreased over the test period (Fig 4.)

Discussion and Conclusions

The results from the current study indicate that both L-PRF and cross-linked HA are biocompatible and induce cellular proliferation of fibroblasts *in vitro*.

L-PRF

It appears as if the effect of L-PRF used in this study is most pronounced in the first 24 hours when compared with any of the other materials tested. This may be due to the high concentration of growth factors present in the material. These include substances such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF-AB), transforming growth factor beta 1 (TGF-β1) and epidermal growth factor (EGF), released from platelets as well as leukocytes.[13-16] This initial "proliferative burst" is similar to that seen by Vahabi *et al.* who reported a statistically significant increase in cellular proliferation of human gingival fibroblasts (HGFs) after 24 hours.[17] However, unlike the Vahabi *et al.* study, which showed a reduction of cell viability of up to 60% after 72 hours, the present study showed sustained cellular viability and proliferation that was statistically similar to the control group for all subsequent test days. The difference in observations in the two studies is

difficult to explain without a direct comparison between the two biomaterials associated with each investigation. Dohan Ehrenfest *et al.* showed that no significant differences were seen in cell viability and proliferation when measured using the MTT assay over 21 days for HGFs cultured with L-PRF [13]. However, the MTT assay was only reported after day three, thereby possibly failing to record any earlier significant findings. Similar to the Dohan Ehrenfest *et al* investigation, the present study showed no statistically significant differences when L-PRF was compared to the control group at day 7, and for subsequent days thereafter.[13] A recent study investigating the *in vitro* effect of a modified protocol to produce L-PRF, also showed no significant cell proliferation after 24 hours compared with the control group, suggesting that the protocol used to produce L-PRF in the current study may result in a L-PRF with a different biological effect compared with the abovementioned reports.[16] A previous investigation by Peck et *al.* showed that the L-PRF used in this study has a distinct fibrin architecture with most of the fibers being of a "larger" diameter.[18] Whether this characteristic is significant in the biological behaviour of the biomaterial is still unclear.

Cross-linked HA

A limited number of studies have reported on the cell stimulating potential of the specific high molecular weight clHA used in the present study.[6,7] Previous research indicates that this formulation is biocompatible and is able to positively stimulate the proliferation of periodontal ligament cells *in vitro*.[6] These results were statistically significant when the biomaterial was diluted at ratios of 1:100 and 1:10. The authors can provide no conceivable reason why the diluted

material evoked a higher proliferation rate than the undiluted material. We speculate, however, that this may possibly be due to the reduced viscosity of the material after dilution, thereby allowing better interaction with the growth medium and cell culture.

In the present study we showed that undiluted clHA resulted in a reduction of cellular proliferation for the first few days after cell culture. This was similar to what was observed by Fujioko- Kubayashee *et al* and colleagues. At day 5, cellular proliferation approximated that of the control group, and, by day 8, was significantly higher than that of the control group ($p < 0.05$). Therefore an augmented proliferative effect was noticed for the present study. Although HA has well-established water retention properties, the exact mechanism of HA and fibroblast interaction is unclear with "fibroblast stretching" cited as the most likely cause.[19] In a recent study evaluating the effect of both clHA and non-cross linked HA on dermal fibroblasts, it was shown that the molecular structure, particularly the type and density of the cross-linkages, play a significant role in the ability of HA to stimulate fibroblast proliferation.[19] Any biological effect of synthesized HA is product dependent and the results of each study should therefore be interpreted with this in mind. This might also explain the variations in clinical efficacy of dental related HA that are reported in the literature.

Hyaluronic Acid and L-PRF

Because HA has previously been used for tissue engineering and is commercially available for the management of several inflammatory-related conditions, the authors evaluated the cell viability and proliferative potential of a

combination of clHA and L-PRF on fibroblasts. As L-PRF has an inherently high concentration of growth factors related to wound healing, it was assumed that the combination of the two biomaterials would stimulate cellular proliferation. The results from the current study indicate that the combined formulation was biocompatible, but had a varied effect on cell proliferation. Similar to clHA, the clHA+ L-PRF mixture showed a significant reduction in cell proliferation after 24 hours. Proliferation then increased over time, and on day 7, it peaked, showing results similar to those of the control group ($p > 0.05$). This proliferation was opposite to that seen for the L-PRF group. It is interesting to note that the clHA+L-PRF combination showed consistently lower cell proliferation when compared with the control group throughout the time period of the study. Although L-PRF on its own stimulated cell proliferation significantly after 24 hours, it seems the addition of clHA constrained this initial stimulatory effect. Whether this is related to the viscosity of the clHA or its concentration is critical to debate. It is known that diluted forms of clHA seem to better stimulate cell growth, and this might therefore explain the results seen in the present study [6].

Conclusion

L-PRF and clHA are unique biomaterials that are being used in tissue engineering. In the present study, we show that these materials on their own, or in combination, are biocompatible and stimulate cell growth. Interestingly, it appears that this specific method of L-PRF preparation results in a material that has a stimulatory effect that peaks within the first 24 hours. Cross-linked HA also stimulates growth positively, but unlike L-PRF this effect is prolonged over 8 days, implying a different, as yet unknown, mechanism of action. The

combination of L-PRF and clHA seems to provide no further advantage to cellular growth when compared with using either of the materials on their own. Based on the above, we can speculate that although L-PRF results in an almost immediate stimulatory effect, that clHA, as a sole treatment choice, might be beneficial in the management of wounds that require more prolonged stimulation. Further research is required to determine the clinical implications of the above findings.

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Figures and Tables
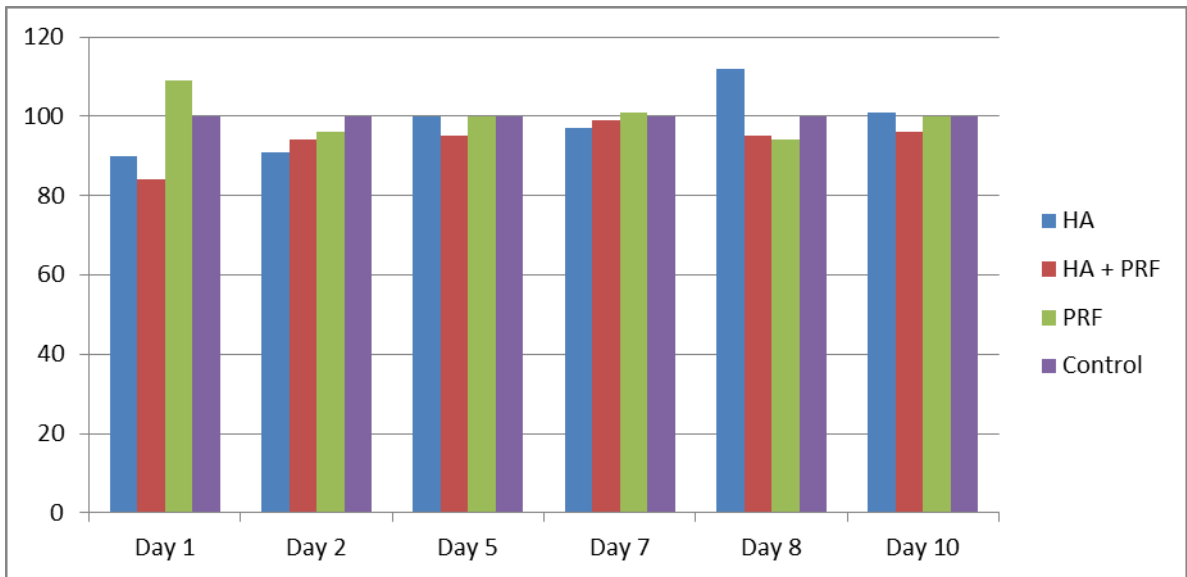
Figure 1: Proliferation and viability assay (MTT) of 3T3 cells. Control is taken as 100%
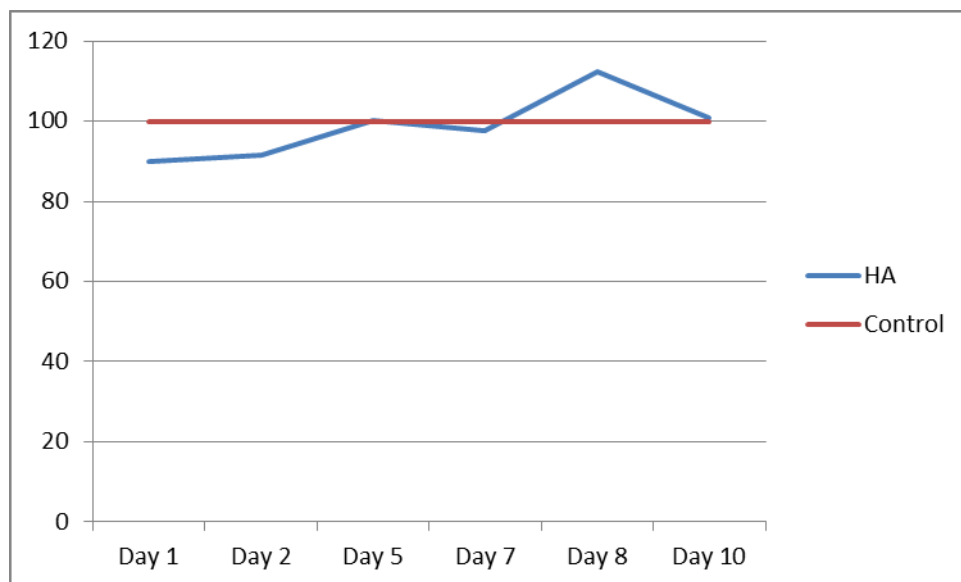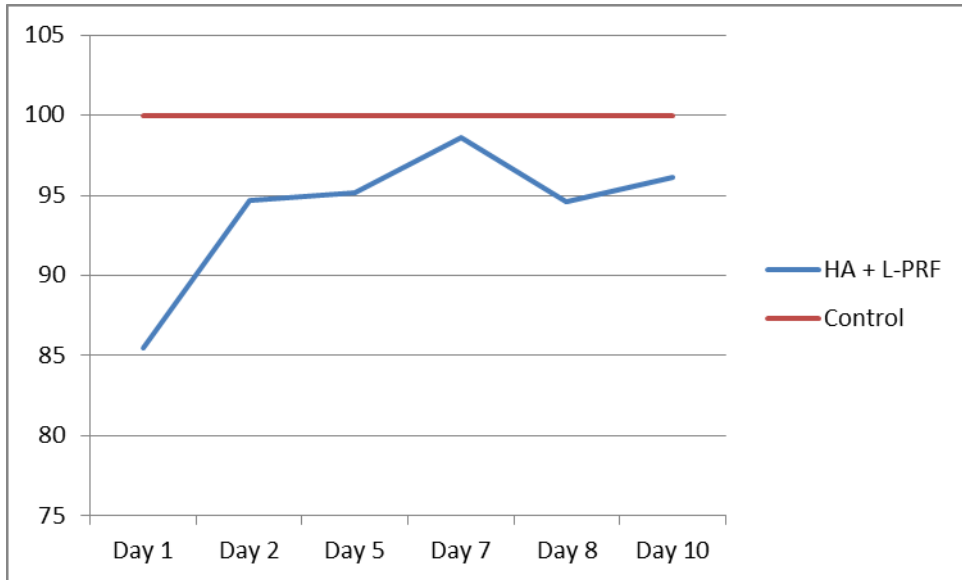

Figure 2: HA vs control (MTT assay)

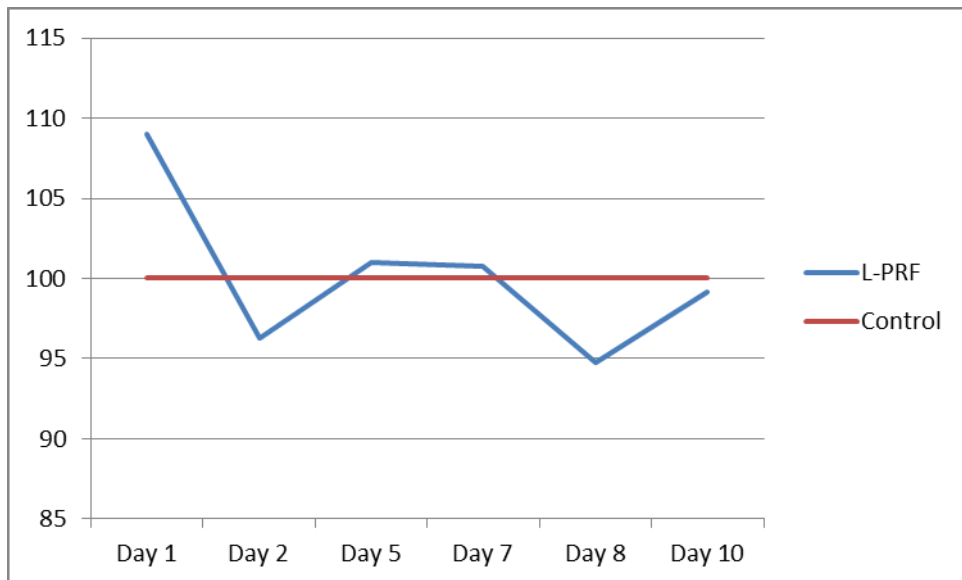Fig 3: HA + L-PRF vs Control (MTT assay)

Fig 4: L-PRF vs control (MTT assay)

Table 1: Comparison between the test groups (one way ANOVA)

	HA		HA + PRF		PRF		
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	P value
Day 1	89.98	9.83	85.44	12.35	108.99	5.43	0.005 *
Day 2	91.41	8.79	94.72	9.89	96.27	4.34	0.632
Day 5	100.08	8.79	95.18	7.29	101.01	7.26	0.47
Day 7	97.47	8.92	98.6	6.15	100.79	5.77	0.757
Day 8	112.2	9.69	94.57	4.09	94.78	9.66	0.007 *
Day 10	100.88	6.46	96.15	2.62	99.15	7.64	0.439

(* p<0.05)

Table 2: Comparison between the test groups (Tukey test)

	HA vs Control	HA-PRF vs Control	PRF vs Control
Day 1	0.02*	0.01*	0.003*
Day 2	0.03*	0.13	0.045*
Day 5	0.49	0.08	0.38
Day 7	0.27	0.31	0.08
Day 8	0.01*	0.009*	0.13
Day 10	0.38	0.005*	0.49

(* p< 0.05)

6.3   Conclusion

The current study highlights several previously unknown properties of a L-PRF prepared using a modified protocol. It demonstrated a direct stimulatory effect of the biomaterial on living cells, with a significant increase in cellular growth observed during the first day. It also showed no negative effect on cell viability, thereby indicating no cytotoxic effect to the cells. The fact that it significantly stimulated cellular proliferation independently, may explain the rapid healing results that were documented in the reported clinical cases (VI and VII). Interestingly, although hyaluronic acid is a known biostimulator (*as was seen in this study*), the combination of the two biomaterials (HA and L-PRF) does not appear to demonstrate any benefit on cellular proliferation when compared to using either of the materials on their own. The exact reason for this is unknown, but may be due to a number of factors, one of those being the high water absorption capacity of HA. HA, because of its unique ability to absorb large amounts of water, when combined with L-PRF, may result in absorbing its associated growth factors, thereby neutralizing their effect on the surrounding cells. Although not cytotoxic, this combination may explain the lower than normal growth rate of the cells seen in the early part of the investigation. This hypothesis is however speculative and requires further rigorous scientific examination.

Within the limitations of the laboratory-based study, this is the first scientific published evidence that demonstrates that L-PRF (as prepared by the author), has a direct proliferative effect on cellular growth and is not cytotoxic. This may have significant implications for the future of this biomaterial.

Chapter 7

7.1 The duration of antibiotic release from leukocyte- and platelet-rich fibrin (L-PRF)

Leukocyte- and platelet- rich fibrin (L-PRF) was initially developed as a simple method of concentrating platelet associated growth factors (Choukroun et al, 2001). These growth factors were then easily transferred to wound sites, due to the platelets being intimately dispersed within the fibrin fiber meshwork of the L-PRF clot (Dohan et al, 2006). This structural configuration had resulted in advantages of L-PRF over previous generations of similar platelet concentrates such as platelet- rich plasma (PRP) in that the growth factors were released over a longer period of time, thereby potentially optimizing wound healing (Dohan et al 2006). Because of its dense concentration of fibrin fibers, we hypothesized that this would facilitate L-PRF as a potential drug delivery system. This was based on the fact that previous studies have successfully used fibrin as a drug carrier. The potential to deliver drugs locally via this method, with specific reference to antimicrobials, would allow for wound sites to resist postoperative infection and therefore optimize the healing. Because the structural properties of L-PRF (as prepared by the author) had already been described (II), we investigated its ability to release a systemically ingested antibiotic over time. The findings were formulated into a scientific article and published in the South African Dental Journal in May of 2018.

Title: Antibiotic release from leukocyte- and platelet-rich fibrin (L-PRF) – an observational study.

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Acronyms

L-PRF: Leukocyte- and platelet-rich fibrin

Abstract

Introduction: Leukocyte- and platelet-rich fibrin (L-PRF), an autologous derived platelet and leukocyte concentrate, was first introduced by Choukroun *et al*. in 2001 and is currently used in a wide range of medical procedures. Although various biological properties have been attributed to L-PRF, nevertheless, when tested for inherent antimicrobial activity, the biomaterial fails to demonstrate a clear and significant effect against a range of oral microbiota. **Aims and objectives:** To determine whether L-PRF prepared after a single oral dose of antibiotic had any significant antimicrobial effect over a 48 hour period. **Methods:** An *in vitro* laboratory study for which L-PRF was prepared from a single healthy volunteer who had previously ingested oral antibiotics. The resultant prepared L-PRF was tested for antimicrobial activity against *Streptococcus mutans (*ATCC 35668) using standard laboratory methods. **Results:** For all samples tested, measurable zones of inhibition were clearly visible after 24 hours, but were absent after 48 hours. **Conclusions:** L-PRF prepared after a single dose of oral antibiotic results in a measurable antimicrobial effect that is sustainable for 24 hours. Although L-PRF will remain structurally intact for a few days, it does not appear to influence the duration of the antimicrobial activity.

Introduction

Leukocyte- and platelet-rich fibrin (L-PRF), an autologous blood-derived biomaterial, was first introduced by Choukroun *et al.* in 2001 as a simple method of introducing autogenous growth factors to a wound site.[1] Since then, L-PRF has been utilized for a number of medical procedures, including the management of diabetic wounds, soft and hard tissue augmentation and dermatological lesions.[2-5] L-PRF is prepared using a single spin protocol that results in an easily manipulated biomaterial that can be applied directly to the site of surgery.[1] The structure of this biomatrix, which consists of a platelet and leukocyte concentrate interwoven within a fibrin mesh, is claimed to be one of the major factors that contribute to its clinical success. Previous studies indicate that the unique characteristic of fibrin make it an ideal drug delivery system that allows the distribution of an active agent directly to the site required.[6] The fact that fibrin undergoes fibrinolysis over a period of time, has the potential of prolonging the drug release and may therefore influence the clinical outcome. Previous studies have combined antibiotics with fibrin (mostly fibrin sealants) for the management of various conditions such as osteomyelitis, endocarditis, and other "difficult to treat" infections.[7-11]

Although various biological properties have been attributed to L-PRF, nevertheless, when tested for inherent antimicrobial activity, the biomaterial failed to demonstrate a clear and significant antimicrobial effect against a range of oral microbiota.[12] We hypothesize that incorporating antibiotics into L-PRF may enhance its antimicrobial profile and that the structure of the L-PRF would allow for a prolonged release of the drug. The aim of this *in-vitro* pilot study was

to determine whether L-PRF prepared after a single oral dose of antibiotic had any significant antimicrobial effect over a 48 hour period.

Materials and methods

The study was conducted at The University of the Western Cape, Cape Town, South Africa, in 2017. Ethical clearance was obtained from the University Research Ethics Committee (reference number: BM 16/3/31). Informed consent was obtained from the blood donor.

Preparation of the L-PRF

Thirty six milliliters of blood were obtained from a 24 year old healthy female volunteer who had undergone dental implant surgery. She had ingested a single dose of antibiotics, as surgical prophylaxis (Amoxicillin, 2 g orally), one hour prior to the surgical procedure. One hour after antibiotic ingestion, blood samples were collected in blood collecting tubes that contained clot activator, i.e., Vacuette® 9-ml serum tubes with Z Serum Clot Activator (Greiner BioOne International AG, Germany). These were then immediately centrifuged at 400 x g for 12 minutes in a standard benchtop centrifuge (PLC-03, HiCare International, Taiwan) as previously described.[12]   Some of the resultant L-PRF clots were then used during the surgical procedure, whilst the remaining clots were used for the current study. These L-PRF clots were then compressed using the PRF Box ™ (Process for PRF, Nice, France) to obtain uniform thickness of the sample specimens, two of which were selected and designated as Sample One and Sample Two respectively.

Microbial Culture

The Oral and Dental Research Laboratory (Faculty of Dentistry, University of the Western Cape) sponsored samples of *Streptococcus mutans* (ATCC 35668, Quantum Biotechnologies, South Africa) for the study. The authenticity of the bacteria was confirmed via Gram stain, growth morphology on blood agar and API STREP 20 system (bioMerieux). Cultures were grown for 24 hours at 37°C. Two colonies were adjusted to 0.5 McFarland's standard (DensiCHEK Plus, bioMerieux) in sterile physiological saline. One millilitre (1 ml) of the adjusted colonies was then spread plated on CASO agar (Merk Life Science GmbH, Germany).

Antimicrobial activity

The samples of prepared L-PRF were of comparable size and thickness due to the standardized protocol used in preparation. Each sample was placed in the centre of a bacteria-streaked agar plate and then incubated for 37°C in a standard laboratory incubation chamber for 24 hours. At that stage the plates were removed and the clear zones surrounding each L-PRF sample (zones of microbial inhibition), were repeatedly measured using a digital vernier caliper (Mastercraft, South Africa). In order to test the antimicrobial efficacy over the subsequent 24 hours, the samples were removed and placed in a fresh bacteria-impregnated agar plate. The plate was incubated for 24 hours as before and the inhibition zones again measured.

Data analysis

Data from the test samples were captured using Microsoft Excel 2010 (Microsoft Corporation, Washington, USA) and statistically analyzed using one-way ANOVA and Tukey's test.

Results

The 24 and 48 hour results were recorded as the observable zones of inhibition in millimeters and are represented in Table 1.

Table 1. Zones of inhibition measured in millimeters

24 Hours (Sample 1)	24 Hours (Sample 2)	48 Hours (Sample 1)	48 Hours (Sample 1)
33.97	29.41	0	0
41.57	42.70	0	0
32.20	31.22	0	0
29.53	44.03	0	0
32.90	31.99	0	0
32.91	47.15	0	0
39.65	47.06	0	0
40.75	31.82	0	0
35.11	42.82	0	0
41.33	31.60	0	0

Zones of inhibition were noted for both L-PRF samples after 24 hours. According to statistical analysis using one-way ANOVA and Tukey HSD Test, no significant differences were noted for the sizes of inhibition zones when the

two samples were compared after 24 and 48 hours, i.e., similar results were recorded at both 24 and 48 hours. No measurable inhibition zones were seen for either sample after 48 hours (Table 2), indicating that all antimicrobial activity had been lost at that time. A statistically significant difference was seen in the data between the two time points.

Table 2: Tukey HSD results

Samples	Tukey Q stat	Tukey p-value	Tukey inference
S1 (24hrs) vs S2 (24hrs)	1.4702	0.7058178	insignificant
S1 (24hrs) vs S1 (48hrs)	26.6181	0.0010053	p<0.01
S1 (24hrs) vs S2 (48hrs)	26.6181	0.0010053	p<0.01
S2 (24hrs) vs S1 (48hrs)	28.0883	0.0010053	p<0.01
S2 (24hrs) vs S2 (48hrs)	28.0883	0.0010053	p<0.01
S1 (48hrs) vs s2 (48hrs)	0	0.8999947	insignificant

Discussion and Conclusions

Repeated systemic use of antibiotics to treat or minimize localized infection has its limitations, especially in areas with constrained blood supply. Furthermore, localized drug delivery systems often require specialized carriers to allow for the delivery of therapeutically sustainable doses of the active agent. In the present study we attempted to determine whether using an antibiotic- laden autologous blood-derived concentrate had any significant antimicrobial effect over a period of time. The results from the current study indicates that L-PRF prepared after a single dose of antibiotics provides measurable antimicrobial activity for at least 24 hours against specific oral microbiota. After 48 hours, the antimicrobial effect is markedly reduced, with no statistically relevant antimicrobial effect seen. This may suggest that although L-PRF has a unique structure, its ability to concentrate antibiotics and release them over time, may be limited. This has been seen in previous studies in which fibrin sealants directly supplemented with antibiotics showed a rapid antibiotic release over a short period of time (85% over 72 hours).[14-16] Woolverton et al. (2001) attributed this to rapid diffusion as a result of the antimicrobial molecules being small, ionic, and designed for oral and parenteral delivery.[6] However, antibiotics having a less soluble nature, unlike the current study, have been shown to exhibit a much longer sustained release from fibrin sealants.[17] Another reason for the rapid diffusion of antibiotics from L-PRF may be the limited binding capacity of the antibiotic used in the present study. Amoxicillin, when orally ingested, is 20% protein bound in the blood and about 60% of the drug is excreted in the urine within 6-8 hours.[18] The protein binding is mostly to albumin (the most abundant

plasma protein) which has a specific binding site but a relatively low affinity for the antibiotic.[18] The drug has no known affinity to fibrin and this may explain the limited antimicrobial effect observed in the present study. It is therefore assumed that the drug was concentrated in the plasma component of the L-PRF and not directly bound to the fibrin or cells associated with the L-PRF matrix.

Unlike previous studies that have directly combined the antibiotic with the fibrin matrix, we attempted to incorporate antibiotics that were already in the blood (at peak plasma concentration) during the process of preparing the L-PRF matrix. This has the advantage of not requiring any additional materials or steps in incorporating the antibiotic into the L-PRF. Interestingly, certain clinical studies involving the use of L-PRF for dental surgical procedures have made use of oral antibiotic surgical prophylaxis prior to preparing L-PRF. [19] In these studies, no mention was made of the influence of antibiotic ingestion in the healing outcomes of the site treated with the L-PRF. The present study indicates that antibiotics are presumably incorporated into L-PRF after a single oral dose and may be active for at least 24 hours. We can therefore presume that the clinical studies that have administered antibiotics prior to surgery, have indeed incorporated antibiotics into the L-PRF, if the L-PRF was prepared at least one hour after antibiotic prophylaxis. A further investigation into the influence of pre-operative antibiotics as a factor influencing the clinical behaviour of L-PRF is therefore required.

Conclusion

L-PRF has restricted intrinsic antimicrobial activity. Within the limitations of the present study, we have shown that L-PRF prepared after a single dose of oral antibiotic, results in a measurable antimicriobial effect that is sustainable for 24 hours. Although L-PRF will remain structurally intact for a few days, this does not appear to influence the duration of the antimicrobial activity. We therefore assume

that the antibiotic is mostly concentrated in the plasma and is not directly bound to the structural components of the L-PRF matrix. Further research is required to determine the significance of these preliminary findings.

Conflict of interest.

The authors declare no conflict of interest regarding the publication of this paper. This paper forms part of the requirements for partial fulfillment of the degree PhD.

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7.2 Conclusion

L-PRF has previously been shown to accelerate wound healing by delivering high concentrations of platelet associated growth factors to the site (Miron et al, 2017c). Patients undergoing oral surgery routinely ingest oral antimicrobial agents as surgical prophylaxis, as a result we hypothesised that L-PRF prepared after the ingestion of antibiotics, could potentially result in incorporating these agents into the biomaterial itself. Because of the slow dissolution of L-PRF, this provided and ideal potential for prolonged local drug delivery. Based on the findings of the above study, it was shown that a single dose of antibiotics, ingested one hour before L-PRF preparation, could be incorporated into the biomaterial and remain active for at least 24 hours after the procedure. It is unknown whether this property is common to all L-PRF variants or whether it influenced previous clinical reports. However, this finding may provide a method of localised antimicrobial protection during the immediate post-operative healing period. This is important, especially in cases where there is a potential for wound dehiscence or when a wound is left open or exposed e.g. an extraction socket. Other potential benefits may include using L-PRF laden with antibiotics in sites of bone grafting, or periodontal regeneration. This allows both growth factors as well as antimicrobials to be delivered directly at the intended site. Based on the above the need for the post-operative use of antibiotics and the adverse effects associated with it, may be negated. This may be relevant to both oral and systemic wound care for difficult to treat conditions such as diabetic ulcers. Even though the current study focussed on the ability of L-PRF to store and release antimicrobial agents, it should be considered that

the biomaterial may have the ability to concentrate and release other drugs. Whether this has any clinical significance, requires further research.

Chapter 8

8.1   The clinical efficacy of L-PRF, prepared using a modified protocol

Platelet concentrates have been used to accelerate healing for several decades (Miron et al, 2017c, Miron et al, 2018). Many of these concentrates require the use of specific equipment and involve several steps to ensure the viability of the final biomaterial (I). Since the introduction of PRP, which was initially used for maxillofacial procedures, platelet- rich concentrates have undergone an expanded scope of clinical use, and are now used in most surgical disciplines. L-PRF which was introduced as a simplified method of producing platelet concentrates, gained popularity after its initial introduction and appears to currently be the preferred method of concentrating platelets. By 2009, L-PRF had gained limited popularity, mostly in Europe, with specific equipment being designed and developed to produce the biomaterial (I). However, at that time, these machines were not available in South Africa. As a result, the authors sought to use locally available equipment to produce L-PRF-like material, using the parameters that were previously published in the literature. Consequently, we were able to produce a biomaterial using a single spin centrifuge protocol that physically resembled L-PRF, as was described in the literature. The material was successfully used in several oral surgical procedures, the results of which were published as case reports in peer reviewed scientific journals. The following are reproductions of those publications;

8.2   Published article 1

Case Reports in Dentistry 2011 (2011): 3450480

Title

Alveolar Ridge Preservation (ARP) using leukocyte and platelet-rich fibrin (L-PRF): A report of a case

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Abstract

In order for a dental implant to be restored optimally, it must be placed in an ideal anatomic position. However this is not always possible, since physiological wound healing after tooth removal, often results in hard and soft tissue changes which ultimately compromises ideal implant placement. With the aim of minimising the need for tissue augmentation, several alveolar ridge preservation (ARP) techniques have been developed. These often require the use of grafting material and therefore increases the risk of disease transmission. Leukocyte and platelet-rich fibrin (L-PRF) is a newly developed platelet concentrate that is prepared from the patient's own blood. Clinical research has indicated that it improves wound healing and stimulates bone formation. We present a case where L-PRF was successfully used in an ARP procedure to facilitate implant placement in a compromised extraction socket.

Introduction

In order for a dental implant to be restored optimally, it must be placed in an ideal anatomic position. However this is not always possible, since physiological wound healing following either tooth extraction, trauma, or pathology, often results in a deficiency of both hard and soft tissue. Unless augmentation procedures are carried out, placing an implant in these tissue deficient sites would ultimately compromise the functional and aesthetic results [1]. Although several different augmentation procedures have been developed, many of them are associated with a number of disadvantages such as increased overall cost, the requirement for a second surgical site, and the use of animal derived products [2].

With the aim of minimising the need for tissue augmentation, several authors have proposed techniques to preserve the anatomy of the alveolar ridge after tooth extraction. These procedures have collectively been termed alveolar ridge preservation (ARP) or socket preservation [2]. Several different ARP techniques exist, most of which include the use of a foreign graft materials. Because ARP is a relatively new procedure, no long term studies regarding the technique have been published and even though several cases reports have been presented, there is no evidence to support the superiority of one technique over the other.

Recently, Choukroun introduced leukocyte and platelet-rich fibrin (L-PRF), a second generation platelet concentrate that improves healing of the both hard and soft tissues [3]. We present a case where L-PRF was used in an ARP

procedure to limit ridge resorption after tooth extraction, in-order to maximise the tissue available for ideal implant placement

Case presentation

A 43 year old healthy female presented for the restoration of her dentition in the upper right jaw. Upon clinical examination, it was noted that several posterior teeth were missing from the 1st quadrant and that the only remaining multi-rooted tooth (the upper right first molar), was severely periodontally compromised (Fig 1). The radiographic examination revealed the presence of unextracted roots in the areas immediately mesial and distal to the remaining molar (Fig 2). Based on the poor prognosis of the molar as well as the presence of the unextracted roots, a treatment plan that involved the extraction of the remaining tooth and roots, and subsequently replacing them with an implant-supported prosthesis, was deemed the best long-term restorative solution. In order to maximise the amount of available bone for implant placement, an ARP procedure was indicated at the time of extraction.

Initial visit

After local anaesthesia had been obtained, the upper right molar together with the residual tooth roots were extracted atraumatically by using a 5mm dental luxator (Dentsply® Ltd, Surrey, United Kingdom). The remaining tooth sockets were curetted and all granulation tissue and socket debris, removed (Fig 3). At the same time, 30ml of blood was drawn from the antecubital fossa of the patient into three separate blood collecting tubes (Vacuette® with Z Serum Clot Activator, Greiner Bio One International AG, Germany).These were then

immediately centrifuged at 400g for 12 minutes, using a standard tabletop laboratory centrifuge (PLC-03®, Hi-care international, Taiwan). Using this method, the blood in the tubes separated into three visible layers i.e. a red blood cell layer (RBC) that occupied the lowermost part of the tube, a cell free layer that occupied the uppermost part of the tube, and a L-PRF layer, that was located between the two (Fig 4). For each tube, the L-PRF layer was removed, and compressed between saline soaked sterile gauze to form a " L-PRF membrane" (Fig 5). A total of three L-PRF membranes were formed and inserted into the extraction socket site. These were then stabilised using 4-0 braided resorbable sutures (Clinisut®, Port Elizabeth, South Africa) that was sutured over the wound site. Oral analgesics and a chlorhexidine 0.2% mouthrinse was prescribed during the healing period and the patient was followed up two weeks later.

Follow-up visit

On the follow up visit, the extraction site showed signs of healing with no evidence of residual inflammation. The site was free of infection and the L-PRF membrane was still clearly visible (Fig 6). Even though it remained exposed to the oral environment, there were no signs of membrane disintegration or infection. The patient also reported minimal pain during the postoperative period. Because of the positive response to treatment, she was scheduled for implant placement four weeks later.

Implant placement

A radiograph taken prior to implant placement confirmed new bone formation in the extraction area (Fig 7). Upon surgical flap reflection, the underlying alveolar ridge was clearly visible. The ridge had retained its morphology with no signs of bone resorption or of the residual socket. At implant insertion, the quality of the newly formed bone was such that it allowed for the implant to be inserted at an insertion torque of more than 35Ncm.

Prosthetic management

Eight weeks after implant placement, the implant was restored with a cement retained crown and has since then remained in function without any complications. At the 3 month follow up after the restorative treatment had been completed, radiographic evidence of bone maturation was present at the peri-implant sites (Fig 9).

Discussion

The healing of an extraction socket is characterised by both internal and external changes that ultimately effects the shape of the alveolar ridge [2]. Studies indicate that during healing, bone does not regenerate to the level of bone crest or to the level of the neighbouring teeth and therefore 100% socket fill does not occur. Using an animal model, Araujo and Lindhe showed that in the first 8 weeks following extraction, there is marked osteoclastic activity, resulting in the resorption of the facial and lingual bone walls, especially in the crestal region [4]. They also noted that bone resorption was greater on the facial

wall and that any loss of ridge height was accompanied by a horizontal loss on both facial and lingual walls of the extraction site.

Alveolar ridge preservation is a relatively new surgical procedure aimed at retaining maximum bone and soft tissue after a tooth has been removed [2]. By maintaining the original ridge morphology, there will a minimal need for augmentation procedures thereby allowing the resultant restoration to be placed in an aesthetically and functionally ideal position.

During the last decade several different ARP techniques have been developed, most of which include the use of a graft material that is placed into the extraction socket [2]. This increases the treatment cost as well as increases the risk of disease transmission. Studies also indicate that in many cases, the graft material is not totally incorporated into the newly formed bone and when compared to sites without graft material, show less vital bone formation. In some cases ARP requires the use of collagen membranes. In these cases a 25% membrane exposure rate has been reported, and this directly affects the amount of bone fill that takes place within the socket [2].

Leukocyte and platelet-rich fibrin (L-PRF) was first described by Choukroun in 2000 [5]. It is considered a second generation platelet concentrate and has been used in various surgical procedures in an attempt to enhance wound healing. It is prepared from the patient's own blood thereby eliminating the possibility of disease transmission or foreign body reactions.

The preparation technique of L-PRF is simple and requires no special equipment. Blood is drawn into standard glass/silica coated blood collection

tubes and centrifuged at a predetermined speed to ensure cell separation. No anti-coagulants are used during the procedure and natural coagulation can therefore take place. This unique preparation technique allows L-PRF to trap at least 95% of the platelets of the collected blood into a fibrin mesh [6]. The fibrin mesh can then be easily manipulated into a membrane that allows it to be transferred to any surgical site. Here, high concentrations of the collected platelets allow for the slow release of growth factors (GFs) from the platelet granules [7]. These GFs include 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 these play a role in replacing lost tissue, resurfacing of the wound, and restoring vascular integrity. Compared to other platelet concentrates, L-PRF releases these factors at a sustained rate over a longer period, thereby optimising wound healing [8]. Recently L-PRF it has also been shown to stimulate the growth of osteoblasts and periodontal ligament cells, both of which are significant for the regeneration of periodontal defects [6], [8-11].

Because of the in-vitro efficacy of L-PRF, several clinical studies have been carried out to determine its clinical potential. Currently, L-PRF has been successfully tested in a number of procedures including maxillofacial surgery, periodontal surgery and implantology [9]. Mazor et al successfully used L-PRF as the only grafting material in a series of sinus augmentation procedures [10]. With this technique Mazor et al was able to demonstrate that L-PRF could stimulate new bone formation in areas that were previously deficient of the

amount of bone required for implant placement [10]. In a similar 6 year follow up study, Simonpeiri et al was able to demonstrate that using L-PRF as a sole grafting agent was a viable long term option in sinus augmentation procedures [11].

L-PRF has also been used successfully to treat periodontal defects. In-vitro studies have confirmed that L-PRF selectively stimulates the growth of osteoblasts and gingival cells [12]. In a series of clinical trials conducted by Pradeep et al is was shown that L-PRF could be used as a guided-tissue-regeneration (GTR) membrane to affect periodontal regeneration in 3-wall bony defects and degree II furcation lesions [9], [13]. Del Corso et al published several case reports showing the successful use of L-PRF membranes in the management of both single and multiple gingival recession defects [14]. The clinical results were maintained successfully for at least one year. Anilkumar et al confirmed this observation and showed that L-PRF could be used for root coverage procedures [15].

Conclusion

In the above case report, we demonstrated the successful use of L-PRF in an ARP procedure. The biomaterial acts by releasing high concentration growth factors to the wound site, thereby stimulating healing and new bone formation [16]. Unlike other ARP procedures, the use of L-PRF is a simple method that requires minimal cost and reduces the need for specialised grafting material. Because it is a completely autologous product, the risk of disease transmission

and graft rejection is negated. Further long-term research is required to determine whether ARP procedures would benefit from the use of L-PRF.

Declaration:

We, the above authors confirm and declare that:-

1.   This manuscript is our original work

2.   The study was completely self funded and no incentives or financial interests was gained from the study

3.   We confirm that this manuscript has not been published previously and it is not currently considered for publication elsewhere.

4.   We have disclosed in our acknowledgments all sources of funding, possible financial interests or incentives in products or services mentioned.

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Figure 1: Initial presentation
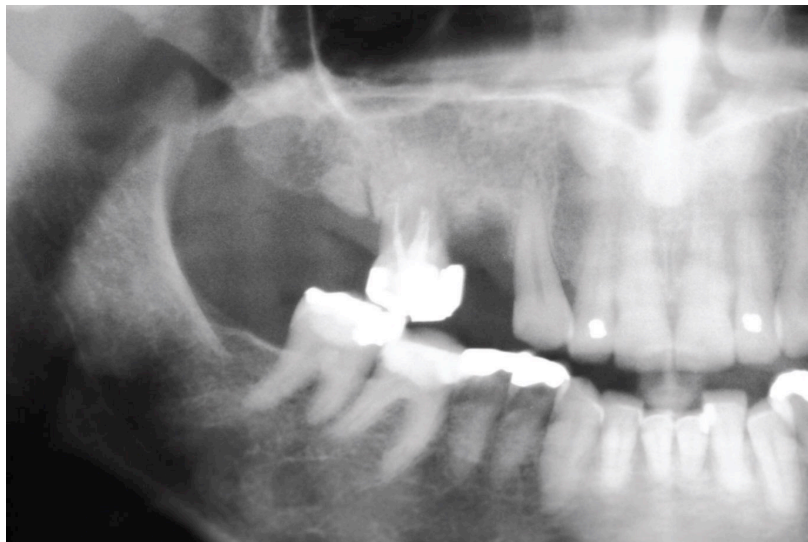
Figure 2: Radiograph shows hopeless upper molar with retained roots both mesial and distal to the tooth
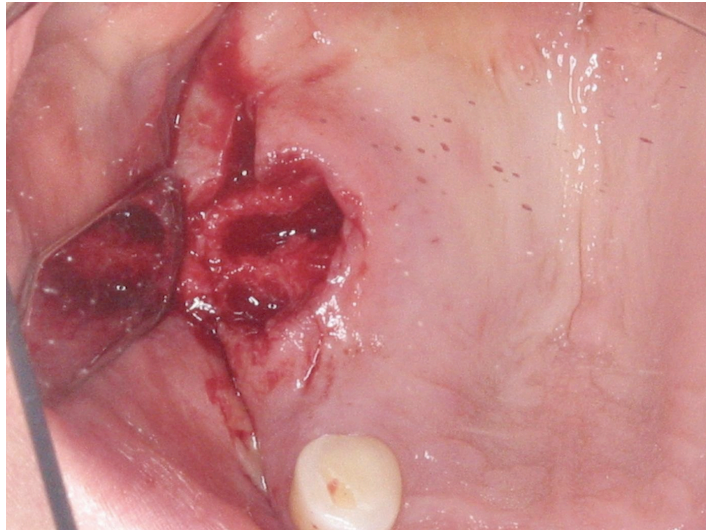
Figure 3: Extraction site immediately after tooth removal.
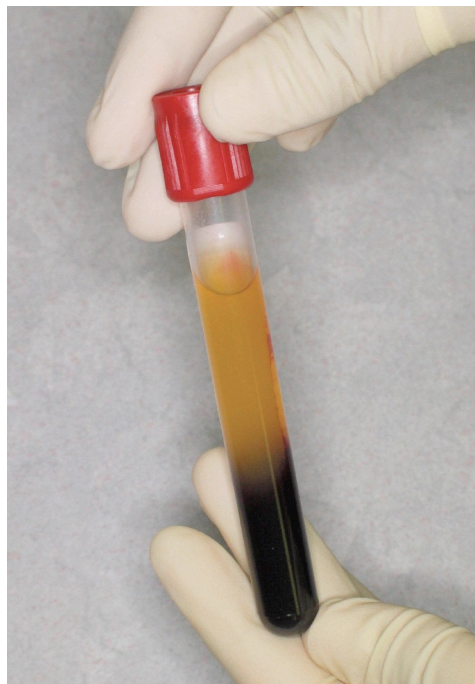
Figure 4: Formation of L-PRF.

Figure 5: L-PRF membrane.
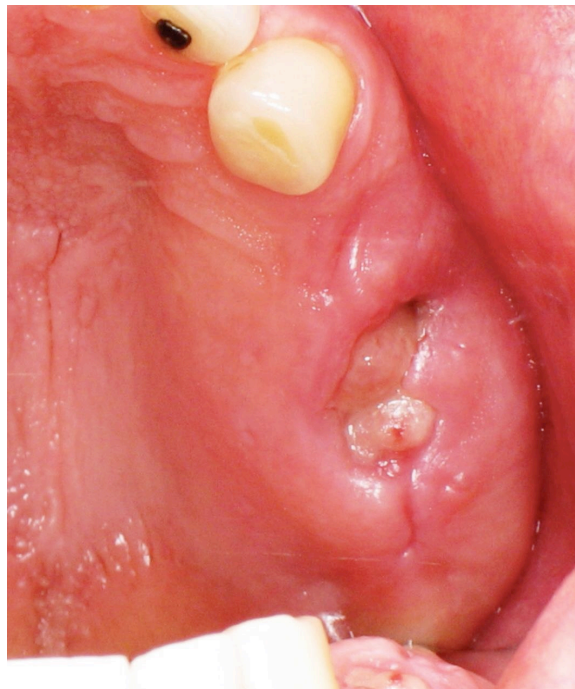
Figure 6: Extraction site healing 1 week after tooth removal (note

the visibility of the L-PRF membrane).
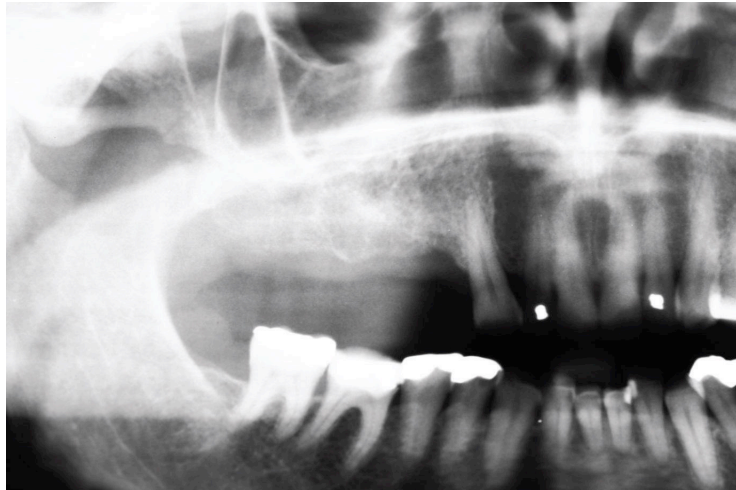
Figure 7: Radiograph showing new bone formation.
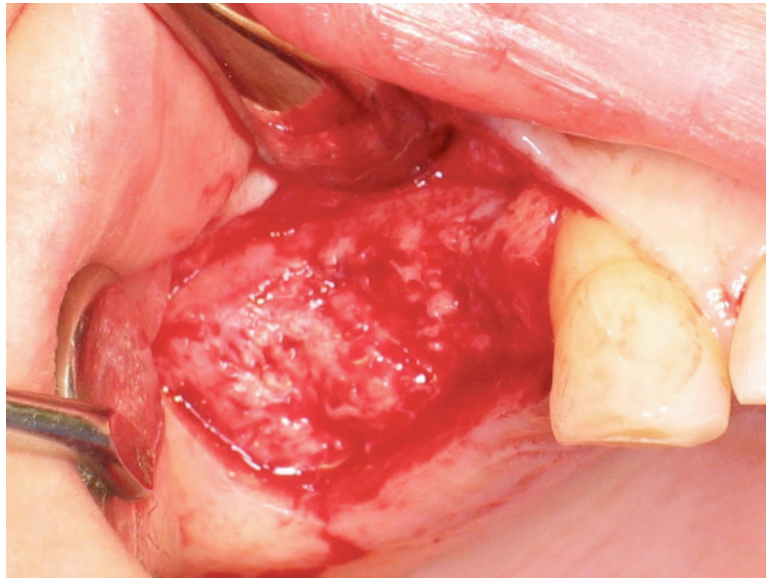
Figure 8: Flap reflection.
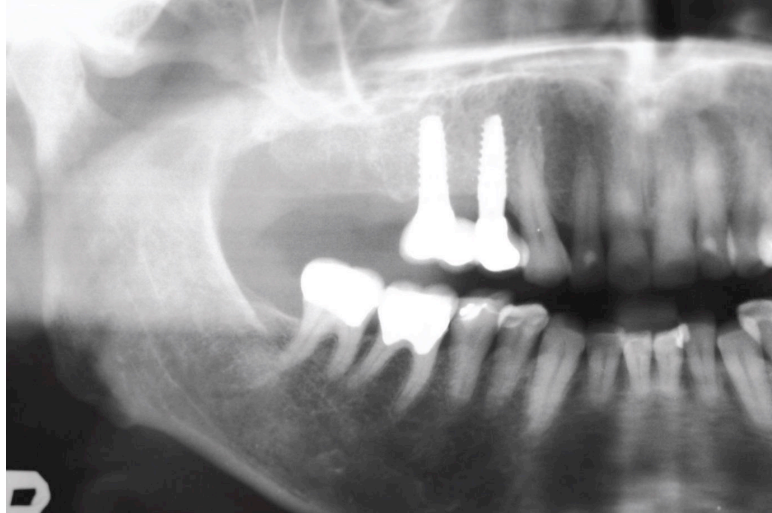
Figure 9: Radiograph showing stable peri-implant bone 3 months after restoration.

8.3   Published article 2

South African Dental Journal, March 2012, 67 (2)

Title

Alveolar Ridge Preservation (ARP) using leukocyte and platelet-rich fibrin (L-PRF): A report of a case

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ABSTRACT

Successful dental implant treatment usually requires that the implant be placed in the ideal anatomic position, so that it will readily facilitate the placement of a functional and aesthetically acceptable restoration. However, this is not always possible, and in many cases augmentation procedures may be required to compensate for lost tissue structures. These interventions often require more complex surgery, as well as the use of graft material derived from animal sources. Leukocyte- and platelet-rich fibrin (L-PRF) is a newly developed platelet concentrate that has successfully been used in a number of surgical procedures to optimise wound healing. Several studies indicate that it may also have the ability to stimulate bone formation. In this article we present two cases where L-PRF was used to stimulate bone formation to facilitate ideal placement of implants.

Keywords: Leukocyte- and platelet-rich fibrin (L-PRF), bone augmentation, osseous regeneration

INTRODUCTION

The use of implant-supported restorations to replace missing teeth has become widely accepted as a viable treatment option in modern restorative dentistry. However, once teeth are lost due to pathology or trauma, the remaining alveolar bone anatomy is often compromised and there may be insufficient bone volume to allow placement of an implant that would be functional. Frequently, aesthetics are also compromised. Several techniques have been developed to overcome this problem, including one or more combinations of guided bone regeneration, osteoconduction, osteo-induction, distraction osteogenesis, and revascularised bone grafts.[1] In this article we present two cases where a novel biomaterial, "leukocyte- and platelet rich fibrin (L-PRF)",[2] was used with the intention to enhance osseous regeneration and to enable the placement of dental implants in previously deficient bony sites.[3]

CASE 1

A 45-year-old female patient presented with complaints of "difficulty when chewing" on her right side. Both upper-right premolars had been extracted by her dentist due to failed endodontic treatment and since then she had not been able to adapt to a removable partial denture. She therefore requested a "fixed" restorative option. Her medical history was unremarkable and she had no history of smoking or evidence of periodontal disease. It was decided that two implant-supported crowns in the region of teeth 14 and 15 would offer her the most stable, long-term restorative solution.

The clinical examination indicated a buccal bony defect in the area of 14 and 15, but the affected area was otherwise healthy and free from signs of pathology.

The pre-operative radiographic examination revealed insufficient bone height secondary to maxillary sinus pneumatisation in the area of extracted tooth 15, with only 2-3mm of vertical bone remaining (Figure 1). A sinus augmentation procedure was indicated to increase the bone available so there may be adequate support for the length of the intended implant.

The two implant sites were prepared simultaneously, as follows:

Surgical management of site 14 (upper right first premolar)

A midcrestal incision was made and a full-thickness mucoperiosteal flap was raised. No vertical releasing incisions were made. The bone in site 14 was judged to be sufficient for implant placement without any additional augmentation procedures, and therefore was prepared according to the standard protocol. During the procedure, intra-oral radiographs were repeatedly taken to confirm the correct depth and direction of the osteotomy site. Once the final width and depth had been reached, a 3.7mm by 10mm dental implant (Uniti®, Equinox, Holland) was inserted into the prepared site and torqued to 35Ncm.

Surgical management of site 15 (upper right second premolar)

As the residual vertical bone was less than 4mm in the 15 area, a 2mm pilot drill was used to prepare the osteotomy site to within 1mm of the sinus floor (Figure 2). A 3mm drill was then used to the same depth so as to allow for the insertion

of osteotomes. Sixty millilitres of blood was then drawn from the antecubital vein of the patient and collected into six 10ml anti-coagulant-free blood collecting tubes (Vacuette® with Z Serum Clot Activator, Greiner Bio One International AG, Germany). These were then centrifuged at 400g for 12 minutes, using a standard laboratory table-top centrifuge (PLC-03®, Hi-care International, Taiwan). Immediately after being centrifuged, one L-PRF clot was separated from the underlying red blood cell layer and introduced into the 15 osteotomy site (Figure 3). The floor of the sinus was in-fractured using a 3mm osteotome (Southern Implants®, Irene, South Africa), and the lining was raised. Once the integrity of the sinus membrane was confirmed using the Valsalva manoeuvre, four more L-PRF clots were then introduced into the sinus with the osteotome to elevate the floor to the desired height. A 3.7mm by 13mm implant (Uniti®, Equinox, Holland) was then inserted, with the previously introduced L-PRF clots acting as a "protective layer" for the sinus lining.

After primary stability had been reached, titanium cover screws were placed on both implants and the remaining LPRF was then applied to the lateral aspect of the alveolar ridge. The site was closed using 4-0 synthetic absorbable braided sutures (Clinisut®, Port Elizabeth, South Africa). An immediate post-operative radiograph was then taken that depicted the 10mm rise in sinus membrane height (Figure 4).

Post-operative healing was uneventful, and after two weeks the sutures were removed. The patient returned after six months having encountered no complications associated with the initial surgery. At this time, the implants were surgically exposed and the healing abutments were placed. No movement or

pain was associated with implant 15 and the radiographic examination revealed evidence of new bone formation extending to the tip of the implant (Figure 5). The implants were subsequently successfully restored with two cement-retained splinted porcelain-fused-to-metal (PFM) crowns (Figure 6).

CASE 2

A 48-year-old healthy male presented with complaints of a pain on the left side of his mouth that was associated with an unrestored dental implant (Mistral®, MIS implants, Tel Aviv, Israel) placed three months previously (Figure 7). Clinically, the patient experienced pain on percussion at the implant in the 27 area. Further clinical investigation revealed that the implant was mobile and had failed to osseointegrate. It was decided to remove the failing implant and to subsequently replace it with another, once adequate healing had taken place. Taking into account the previous history of implant failure together with the risk of potential bone resorption, it was decided that an alveolar ridge preservation (ARP) procedure was to be carried out immediately after removal of the failed implant at the same operative procedure.

Surgical procedure for simultaneous implant removal and ARP procedure

Under local anaesthesia, the implant in the area of 27 was removed and the resulting bony socket thoroughly curetted to remove all granulation tissue and fibrous debris. Blood was then drawn from the vein of the antecubital fossa and L-PRF prepared in a similar manner as described above. Three (3) L-PRF clots were then inserted into the 27 socket site and secured in place using 4-0 synthetic absorbable braided sutures (Clinisut®, Port Elizabeth, South Africa).

Implant placement

Healing was uneventful and three months after initial introduction of the L-PRF clot into the failed implant site, a follow-up radiograph revealed substantial new bone formation that was more radio-opaque compared with the immediate adjacent bone (Figure 8). The patient was then scheduled for implant surgery one week later to replace the initial failed implant. At surgical exposure of the 27 area, substantial new bone formation was evident with only minimal signs of the failed implant site still visible (Figure 9). A 4.2mm by 10mm dental implant (Screwvent®, Zimmer, California, USA) was then placed into the area without any complications being noted.

DISCUSSION

Leukocyte- and platelet-rich fibrin (L-PRF), a second generation platelet-rich-plasma (PRP) concentrate, was first described by Choukroun in the early part of the 21st century.[2] It is considered a biomaterial that actively promotes wound healing in both hard and soft tissues.[3] It is prepared from the patient's own blood and requires no additional additives or specialised equipment for its preparation.[4-8] Blood is drawn from the patient directly into glass or silica coated plastic blood-collecting tubes and centrifuged at low speed to separate the platelets from the red blood cells (RBCs).[6] The absence of any anticoagulant during the centrifuge process allows for the unimpeded activation of platelets, thus triggering the coagulation cascade.[5] This results in the formation of a fibrin clot that contains a high concentration of leukocytes and platelets.[9] This L-PRF clot can then be moulded into a usable "membrane" that

acts as a simple method of transferring high concentrations of platelets to any wound site. A three dimensional analysis of the L-PRF membrane indicates that it has a unique structure that traps platelets, leukocytes and cytokines in a mesh of fibrin strands. This imparts structural integrity to the biomaterial and allows it to be manipulated for use in variety of clinical procedures.[3]

The L-PRF membrane traps at least 95% of the platelets of the collected blood, allowing for the natural release of several growth factors (GFs) to the surgical area.[3,10,11] These GFs are released from the platelet granules, and include 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), platelet-derived-growth factor (PDGF) 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.[4,12] PDGF 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 also induces osteoconduction and promotes the production of hyaluronic acid, an essential component for wound contraction and remodelling. Although similar GFs may be released by platelet-rich plasma (PRP), the unique structure of L-PRF allows it to release these factors over a longer period of time, potentially optimising wound healing.[12,13] The presence of leukocytes (absent from PRP) also contributes to the release of TGF-beta and VEGF, further increasing the concentration of available GFs.[3,13]

L-PFR has been exploited in various clinical procedures including maxillofacial surgery, periodontal surgery, implantology and plastic surgery.14,15 In a clinical study of maxillary sinus lift procedures in which L-PRF clots and L-PRF membranes were used as the only augmentation material, Mazor et al showed that L-PRF was capable of stimulating complete bone regeneration in the areas that initially had inadequate vertical bone height.8 In a similar six-year follow-up study, Simonpeiri et al were able to demonstrate that using L-PRF as a sole grafting agent was a viable long-term option in sinus augmentation procedures.16

Case 1 demonstrated the use of L-PRF in osteotome mediated sinus augmentation procedures. By applying L-PRF to the osteotomy site, before fracturing the sinus floor, the biomaterial acts as a "protective cushion" that facilitates the safe upward displacement of the sinus membrane.9 Studies indicate that using L-PRF in osteotome sinus floor elevation techniques, together with immediate implant placement, reduces the healing time between surgical placement and prosthetic restoration of the implants.9

L-PRF has been used to treat periodontal hard and soft tissue defects.14,15 In vitro studies have confirmed that L-PRF selectively stimulates the growth of osteoblasts and gingival cells.17 In a series of clinical trials conducted by Pradeep et al, it was shown that L-PRF could be used as a guided-tissue-regeneration (GTR) membrane to effect periodontal regeneration in three-wall bony defects and degree II furcation lesions.14,15 Del Corso et al published several case reports showing the successful use of L-PRF membranes in the management of both single and multiple gingival recession defects.18 Using

multiple L-PRF membranes instead of connective tissue grafts, it was shown that the lost soft tissue could effectively be restored by layering the L-PRF membranes over each other, thereby increasing gingival height and thickness. The clinical results were maintained for at least one year.18 Ramakrishnan et al confirmed this observation and showed that L-PRF could be used for root coverage procedures.5

L-PR F vs PRP (Where is the evidence?)

Since the initial development of PRP in the late 1990's, the platelet concentrate has undergone significant research, especially in the field of oral and maxillofacial surgery.19 Although widely used in a variety of clinical fields, the ability of PRP to enhance wound healing has recently been questioned.20 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 with controls and suggested that no substantial evidence existed for its use in other dental procedures.19 The heterogeneity in studies that have examined PRP is regarded as a major reason for the lack of conclusive evidence supporting its use.

L-PRF is a new form of platelet concentrate which has features that are distinctly unique when compared with PRP and therefore cannot be regarded as the same biomaterial.4,6,10 However, as with PRP, there is a lack of long-term controlled trials that would endorse the use of L-PRF in routine surgical procedures. Recently, though, five-year follow-up reports have been published

which demonstrate the clinical safety and efficacy of using this new biomaterial. Further reports are awaited.

CONCLUSIONS

In the cases presented above, the clinical application of L-PRF in bone regeneration procedures was demonstrated. These potential effects are directly related to the biological properties of the material, which allow it to act as a "membrane-laden leukocyte- and platelet-rich concentrate". Its preparation is inexpensive, requires no specialised equipment or the addition of animal derived products, and it could promise a new era in the field of wound healing. Further research is required to fully explore the broad potential of this biomaterial.

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Figure 1: Initial presentation
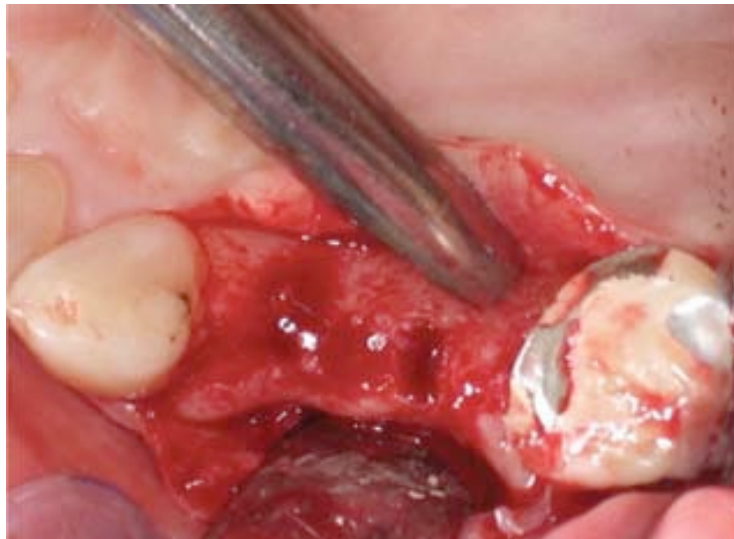
Figure 2: Osteotomy site preparation for the placement of two implants in 14, 15 area.

Figure 4: Immediate post-operative radiograph showing the distal implant in

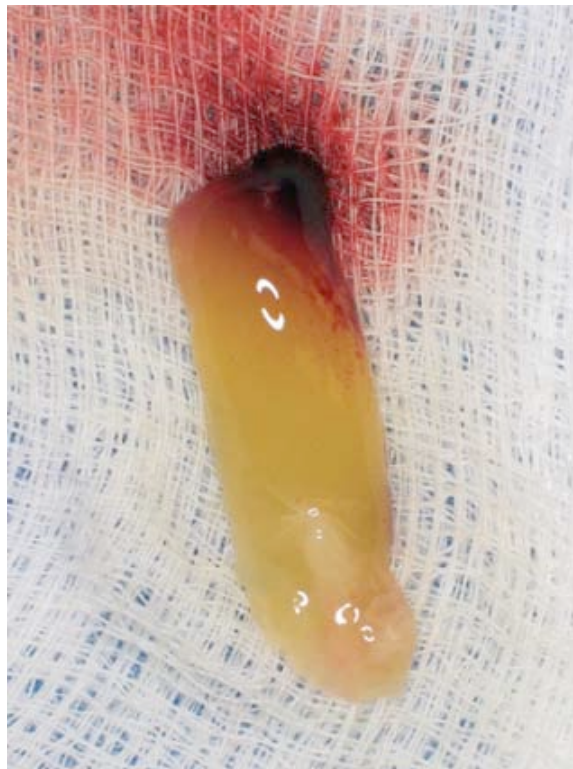the maxillary sinus surrounded by L-PRF.

Figure 3: Leukocyte- and platelet-rich fibrin (L-PRF) clot obtained from patient's blood.

Figure 5: Although taken at a slightly different angle, this six month postoperative radiograph reveals increased radio-opacity indicating the presence of new bone extending to the tip of the implant.

Figure 6: Cement-retained implant-supported PFM crowns (14, 15) in place.

Figure 7: Initial presentation of patient with a painful, failed implant in 27 area.
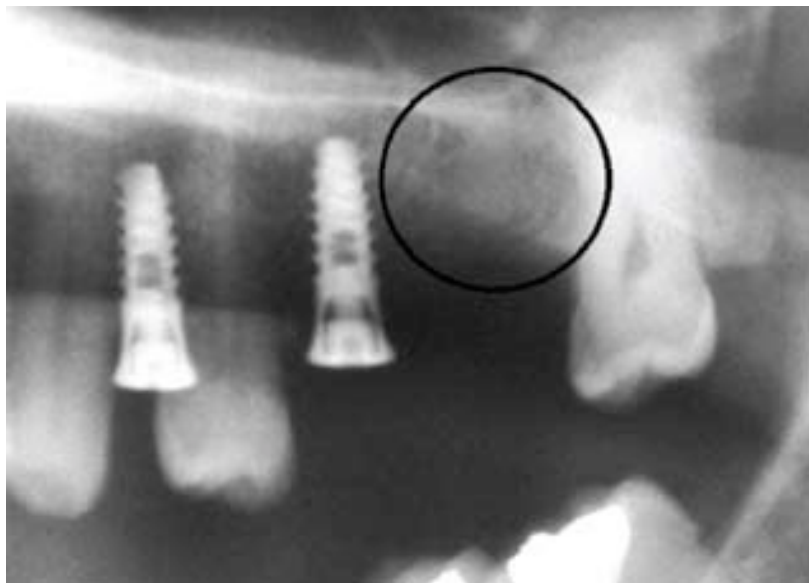
Figure 8: Radiograph indicating increased opacity at the failed implant site 3months after the implant removal and placement of L-PRF clot in the area.

Figure 9: Presence of new bone formation as seen clinically at the time of

implant placement in 27 area.

8.4 Conclusion

The publications that are presented, are according to the authors knowledge, some of the first English scientific peer reviewed reports of the use of L-PRF (prepared using a modified protocol), for alveolar ridge preservation. The significance of the above findings indicate that this method of preparing L-PRF can result in positive clinical outcomes for both alveolar ridge preservation and sinus augmentation. Since this protocol was developed, the biomaterial has successfully been used to treat a variety of clinical cases involving oral surgical procedures over the past eight years. This implies that a clinically effective platelet concentrate can be produced using standard equipment and a simple cost-effective protocol.

Chapter 9

9.1 Discussion and conclusions

The use of platelet concentrates in order to accelerate or stimulate wound healing has become a common technique used in the management of a range of medical procedures. This is based on the biological principle that platelets contain a large number of growth factors and cytokines that play a crucial role in wound healing. Nevertheless, the practice remains controversial with several contradictory reports either advocating or criticising the procedure. Since the introduction of Choukroun's method of concentrating autologous platelet concentrates, the process has been widely adopted, especially in the field of oral surgery (Dragonas et al, 2018). The fact that specific parameters are required to prepare L-PRF via Choukroun's method, did not dissuade others from attempting to duplicate the procedure using non-standard techniques (Miron et al, 2018). With this in mind, we sought to analyse whether an alternative method of producing a human leukocyte and platelet- rich concentrate, using standard laboratory equipment, could result in a biomaterial that was biologically stable and clinically effective. From a review of the literature as well taking into consideration of the limitations associated with the studies presented, the following comments can be made;

- Variation from the original protocol developed by Choukroun et al (2001), results in variants of the original biomaterial and hence,

- L-PRF, as presented in the literature, is not a single entity but represents a spectrum of leukocyte and platelet rich concentrates that have the following features in common (I);

  a. They are all autologous i.e. derived from the host

  b. They all are prepared using a single spin protocol i.e. whole blood is centrifuged once to obtain the desired biomaterial

  c. No added chemicals are used in the process of preparing the biomaterial

  d. They all appear macroscopically similar i.e. a yellow fibrin clot

  e. The can all be compressed mechanically to yield various shapes

- Technical variations of preparation method mean that a single universally accepted protocol for preparing L-PRF does not exist (I).

- Variation from the original protocol has a direct effect on the microscopic structure and biological properties of the final biomaterial (II).

- L-PRF variants have shown both positive and neutral clinical results (I).

- The variation of preparation methods may explain contradictory clinical results (I).

- Clinical comparisons between variants are difficult due to the heterogony of preparation methods that exist (I).

- L-PRF prepared the way the candidate proposes;

  a. concentrates 97% of plasma platelets (Peck, 2011)

  b. is not cytotoxic to living cells *in-vitro* (III)

  c. can be stored for at least 1 hour after preparation, within the blood collecting tube (IV).

d. has a unique morphological structure but has similarities to classical L-PRF as presented by Choukroun (II).

e. has an early accelerative effect on cellular proliferation that lasts for at least 24 hours *in-vitro* (IV).

f. can contribute to the localized release of systemic antibiotics for at least 24 hours *in-vitro* (V).

g. has been proven to be clinically effective for the management of alveolar bone defects and sinus augmentation procedures (VI and VII)

h. is cost effect

i. is easy to produce (II).

j. requires no specialised equipment (II – VII)

k. may be a way of producing L-PRF in resource-poor healthcare settings.

The fact that our research has shown that a biologically effective platelet concentrate can easily be produced, has a direct important implication for clinical practice. It allows the technology to be made available to wide range of health care practitioners who do not have access to specialised equipment, as well as reduces the burden on them for purchasing new equipment. Platelet concentrates can now also be available not only for oral surgical procedures, but also for those seeking to use the biomaterial in the management of other conditions such as diabetic ulcers or chronic slow healing wounds (Pinto, et al, 2018). Although this specific biomaterial has not been tested in these scenarios, the evidence presented here suggests that it has the potential to accelerate

wound healing and cell growth (IV, VI-VII). Further large scale clinical trials are

therefore required to verify the findings presented in this thesis.

Chapter 10

10.1 References

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Appendix

Factors affecting the preparation, constituents, and clinical efficacy of leukocyte- and platelet- rich fibrin (L-PRF).

ABSTRACT

Platelet-rich fibrin (PRF) was first introduced by Choukroun *et al*, in 2001 as a method of concentrating autologous human leukocytes, platelets and fibrin for autotransplantation into surgical wound sites to accelerate healing. Even though several clinical reports have documented the use of L-PRF, controversy still exists with regards to many aspects of this biomaterial. Diverse publications report the use of non-standardised methods to prepare L-PRF, resulting in variable clinical results. The impact of the type of centrifuge, as well as of the growth factor release kinetics, have recently been studied and have yielded new insights into the structure and function of L-PRF. The presence of bone morphogenetic proteins as well as stem cells has also been documented. In this report we analyse various factors affecting L-PRF preparation and its constituents and highlight some of the controversies surrounding the biomaterial.

INTRODUCTION

Platelet-rich fibrin (PRF) was first introduced by Choukroun *et al*. in 2001 as a method of concentrating autologous human leukocytes, platelets and fibrin for autotransplantation into surgical wound sites to accelerate healing.[1] This method of concentrating blood platelets was different to previous techniques in that it centrifuged the collected blood only once, no anticoagulant agents were added, and leukocytes and fibrin were deliberately included in the final product. Previous similar techniques had sought

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ACRONYMS

BMPs:	bone morphogenic proteins
G-CSF:	granulocyte colony-stimulating factor
HUVEC:	human umbilical vein endothelial cells
IGF-1:	insulin-like growth factor-1
MSC:	mesenchymal stem cells
PRF:	platelet-rich fibrin
PDGF-AB:	platelet derived growth factor AB
RCF:	relative centrifugal force
RPM:	revolutions per minute
VEGF:	vascular endothelial growth factor

to concentrate platelets only, with little consideration for the other constituents.[2,3] Choukroun's protocol (Process protocol, Nice, France) was simple and essentially consisted of collecting venous blood into dry glass tubes, after which the tubes would be spun at a low centrifuge speed to allow the blood to separate into the constituents.[4] This resulted in three distinct layers forming in the blood collecting tube, i.e. a red blood cell layer at the bottom of the tube, an acellular layer at the uppermost part of the tube, and a leukocyte- and platelet-rich fibrin (L-PRF) layer formed in the middle of the tube.[4] The L-PRF layer was considered as the active biomaterial and has, since its development, been promoted as an agent that accelerates wound healing and tissue regeneration.[5] Even though several clinical reports have documented the use of L-PRF in oral and extra-oral surgical procedures, controversy still exists with regards to several aspects of this biomaterial. In this report we set out to highlight some of these debates.

The terminology and classification of L-PRF

In an attempt to distinguish various platelet concentrates from each other, Dohan Ehrenfest *et al*. used three key parameters i.e; the preparation process, the pharmacological properties, and the characteristics of the final material to establish a functional classification.[6] By applying specific criteria to these parameters, the authors were able to classify platelet concentrates into four distinct categories (Table 1).[6]

Table 1: Categories of platelet concentrates as proposed by Dohan Ehrenfest *et al*,[6]

1. Pure platelet-rich plasma (P-PRP)
2. Leucocyte- and platelet-rich plasma (L-PRP)
3. Pure platelet-rich fibrin (P-PRF)
4. Leukocyte- and platelet-rich fibrin (L-PRF)

Although this classification elucidates and simplifies the distinction of various platelet concentrates, it is not the only existing proposed system to classify platelet concentrates.[7] However, Dohan Ehrenfest's classification is widely quoted in the literature and at the time of its publication, in 2009, Choukroun's PRF was the only platelet concentrate included in the category of L-PRF.

As the popularity of Choukroun's protocol for the production of L-PRF grew, publications appeared describing processes that purported to produce L-PRF. However, none had applied the exact criteria as described by Choukroun (Process protocol, Nice, France).[8-11] It is unclear whether L-PRF produced by other than the Choukroun method can be classified as a true L-PRF.[4] Publications incorrectly use the terms L-PRF and Choukroun's PRF interchangeably, even though the exact method as described by Choukroun has not been used to produce the platelet concentrate.[12] This has lead to incorrect assumptions and a clear, unequivocal, classification of platelet concentrates that is universally accepted is therefore sought. For the purposes of clarity, the following proposed terminology will be used throughout this article:

- L-PRF – Leukocyte- and platelet-rich fibrin. Defined as a broad and all-inclusive category that is used to describe a mixed platelet, leukocyte and fibrin concentrate prepared using no-anticoagulants and a single spin centrifuge technique.
- L-PRF (C) – Leukocyte- and platelet-rich fibrin (Choukroun type). Defined as a specific leukocyte and platelet-rich fibrin prepared using Choukroun's protocol i.e. (*the equipment and the preparation method follows the exact recommended protocol as outlined by Choukroun[1]*).
- L- PRF (I/E) – Leukocyte- and platelet-rich fibrin (Intraspin/ EBA 20 type). Defined as a specific leukocyte and platelet-rich fibrin prepared using either an Intraspin (Intra-Lock International, Boca-Raton, FL, USA), or EBA 20 (Andreas Hettich GmbH & Co KG, Tuttlingen, Germany) centrifuge and following the recommended protocol as outlined by Dohan Ehrenfest *et al*.[4]
- L-PRF (O) – Leukocyte- and platelet-rich fibrin (Other). Defined as a leukocyte- and platelet-rich fibrin prepared in a manner similar to L-PRF (I/E) and L-PRF(C) production, but using a non-purpose-built centrifuge.

TECHNIQUES AND METHODS OF PRODUCING L-PRF

Choukroun's method of producing L-PRF was intended to be a simple technique that would allow for the production of high quality platelet and leukocyte concentrates, which could be prepared easily and used in everyday healthcare facilities.[4] This method specified the use of a PC-02 table centrifuge and a collection kit from Process (Nice, France).[4] Further, the blood sample was to be taken without anticoagulant in 10-mL blood collecting tubes which were then immediately centrifuged at 3000 revolutions per minute (RPM) (approximately 400g of relative centrifugal force (RCF)) for 10 minutes. The formed L-PRF clot could then be removed from the blood collecting tube and used as required.

The influence of centrifuge type and RCF on L-PRF

Even though Choukroun's protocol was clearly outlined, a number of publications were subsequently produced that reported on procedures which did not follow the prescribed methods.[13-15] Key to these differences was the failure to use a specific centrifuge (PC-O2, Process, Nice, France) and a specific RCF (400g). In many publications, the RCF was not reported, and instead the centrifuge speed and time was quoted.[13-15] This is a significant deviation from the protocol since the influence of the RCF is underestimated and not considered.

Relative centrifugal force (RCF) can be defined as the amount of accelerative force applied to a sample in a centrifuge.[16] It is not equivalent to revolutions per minute (RPM) and the terms cannot be used interchangeably. Centrifuges work by putting samples in rotation around a fixed axis, thereby applying an accelerative force perpendicular to the axis.[16] This resultant force causes the separation of various elements in the sample based on the individual weight of the sample elements and is the basis for blood separation techniques carried out by laboratory centrifuges. RCF is measured in multiples of the standard acceleration due to gravity at the Earth's surface (x g) and is based on two specific variables i.e. how wide the rotor is and how fast it is moving.[16] The radius of the centrifuge or rotor is as critical to the process of producing a specific RCF as is the RPM. Only those processes where the RPM and the rotor radius are identical are comparable and any deviations from these criteria may result in inaccuracies. Consequently, RCF will only be constant for centrifuges with the same rotor radii. Results derived from investigations using centrifuges with different radii will produce differing RCF's.[16] Therefore one cannot assume that all centrifuges used for producing L-PRF and spinning at 3000 RPM will produce an RCF of 400g. This is a significant parameter that is often misunderstood. RCF is critical to the production of L-PRF and must be calculated for each centrifuge used, especially if this parameter is not pre-set on the machine.

The effect of varying RCF's during platelet concentrate preparation was recently reported.[17] Amable, Carias, Teixeira *et al*. analysed various factors affecting the preparation of Platelet-rich plasma (PRP), and showed that changes in RCF significantly influenced the platelet yield even though other parameters such as period of centrifuge time as well as temperature remained constant. Dhurat and Sukesh reviewed several PRP preparation methods.[18] Based on their analysis, it was shown that the use by authors of different RCF parameters resulted in variations in the platelet yields of the PRP produced. More pertinently, scrutiny of the literature reveals that although PRP has been clinically used for several years, no standardised preparation protocol has yet been documented. With regards to the preparation methods of L-PRF that are published in the literature, similar inconsistencies exist.

Other centrifuge parameters that may influence L-PRF preparation.

A series of articles recently published by a team of authors reported on investigations into the effect of various parameters on the quality of the resultant L-PRF's.[19-21] Using the same centrifugal force (400g) as well as the same type of blood collecting tubes, the authors tested four different commercially available L-PRF centrifuges. The results indicated that centrifuge vibration as well as centrifuge type significantly affect the quality and quantity

of the L-PRF clot produced. Under scanning electron microscope (SEM) analysis, the L-PRF clots produced from the different centrifuges showed variations in cell morphology and fibrin architecture, with some cells showing signs of significant damage. These differences were attributed to the type of centrifuge used.[19-21] The Intra-Spin L-PRF centrifuge (Intra-Lock International, Boca-Raton, FL, USA) produced clots displaying cells with the most stable and normal shape.[20] It is therefore critical that identical processes should be followed if the biomaterial product is to be standardised. Researchers cannot simply recreate the biomaterial by using any centrifuge with a setting of 400g RCF even at the appropriate spin time.

Growth factors and their release kinetics

The preferred use of L-PRF in clinical practice is largely due to its reported release of autogenous growth factors. It is assumed that the high concentration of these growth factors results in reduced healing time as well as the stimulation of tissue regeneration.[8] These growth factors have been well documented in the literature.[22] Recently however, the release kinetics of these growth factors has been questioned.[23] Schär et al. prepared L-PRF (I/E) with a single spin protocol at 400g for 12 minutes using an EBA 20 (Andreas Hettich GmbH & Co KG, Tuttlingen, Germany) centrifuge.[23] This is the same machine, recently upgraded, as the Intra-Spin L-PRF centrifuge (Intra-Lock International, Boca-Raton, FL, USA).[24-26] The authors compared the release of various growth factors from L-PRF, L-PRP and a coagulated blood clot. The results demonstrated that the total growth factor release of vascular endothelial growth factor (VEGF) as well as of interleukin-1β (IL-1β) was higher from the blood clot than from any of the platelet concentrates. Furthermore, no statistically significant differences could be established between the blood clot and the various platelet concentrates as regards the amounts of insulin-like growth factor-1 (IGF-1) and of platelet-derived growth factor AB (PDGF-AB) that were released. The L-PRF (I/E) clot released the highest concentrations of transforming growth factor β1 (TGF-β1). When the release kinetics of L-PRF (I/E), L-PRP and the blood clot were investigated, the researchers found that the various growth factors were released at different times as well as for different lengths of time. An examination of this effect on the migration on human bone marrow-derived mesenchymal stem cells (MSC) and human umbilical vein endothelial cells (HUVEC) found no significant differences in the overall patterns of migration for any of the groups tested. However it was reported that IGF-1 had a positive correlation with the migration of both cell types whereas PDGF-AB had a negative correlation with both cell types i.e. MSC and HUVEC. It may be of relevance that IGF-1 had the highest concentration in the blood clot and that there were no differences in the release kinetics of this growth factor when compared with those of L-PRF and L-PRP.[23]

In a similar study, in which the release of growth factors as well as the effect of platelet concentrates on tendon cells were compared with those of a whole blood clot, it was shown that the platelet concentrates had the ability to significantly increase cell proliferation as compared with that of the blood clot.[27] However, it must be pointed out that the techniques of preparing these platelet concentrates were completely different between the two studies, thereby influencing the final architecture and possible biological properties of the various concentrates.

Bone morphogenic proteins

Bone morphogenic proteins (BMPs) are low molecular weight glycoproteins that are responsible for ectopic bone formation.[28] First described in the 1960's, these proteins play a critical role in various aspects of cell function, differentiation and tissue repair. More significantly, they are crucial in the maintenance of skeletal integrity and bone fracture healing. BMPs are released and synthesised by a number of cells including osteoblasts, osteoprogenitor cells, chondrocytes, platelets and macrophages.[28,29] It is therefore clear that the synthesis of these key proteins is not restricted to bone forming cells.

It has recently been shown that L-PRF (I/E) releases BMP-2 over a period of seven days, but that the amounts released are relatively small.[21] Dohan Ehrenfest et al. found it difficult to explain the exact origin of these BMPs, but suggested it was related to the presence of leukocytes in the platelet concentrate.[21] However it had been shown previously that the platelets themselves contain significant amounts of BMP-2 and that the release of these proteins is pH dependent.[30] As a result, it has been suggested that the release of BMP-2 by platelets may play a significant role in the initial stages of bone fracture healing, since the pH in this environment is optimal for platelet activation.[30]

Other researchers have found that other BMPs such as BMP-6, BMP-7 and BMP-4 are also released by platelets, and, further, that the concentration of BMPs contained in platelets is patient dependent.[31,32] It has also been shown, by genome-wide micro analysis, that lysed platelets have the ability to upregulate proliferative pathways of osteoblast like cells in-vitro.[33]

The potentially ground-breaking findings from various studies investigating the BMP potential of L-PRF as well as its variants must be seen in the light of patient variation as well as the pH of the test environment.[21,28,30,32] Further research into these factors may have clinical implications and could explain the reasons for the inconsistent clinical outcomes experienced when using platelet-rich concentrates for bone grafting or regeneration. By implication then, it would currently be difficult to control the amounts of BMP's released from platelets when used in a clinical setting.

Stem cells

Stem cells are undifferentiated cells that can differentiate into specialized cells, including more stem cells or other cell types during development.[34] Recently, a variant of L-PRF(C) has been analysed, thought to contain haematopoetic stem cells (HSC).[35] The presence of these HSC cells is mostly determined using immunohistochemical analysis for the detection of specific cell markers, in this case, CD34. This is a transmembrane phosphoglycoprotein that is predominantly used as a marker for HSC as well as haematopoetic progenitor cells.[36] Although traditionally linked to cells of haematopoetic cell origin, CD34 has recently been linked to other non-haematopoetic cells types such as mesenchymal stem cells (MSC), endothelial progenitor cells and interstitial dendritic cells.[36] Therefore, the mere presence of CD34 positive cells does not allow the assumption that a specific cell type such as HSC, exists. In order to verify the existence of HSC, the cells should, in addition to the proven presence of CD34, display other traits such as a low expression of CD90, a lack of expression of CD38 and human leukocyte antigen-DR (HLA-DR), as well as a panel of mature lineage markers (lin-).[36]

The potential of CD34 positive cell types in L-PRF(C) and its variants appears promising, but requires further investigation due to the variation in CD34 detection methods. Almost all CD34 detection methods use antigen-antibody interactions. These interactions are non-covalent and are reversible, with the potential of affecting the detection of the CD34 marker. Because of this, it is suggested that multiple methods be used to verify the presence of CD34.[36]

Although peripheral blood has been used as a source for CD34 positive cells in many forms of therapy, the baseline concentration of these cells in peripheral blood is relatively low.[37,38] As such, most therapies that require the use of CD34 positive cells enrich the presence of these cells in the vasculature by using granulocyte colony-stimulating factor (G-CSF).[39] This allows for an adequate amount of cells to be harvested for local or systemic transplantation. Whether the levels of CD34 positive cells in L-PRF and its variants are at therapeutic concentrations, requires further analysis.

Clinical results from studies using L-PRF

Several randomised controlled trials have been published that involve the use of L-PRF in the clinical management of a variety of disorders.[40-43] These trials have contradictory results, which may be related to a variation in the techniques and equipment used to prepare L-PRF.[41-45] Nevertheless, several articles report positive clinical outcomes even when standardised preparation techniques have not been used.[46-49] Whilst most of these publications are in fact case reports, nevertheless even randomised controlled trials where the RCF has not been verified, have shown positive clinical outcomes.[43] This is similar to reports about the use of PRP, which show a variety of clinical results based on the various methods of producing PRP.[18] Further research is therefore required to verify clinical differences which may be associated with the various methods of preparing L-PRF.

Does generic L-PRF exist?

L-PRF (O) is prepared using standard blood collecting tubes and a non-purpose built table top centrifuge delivering an RCF of 400g with a centrifuge time of 12 minutes. This method allows for the preparation of a platelet and leukocyte concentrate without the need for specialised equipment. Several case reports have demonstrated positive clinical results when using this preparation method.[50,51] However one cannot assume that this generic type of L-PRF has properties similar to those of L-PRF(C) or to L-PRF(I/E) since it has previously been shown that the centrifuge type may play a significant role in the final morphological features of the end product.[19,20] Further analysis of this biomaterial is therefore required to determine equivalence to the established protocols for L-PRF preparation.

CONCLUSIONS

The concept of L-PRF as a bioactive material with possible regenerative properties has resulted in it being adopted for use in various clinical procedures. However, the widespread use of this material has resulted in the production of several variants. Whether the biological properties of all these variants are similar, is unknown. Contradictory clinical results are reported in the literature with several generic types of L-PRF showing diverse results. In order to minimise the controversies associated with L-PRF, further research is required to determine which factors affect the biological properties of the material and whether these factors are clinically beneficial and relevant.

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The effect of preparation method on the fibrin diameter of leukocyte- and platelet-rich fibrin (L-PRF).

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ABSTRACT

Introduction
The use of leukocyte and platelet- rich fibrin (L-PRF) in regenerative surgery has increased exponentially in the last decade. That success has lead various centres to have introduced the biomaterial as a routine inclusion in oral grafting procedures. Evidence suggests that the physical properties of fibrin play an essential role in homeostasis of blood clots. Only a limited number of studies have investigated the features of fibrin fibre diameter of L-PRF concentrates.

Aims and objectives
To investigate and compare the fibrin network and fibrin fibre diameter of L-PRF prepared by two different methods.

Methods
Blood was collected from a single volunteer using established protocols. The resultant L-PRF clots were then prepared and examined using scanning electron microscopy. The results were subjected to statistical analysis.

Results
L-PRF prepared using the modified method had larger diameter fibrin fibres than that prepared using the official protocol. The difference was statistically significant (P=0.001). There was also a larger amount of thicker fibrin fibres observed in the modified L-PRF group.

Conclusions
Preparation methods affect the fibrin fibre diameter of L-PRF. This may have consequences that influence the biological properties of the biomaterial.

INTRODUCTION

The use of leukocyte and platelet- rich fibrin (L-PRF) in regenerative medicine has increased exponentially in the last decade.[1] First introduced by Choukroun et al. in 2001,[2] this autologous, blood derived platelet concentrate that has been extensively used in oral surgical procedures, with clinical success in the fields of

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ACRONYMS

A-PR: Advanced PRF.

L-PRF: Leukocyte and platelet-rich fibrin

SEM: Scanning electron microscopy

surgical implantology and regenerative periodontology.[1,2] Because of its popularity, various centers centres have introduced the biomaterial as a routine inclusion in oral grafting procedures[1]. Although Choukroun had specified the original method of L-PRF preparation, this protocol had been modified over time, with several variants of L-PRF now being made available.[3,4] The modification of the original protocol included updating the equipment and materials used, as well as varying the centrifuge time and force.[4] This resulted in two further variants of L-PRF being introduced i.e. advanced PRF (A-PRF) and advanced PRF+ (A-PRF+).[4] Both of these variants use a lower centrifugal force when compared to the original protocol and this is thought to have distinct advantages over the original preparation method.[3,4]

Figure 1: Modified L-PRF (left) and A-PRF (right)

An important component of the biological advantages of L-PRF concentrates, is its specific structure which comprises a dense fibrin network, with high concentrations of platelets and leukocytes interspersed within the mesh of fibrin fibres.[6] It is thought that this allows growth factors to be trapped, and released over a prolonged period thereby enhancing healing and regeneration.[6,7] Recently, the role of fibrin structure has been researched for its role in blood clots.[8-11] Evidence suggests that the physical properties of fibrin fibres play an essential role in homeostasis of blood clots by affecting their mechanical and biological contribution properties. [8, 12] However, only a limited number of studies have investigated the features of fibrin fibre diameter of L-PRF concentrates.[13,14]

With the increase in popularity of L-PRF, several authors have reported positive clinical results using L-PRF that is prepared using non-standardised equipment or materials.[15-17] An example of this type of "modified" L-PRF has been clinically documented in previous publications by Peck et al.[18, 19] This deviation from the proposed protocol may result in structural and functional changes to the consequences to the biomaterial that is being produced.[20-22] In this report, using scanning electron microscopy (SEM), we analyse and compare the fibrin network and fibrin fibre diameter of two different methods of L-PRF preparation i.e. our modified protocol for the preparation of L-PRF as previously described by Peck et al, and A-PRF+, prepared via the recommended protocol as previously outlined in the literature.[4, 18, 19]
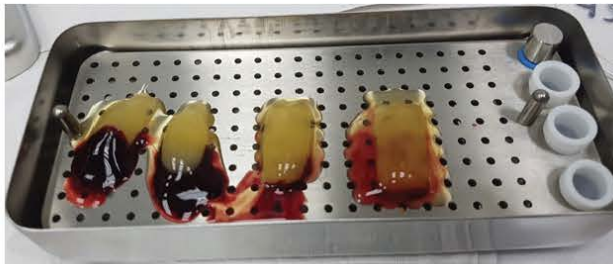
Figure 2. PRF Box with prepared L-PRF before compression
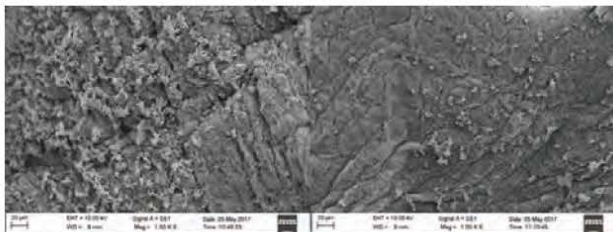
Figure 3: Modified L-PRF and A-PRF+ (Low magnification)

MATERIALS AND METHODS

The study was conducted at the Dental Faculty, University of the Western Cape, Cape Town, South Africa in 2017. Ethical clearance was obtained from the Research Ethics Committee of the university with the following reference number: BM 16/3/31

Preparation of the L-PRF samples

Thirty six milliliters of blood was obtained from a single 30-year-old healthy male volunteer via venipuncture of the left antecubital vein. Two methods were used to prepare L-PRF, i.e. :

1. Modified L-PRF: Blood samples were collected in 9ml blood collecting tubes that contain clot activator i.e., Vacuette® 9ml serum tubes with Z Serum Clot Activator (Greiner BioOne International AG, Germany). The blood samples were then centrifuged at 400g for 12 minutes in a standard benchtop centrifuge (PLC-03, Hicare International, Taiwan).
2. A-PRF+:Blood samples were collected in 10 ml A-PRF+™ tubes (Process for PRF, Nice, France), and centrifuged for 1300RPM for 8 minutes, using a dedicated tabletop centrifuge (PRF DUO™, Process For PRF, Nice, France).

After being centrifuged, the blood from both groups separated into 3 distinct layers (Fig 1). From previous reports, the layers could be distinguished as a topmost layer consisting of acellular platelet poor plasma, Modified L-PRF/A-PRF+ in the middle, and red blood cells at the bottom of the test tube.[5, 6] In order to standardise L-PRF thickness, the resultant L-PRF clots from both groups were removed from the tubes, and subjected to controlled compression, using a specifically designed tool i.e., the PRF Box® (Process for PRF, Nice, France) (Fig 2).[23] All the samples were then transferred for preparation for scanning electron microscopy analysis.

Preparation for scanning electron microscopy (SEM)

All the samples were fixed using 2.5% gluteraldehyde in phosphate buffered saline (PBS) for 1 hour. Each specimen was removed and washed in PBS for 5 minutes (twice) and thereafter distilled water for 5 minutes (twice). The specimens were then dehydrated serially with 50%, 70%, 90% and 100% ethanol, with each dehydration step taking 10 minutes. The samples were then transferred to the Electron Microscope Unit, Department of Physics, University of the Western Cape, for critical point drying, gold-palladium coating, and mounting for SEM analysis.

Scanning electron microscope observation

The surface microstructure of each specimen was analysed using the same scanning electron microscope (AURIGA Field Emission High resolution SEM, Carl Zeiss Microscopy GmbH, Jena, Germany). All measurements of the fibrin networks were carried out at 10 000 times magnification. At least 200 fibres were randomly selected from each group and measured for
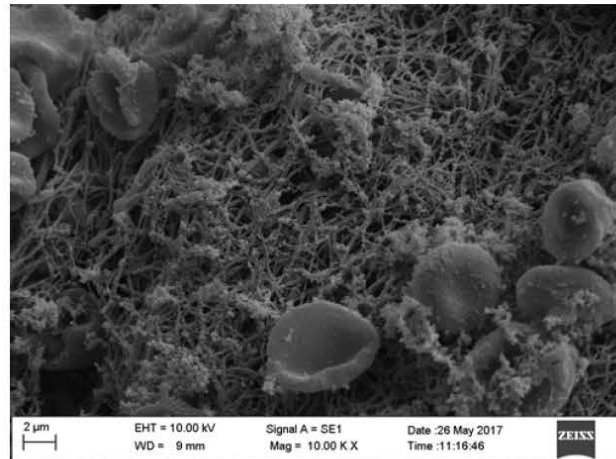

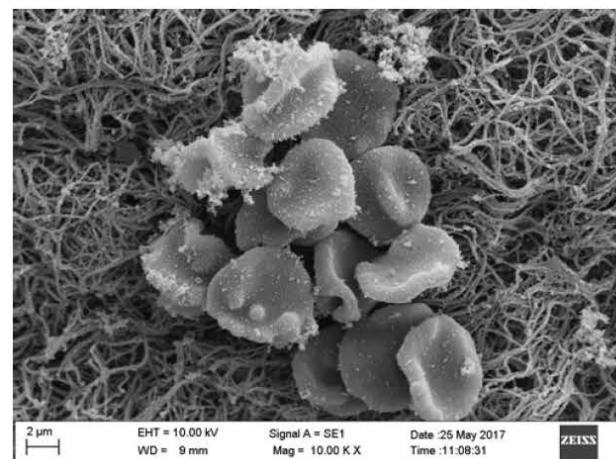**Figure 4:** Modified L-PRF (High magnification)

Figure 5: A-PRF + (High magnification)

analysis using ImageJ version 1.51o software developed by Wayne Rasband (National Institutes of Health, USA). The data was captured using Microsoft Excel 2010 (Microsoft Corporation, Washington, USA) and statistically analysed using the Mann-Whitney U and Wilcoxon W tests.
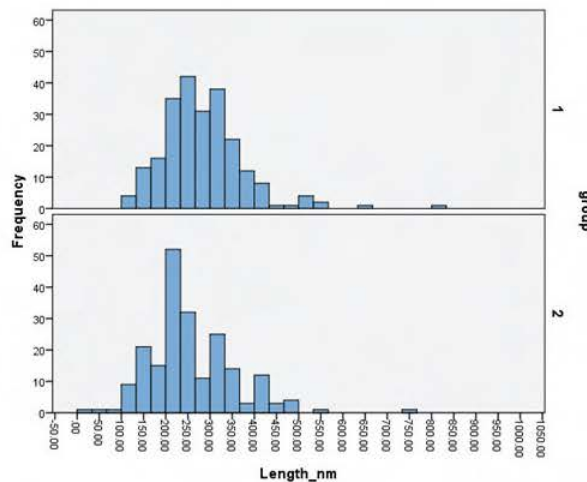
Figure 6: The distribution of fibrin fibre diameters for Modified L-PRF (group 1) and A-PRF+ (group 2) measured as length in nm

RESULTS

Descriptive analysis of Modified L-PRF and A-PRF+ Fibrin fibre networks

Under low magnification (1000 times) both samples show similar morphological characteristics. A dense mesh of fibres was evident with scattered cells seen on the surface for each sample (Fig 3). For the Modified L-PRF sample, more irregular bodies were present, possibly signifying the presence of platelets (Fig 3).[24] This may be characteristic of the region observed and may not be representative of the sample as a whole.[6] No distinction in fibre architecture for the 2 groups was seen at this level of magnification. When observing the samples at higher magnification (10 000 times), the fibre mesh showed distinct characteristics (Figs 4 and 5). For both groups, the fibres were densely arranged in a netlike structure with clear spacing and crosslinking seen. Distinct differences could be noted with regards to fibre diameter, with both groups showing non-uniform fibre thickness. Thicker fibres were interweaved with thinner fibres and many of the fibres showed irregular surface characteristics indicative of platelet-fibrin fibre interaction.[24] The presence of cellular bodies could be noted for both groups. Distinguishing between both groups solely on the basis of SEM analysis, was difficult.

Table 1 a,b: Results of testing the difference in fiberfibre diameter (Length in nm) between both groups with the Mann Whitney test:

	Group	N	Mean Rank	Sum of Ranks
Length_nm	M-PRF	231	238.06	54992.00
	A-PRF+	206	238.06	40711.00
	Total	437		

Table 2

Test Statistics[a]

	Length_nm
Mann-Whitney U	19390.000
Wilcoxon W	40711.000
Z	-3.343
Asymp. Sig. (2-tailed)	.001

a. Grouping Variable: Group

Analysis of Modified L-PRF and A-PRF+ fibrin fibre thickness

A total of 437 fibrin fibre samples were analysed from both groups. The fibres were measured in nanometres (nm) with a range of 100-808nm and a mean of 281nm being observed for the Modified L-PRF group. For the A-PRF+ group, fibre diameters ranged from 43-742nm with a mean diameter of 256nm. For both the groups, statistical analysis revealed that the mean fibre diameter was not a

clear reflection of the various fibre groups present. It also showed that the fibrin fibre diameters were not equally distributed in each of the groups and that a wide range of fibre diameters were present (Fig 6). These fibre groups were present in both Modified L-PRF and A-PRF+, but were significantly different in their distribution for the two versions of L-PRF. Of the two groups tested, Modified L-PRF was characterized by larger diameter fibres and had a higher amount of thicker fibres when compared to A-PRF+ (Fig 6). Because of the wide range of fibre diameters noted in each of the sample groups tested, arbitrary size classes were selected to better illustrate the range of fibre diameters seen. This size selection was based on a previously published model which analysed human fibrin networks.[25] Consequently the fibre diameters were categorised into 3 groups based on "thickness" i.e. thin (lowest thickness to 85nm), intermediate (86 - 202nm), and thick (203nm to highest). This is represented by histogram A.
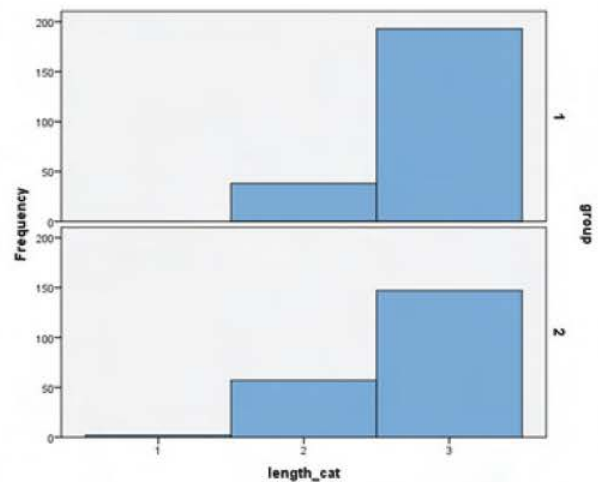
Histogram A: The distribution of "thin, intermediate and thick" fiberfibres of Modified L-PRF (group1) and A-PRF+ (group 2)

DISCUSSION

L-PRF in its various forms has shown promising results in both in-vitro and in-vivo studies.[1, 15-19] This is attributed to the structural integrity of the biomaterial which results in a matrix that contributes to the prolonged release of various growth factors as well as provides a network for the migration of cellular components.[2-6] The process of preparing L-PRF was initially documented by Choukroun in 2001.[2] However since the original protocol was introduced, several authors have attempted to replicate the platelet concentrate using non-standardized methods.[16-19] Although clinically successful, the ultrastructure of these L-PRF variants has not been reported upon extensively.

The data from this study indicates that using both the A-PRF+ protocol as well as the modified protocol for the preparation of L-PRF that we used, may yield L-PRF clots that appear similar macroscopically. Both preparation methods resulted in separation of the centrifuged blood into 3 three distinct layers as previously described. When examining L-PRF clot size, the resultant L-PRF clots appeared smaller for the Modified L-PRF group, however this was not quantified statistically. We speculate this may be due to using a 9ml blood collecting tube as opposed to 10ml in the A-PRF+ group. However, the size of the collecting tube may not be the only factor affecting L-PRF size, since recent research indicates that using different centrifuges may affect L-PRF clot size.[20-22]

Compressed L-PRF clots from both groups appeared similar under low magnification (x1000), with a dense fibre network and large range of fibre diameters being seen. The density of the fibre networks seen in this study has previously been reported upon and is thought to be related to the compression of the clots in the PRF-Box™.[23] When the fibre diameters were analysed as thin, intermediate, and thick, the majority of fibres in both groups were classified as thick. This differs from the findings of Vieira et al.

2009,[25] who reported that the predominant fibre patterns were thin.

Previous studies that have examined the features of fibrin associated with L-PRF, have often used the mean diameter of the fibres to describe the characteristics of the L-PRF clot or are limited to a subjective description of the fibre diameter.[13, 20] In the present study, statistical analysis revealed that using the mean diameter, did not reflect the range and frequency of the fibre types observed. Therefore when analyzing fibre diameters for Modified L-PRF and A-PRF+, we identified clear groupings (Fig 6). These were statistically significantly different for the two L-PRF groups, with the Modified L-PRF showing a higher number of larger diameter fibres as compared to A-PRF+ (Table 1). The exact reasons for this difference in not known, but is probably due to differences in the centrifugal force, centrifuge time, centrifuge make, agents present in the blood collecting tubes and shape of the blood collecting tubes.[20, 26] Because all the samples were taken from the same individual in a 15 minute period, the above may serve as an adequate explanation for the differences in fibre diameters seen. However one must consider that for different individuals or in instances where blood is collected from the same individual at different times, that other factors such as pH, the presence of zinc, ionic strength, concentrations of calcium, polyphosphate, fibrinogen, fibrinogen binding proteins and thrombin, may also influence fibre thickness and network density.[9,12]

The tubes used to collect blood to prepare Modified L-PRF, are coated with silica. Silica is a known procoagulant. It binds to plasma proteins and has the ability to cause reversible structural changes to these molecules.[27,28] This is dependent on the size of the silica particle, with smaller silica particles demonstrating the ability to shorten coagulation time and increase the activation of factor X, whilst at the same time activate platelets.[28] The fact that silica coated tubes were used in the preparation of the Modified L-PRF, may further contribute to the morphological differences seen between the 2 groups.

The individual fibre diameters seen in the samples tested may have implications for the mechanical stability of each L-PRF clot.[29] Clots with thicker fibres tend to be less elastic than those with thinner fibres and may be more readily degraded by the fibrinolytic system.[8, 29] This may have consequences for the biological behaviour of L-PRF since structural integrity and the controlled release of growth factors is thought to be a major contributing factor to its clinical success. Therefore, one might assume that because A-PRF+ has a lower amount of thick fibres than Modified L-PRF, that its rate of dissolution may be slower than that of the Modified L-PRF. As such, its ability to remain intact may be prolonged as compared with other platelet concentrates and may explain the extended release of growth factors as recently reported.[3,4]

CONCLUSIONS

To the author's knowledge, this is the first presentation of the morphological fibre characteristics of an L-PRF clot prepared using a specific modified protocol as previously described by the authors. When compared to an established protocol, the resultant L-PRF clot appears morphologically similar to its A-PRF+ counterpart with a dense fibrin network interspersed with platelets and other blood cells. However, clear differences are noted for the fibrin fibre diameter with Modified L-PRF showing a higher proportion of larger diameter fibres. The reason for these differences is speculated to be associated with the different protocols used to prepare the platelet concentrates. One might therefore assume that changes in the protocol of preparation of L-PRF may directly affect it morphological structure. Whether these differences affect the clinical efficacy of these biomaterials is unclear, and warrants further research.

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Platelet- Rich Fibrin (PRF) - The effect of storage time on platelet concentration

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ABSTRACT

The aim of this study was to determine whether storage time had a significant effect on the platelet concentration of platelet-rich fibrin (PRF). Three blood samples were drawn from each participant into a sterile blood sampling tube. Two of the blood samples were centrifuged to form PRF. The third non-centrifuged sample was used to measure the baseline blood platelet concentration. After PRF had formed, it was removed from the respective test tubes at different time intervals i.e. immediately after centrifugation (Group A) and after 60 min of storage time in the blood collecting tube (Group B). The residual blood from each group was tested for platelet concentration and compared with the baseline reading (as an indirect measure of the platelet concentrate of PRF). The PRF produced in Group A (PRF A) had a mean platelet concentration of 274 $\pm 57.8 \times 10^9$/L, whereas the PRF of Group B (PRF B) was 278 $\pm$ 58.2$\times 10^9$/L. A statistically significant difference was seen between the groups (p < 0.001).

Conclusions: Storage time has a significant effect on the platelet concentration of PRF. Further research is required to determine whether this has any clinical relevance.

INTRODUCTION

Wound healing is a complex process characterised by the repair and reconstitution of lost or damaged tissue. Identification of the pathological and biochemical mechanisms that regulate tissue repair and homeostasis has long been regarded as central to their therapeutic exploitation in the clinical setting. By the mid-1990s, several methods

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ACRONYMS

GF:	Growth factors
PRF:	Platelet-rich fibrin
PRP:	Platelet -rich plasma

were proposed to enhance wound healing, including the administration of high concentrations of human platelets to affected areas.[1-6] It was assumed that platelets optimised wound healing by promoting the secretion of growth factors (GFs) necessary for tissue repair.[7-9] The most common platelet concentrate used in these procedures is platelet-rich-plasma (PRP) and by the early part of the 21st century, its use in various surgical procedures was commonplace.[1,4,10,11] However, the preparation of PRP often requires the use of specialised equipment, chemicals and animal-derived additives. This increases the risk for complications secondary to allergic reactions to certain animal-derived additives.[12-14] As a result, researchers have sought more efficient and safer methods of concentrating platelets for surgical use.[1] This led to the production of platelet-rich-fibrin (PRF), a platelet concentrate that neither contained additives nor required the use of specialised equipment during its preparation. First introduced by Choukroun et al (2001), PRF has been studied extensively and is now regarded as a biological scaffold different to PRP.[15] However, even though the use of PRF is gaining widespread clinical acceptance, several questions regarding its biological stability remain unanswered.[16,17] The aim of this this study was to analyse the effect of storage time on the platelet concentration of this unique biomaterial.

MATERIALS AND METHODS

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 participants were fully informed of the research protocol and were required to sign a declaration of informed consent before being allowed to participate in this cohort analytical study. A total of 30 healthy participants (16 females and 14 males) were enrolled into the study. Participants were drawn from the current patient pool as well as staff members based at the Dental Faculty, University of the Western Cape. All participants had three separate blood samples collected by venipuncture. Two of the samples were acquired using tubes containing clot
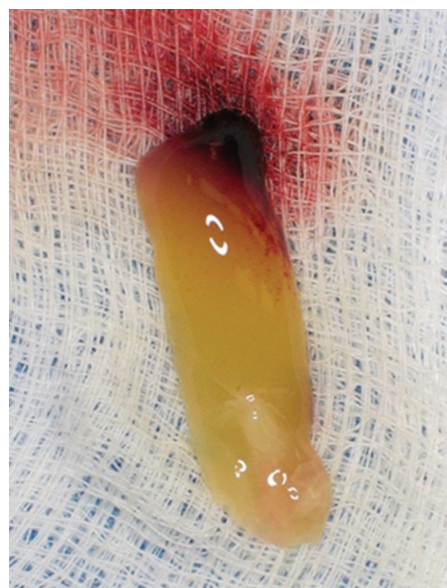
Figure 1: Benchtop centrifuge

Figure 2: Prepared PRF

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.

Platelet concentrations obtained from analysis of all 30 participants were within the normal laboratory reference range of $170\text{-}400\times10^9$L of circulating blood. None of the participants displayed any significant haematological disease. The mean blood platelet concentration was 282.8×10^9/L (Table 1). There was no significant difference between the genders.

activators, i.e., Vacuette® 10ml serum tubes with Z Serum Clot Activator (Greiner BioOne International AG, Germany), whereas the remaining blood sample was drawn into a 10ml BD Vacutainer® tube that contained dipotassium-EDTA, an anticoagulant (BD Diagnostics, New Jersey USA).

All the blood samples collected in the anticoagulant-containing tubes were used to measure baseline platelet concentrations, whereas the remaining blood samples (collected in the Clot Activator containing tubes) were used to prepare PRF by centrifugation (400g for 12 minutes) in a standard benchtop centrifuge (PLC-03, Hicare International, Taiwan) (Figure 1). Therefore, from each study participant, two samples of PRF were obtained (Figure 2). The two PRF samples were then randomly allocated into either Group A or Group B, using a simple coin toss.

For Group A, the PRF was removed from the tube immediately after preparation (0min), whereas for Group B, the PRF remained in the tube and was only removed after 60 minutes. The PRF produced from each group was designated PRF A and PRF B respectively. Because a direct measurement of platelet concentration of PRF is not yet possible, we calculated the PRF concentration for each group indirectly, by determining the numerical difference between the residual platelet concentration (of the remaining serum after removal of PRF) and the baseline platelet concentration for each specific study participant. In this study, platelet concentration analysis was carried out using an electronic automated cell counter (Advia 2120, Siemens AG, Erglangen, Germany).

Data was collected and entered into a spreadsheet (Microsoft Office 2010 Excel, Microsoft Corporation, Washington). The results were compared and analysed statistically using SPSS® Version 13 for Windows.

RESULTS AND DISCUSSION

A total of 30 participants (16 females and 14 males) were entered into the study. The mean age of the participants

ed minimal concentrations of platelets. The mean concentration of remaining platelets was 7.9×10^9/L (Table 2). For Group B, serum platelet concentrations were also minimal after removal of the prepared PRF with the mean concentration of the platelets being 4.0×10^9/L (Table 2).

The residual mean platelet concentration of Group A was higher than that determined for Group B with the mean difference in platelet concentrates between the two groups being 3.90×10^9/L. Using a non-parametric Signed Rank Test, the statistical significance of the differences in platelet concentrates between the Groups A and B was analysed. The difference between the two groups was statistically significant ($p < 0.001$).

The platelet concentration of PRF for both groups was calculated using the difference between baseline and residual platelet concentrations (Table 3). A paired t-test showed a statistically significant difference between the two groups ($p < 0.001$) (Table 4).

Table 1: Mean baseline platelet concentration in cells x 10^9/l of blood

	Baseline	
	Mean	**SD**
Males	275.14	43.13
Females	289.50	69.64

Table 2: Mean baseline platelet concentration of residual blood in cells x 10^9/l of blood

	Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Group A	7.9	30	3.03	0.55
Group B	4	30	1.93	0.35

Table 3: Mean calculated PRF platelet concentration in cells x 10^9/l of blood

	Mean	**Std. Deviation**	**Minimum**	**Maximum**
PRF A	274.9	57.8	169	387
PRF B	278.8	58.2	171	390

Table 4: Statistical analysis of PRF A and PRF B

Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	Significance (2-tailed)
			Lower	Upper			
-3.9	3.133	0.572	-5.07	-2.73	-6.818	29	0.001

Paired t-test performed, P value is significant

DISCUSSION

The purpose of this study was to determine whether storage time had any significant effect on the platelet concentration of 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. This is in accordance with a previously published method.[18] 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. The results from this study indicate that there is a significant difference in the mean platelet concentration of PRF when stored for 0 and 60 minutes.

From the platelet counts obtained in both test groups it was clear that a significant proportion 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 97%-98% of the platelets were concentrated in the PRF clot (Table 3). This observation is similar to results published for previous studies (97%).[19] 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 acts as a reservoir for a concentration of growth factors (GFs) required in the initial stages of wound healing.[20]

Although the physiology of PRF has been studied extensively, very few reports adequately document the ability of this platelet concentrate to be stored.[21] Previous research has indicated that storing PRF under certain conditions may affect its ability to clinically yield positive results.[22] 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.[22] Instead, several authors propose storing the biomaterial in a metal dish or a proprietary designed storage box.[17] Data regarding the maximal storage time and ideal storage temperature of PRF are, however, largely lacking.

In this study, we used standard blood collecting tubes with clot activators to store PRF for at least 60 minutes at room temperature. This particular duration of time was chosen based on the average time of typical periodontal surgical procedures at the Faculty of Dentistry, University of the Western Cape. The results of the study indicate that by using the presented 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, there was no detrimental effect on the platelet concentration of PRF. Indeed, storing the concentrate for 60 minutes resulted in a form of PRF that had significantly higher concentrations of platelets compared with non-stored PRF. The reason for this phenomenon is unclear, 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.[22] As a result, a longer contact time between blood and these activators may enhance fibrin activation and clot formation, with subsequent platelet entrapment.[18]

Another reason for the variation in platelet concentration seen in this study may be that the recommended centrifuge time is too short to allow for complete clot formation to occur. 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 PRF to sustain its platelet concentrate over the tested time may have significant clinical implications.[8,9,22-24] Rather than having to draw blood during the surgical procedure, it allows for blood to be drawn before the start of the procedure, thereby improving patient comfort and saving operator time. The option 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.[18,19] In order to sustain cell viability over time, an isotonic solution is required for storage. The blood collecting tubes, although not designed to store blood, act as containers for the PRF and the remaining formed elements and serum. Consequently, when PRF is stored in the blood that it was derived from, the remaining serum acts as a natural isotonic solution that sustains cell survival.

The release of growth factors is a significant property of blood platelets.[24] Previous studies indicate that several of these factors play an essential role in osteogenesis and periodontal regeneration.[20,25] When PRF is used, the release of these growth factors appears to be constant, and over a longer period of time when compared with that seen with PRP.[23] 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.[23] 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.[18,19,26] 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 it has been suggested that storing it in near-freezing temperatures is not advisable. In this study, all the samples were stored at room temperature. It was clear that storage under these conditions had no detrimental effect on the platelet concentration of the PRF clot.

Although the present study showed statistically significant differences between the two groups tested, some 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 further research is warranted to determine 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 the above 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 in 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. Further investigations are warranted to determine whether this has any clinical implications.

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The *in vitro* effect of leukocyte- and platelet-rich fibrin (L-PRF) and cross-linked hyaluronic acid on fibroblast viability and proliferation.

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ABSTRACT

Introduction: Leukocyte- and platelet-rich fibrin (L-PRF), an autologous derived platelet and leukocyte concentrate, was first introduced by Choukroun et al. in 2001 and is currently used for several oral and maxillofacial procedures. Hyaluronic acid (HA) is an anionic, glycosaminoglycan that shows a wide range of physiological actions, acting as a cellular scaffold, stimulating cell adhesion and migration, as well as having anti-inflammatory effects.

Aims and objectives: To investigate the effect of cross-linked HA and L-PRF on fibroblast viability and proliferation.

Methods: An *in vitro* laboratory study was conducted at The University of the Western Cape. L-PRF was prepared from a single healthy volunteer and cross-linked HA was obtained from a commercially available product. Analysis was carried out on 3T3 cells using MTT assay.

Results: Both L-PRF and cross-linked HA are associated with cell viability and increased cell proliferation. L-PRF had it strongest proliferative effect after 24 hours whereas HA had it strongest effect after eight days ($p < 0.05$). The combination of HA and L-PRF was not significantly better than the control ($p > 0.05$).

Conclusions: L-PRF prepared in a specific manner as outlined, as well as commercially available cross-linked HA, is compatible with cell growth and proliferation.

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ACRONYMS

3T3 cells:	mouse fibroblast cell line
bFGF:	basic fibroblast growth factor
clHA:	cross-linked Hyaluronic acid
DMEM:	Dulbecco's Modified Eagles Medium
EDTA:	ethylene-diamine-tetra-acetic acid
EGF:	epidermal growth factor
FBS:	foetal bovine serum
HA:	hyaluronic acid
HGF:	human gingival fibroblasts
L-PRF:	leukocyte- and platelet-rich fibrin
PDGF-AB:	platelet-derived growth factor
TGF-β1:	transforming growth factor beta 1
VEGF:	vascular endothelial growth factor

NTRODUCTION

Leukocyte- and platelet-rich fibrin (L-PRF), an autologous derived platelet and leukocyte concentrate, was first introduced by Choukroun et al. in 2001 and is currently used for a number of oral and maxillofacial procedures.[1] The use of L-PRF has gained popularity in the field of surgical implantology, specifically for procedures involving soft and hard tissue augmentation.[2-5] The preparation of L-PRF differs from those of previous platelet concentrates in that it involves the use of a single spin protocol that results in an easily manipulated biomaterial that can be applied directly to the site of surgery.[1] It is assumed that the concentrated growth factors associated with L-PRF optimize wound healing and decrease surgical recovery time.[2,5] Consequently, L-PRF has been used in various fields, including dermatology and periodontal regeneration.[2-5]

Hyaluronic acid (HA) is an anionic, glycosaminoglycan.[6,7] It is a naturally occurring molecule that is found in high concentrations in the extracellular matrix of skin, cartilage, bone and periodontal ligament.[7] Even though the exact function of HA has not yet been established, the molecule shows a wide range of physiological actions, acting as a

cellular scaffold, stimulating cell adhesion and migration, as well as having anti-inflammatory effects.[7] Commercially synthesized HA, in various forms, has been used in tissue engineering, dermatology, orthopaedics, and more recently, periodontal regeneration.[7-10] Traditionally, two forms of commercially synthesized HA exist, i.e., non-cross-linked HA, and cross-linked-HA, resulting in different properties and indications for each form of HA.

The aim of the study was to investigate the effect of cross-linked HA and L-PRF on fibroblast viability and proliferation.

MATERIALS AND METHODS
The study was conducted at The University of the Western Cape, Cape Town, South Africa, in 2017. Ethical clearance was obtained from the Research Ethics Committee of the above university (reference number: BM 16/3/31). Informed consent was obtained from the blood donor.

Preparation of the L-PRF
Thirty six milliliters of blood was obtained from a single 56 year old healthy female volunteer. The blood samples were collected in blood collecting tubes that contained clot activator i.e., Vacuette® 9-ml serum tubes with Z Serum Clot Activator (Greiner BioOne International AG, Germany). These were then immediately centrifuged at 400xg for 12 minutes in a standard benchtop centrifuge (PLC-03, Hicare International, Taiwan) as has been previously described.[11] Centrifugation separated the blood into three distinct layers (Figure 1). The layers could be distinguished as a topmost layer consisting of platelet poor plasma, L-PRF in the middle, and red blood cells below. L-PRF was then removed with a sterile forceps, separated from the underlying red blood cells and shredded into smaller fragments using surgical scissors.

Hyaluronic Acid (HA)
A commercially available cross-linked HA (hyaDent BG, Bioscience GmbH) was used in the present study. It is sold as a clear gel contained in a dental anaesthetic-type cartridge and is applied using a dental syringe and a specifically supplied needle. This specific formulation contains 2.0 mg/ml of sodium hyaluronate that is cross-linked with butanediol digylcidyl ether, resulting in a HA molecule with a unique chemical structure.

Cells and Cell Culture
In this investigation, the 3T3 fibroblast cell line was obtained from The National Repository for Biological Materials (Sandringham, South Africa). The 3T3 cells were incubated at 37°C in 5% carbon dioxide and 95% humidity in Dulbecco's Modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS) and 1% penicillin– streptomycin mix. Cells were grown to about 80% confluence and then trypsinized using trypsin-ethylene-diamine-tetra-acetic acid (EDTA). The cells were then seeded into 3 x 96-well plates and treated as follows;
- Plate 1 – 1 drop of HA (HA group)
- Plate 2 – 1 drop of HA added to a 1 mm x 1 mm fragment of L-PRF (HA + L-PRF group)
- Plate 3 – 1 mm x 1 mm fragment of L-PRF (L-PRF group)

All the plates had an equal number of controls containing only DMEM. After a culture period of 24 hours to 10 days, each group was removed and proliferation and viability evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[12] A minimum of 5 replicate experiments for each group was performed to ensure reproducibility.

Cell Viability and Proliferation Assay
Cell viability (the metabolic activity of the cell) and cell proliferation (rate of cell division) were measured using the standard MTT assay (MTT, Sigma Chemical Co., Baltimore, MD, USA). The MTT assay or test is a sensitive and quantitative colorimetric assay that measures these parameters.[12] It reflects the capacity of the mitochondria in living cells to convert MTT to purple formazan crystals that can be solubilized with dimethylsulphoxide (DMSO) or isopropanol.[12] This effect is directly proportional to the cell number and is measured with a spectrophotometer at a wavelength of 540 nm. Rapidly dividing cells i.e. cells which show high rates of proliferation, reduce MTT at a faster rate than cells with low proliferative potential. The MTT assay therefore measures the ability of the cell to reduce MTT (viability) as well as the rate of cell division (proliferation). As a result, the MTT assay can also be used to measure cytotoxicity (loss of viable cells) and cytostatic activity (shift from proliferation to quiescence). For the current study, readings were taken from all groups with the control group set at 100%.

Data Analysis
Data from the MTT assay were captured using Microsoft Excel 2010 (Microsoft Corporation, Washington, USA) and statistically analyzed using the one-way ANOVA and Tukey's test.
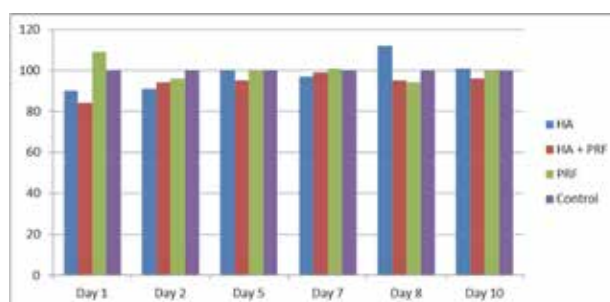

Figure 1: Proliferation and viability assay (MTT) of 3T3 cells. Control is taken as 100%
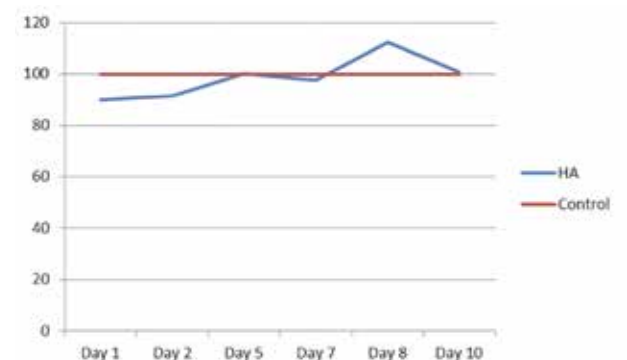

Figure 2: HA vs control (MTT assay).

RESULTS

With the control group set at 100%, the mean optical densities of each of the test groups were divided by that of the control group and expressed as a percentage of the control value. The data from the MTT assay was correlated, and is presented in Figures 1-4. Statistical analysis is represented by Tables 1 and 2.

Table 1: Comparison between the test groups (one way ANOVA)

	HA		HA + PRF		PRF		P value
	Mean	SD	Mean	SD	Mean	SD	
Day 1	89.98	9.83	85.44	12.35	108.99	5.43	0.005 *
Day 2	91.41	8.79	94.72	9.89	96.27	4.34	0.632
Day 5	100.08	8.79	95.18	7.29	101.01	7.26	0.47
Day 7	97.47	8.92	98.6	6.15	100.79	5.77	0.757
Day 8	112.2	9.69	94.57	4.09	94.78	9.66	0.007 *
Day 10	100.88	6.46	96.15	2.62	99.15	7.64	0.439

(* p<0.05)

Table 2: Comparison between the test groups (Tukey test)

	HA vs Control	HA-PRF vs Control	PRF vs Control
Day 1	0.02*	0.01*	0.003*
Day 2	0.03*	0.13	0.045*
Day 5	0.49	0.08	0.38
Day 7	0.27	0.31	0.08
Day 8	0.01*	0.009*	0.13
Day 10	0.38	0.005*	0.49

(* p<0.05)

According to the results obtained, both L-PRF and cross-linked HA (clHA) as well as the combination of the two materials were compatible with cell viability and proliferation (i.e. none of the tested biomaterials tested displayed evidence of being cytotoxic or cytostatic). Cross-linked HA showed a statistically significant reduction in cell number when compared with the control group at 24 hours (89.98% ± 9.83, p < 0.05). Thereafter cell viability steadily increased, reaching 100.88% ± 6.46 at day 10. Peak cell proliferation associated with HA was seen on day 8 and recorded as 112.20% ± 9.69. This was significantly greater than the proliferation seen in both other test groups for the same time period (p < 0.05). The HA + L-PRF group showed a cell viability of 85.44% ± 12.35 at 24 hours. This was statistically significant (p < 0.05) when compared with

the control group, indicating a negative effect on cellular proliferation in the first 24 hours. This difference was not significant for days 2, 5 and 7, but was again recorded on days 8 and 10 (p < 0.05).

The L-PRF group showed the highest cell viability and proliferation after 24 hours (108.99% ± 5.43). This was significantly higher than the other groups tested as well as higher than the control group (p < 0.05). At day 10, no significant differences were seen between all the test groups, however the HA + L-PRF group showed a significantly reduced number of cells as compared with the control (p < 0.05).

A comparison of the proliferative effects of the test groups indicate that both groups containing clHA showed a pattern of cellular proliferation that steadily increased from day 1 to peak at day 8 (clHA group) and day 7 (HA+L-PRF group) respectively. This was different to that observed for the L-PRF group where peak cell proliferation was seen after 24 hours and steadily decreased over the test period (Figure 4.)

DISCUSSION AND CONCLUSIONS

The results from the current study indicate that both L-PRF and cross-linked HA are biocompatible and induce cellular proliferation of fibroblasts *in vitro*.

L-PRF

It appears as if the effect of L-PRF used in this study is most pronounced in the first 24 hours when compared with any of the other materials tested. This may be due to the high concentration of growth factors present in the material. These include substances such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF-AB), transforming growth factor beta 1 (TGF-β1) and epidermal growth factor (EGF), released from platelets as well as leukocytes.[13-16] This initial "proliferative burst" is similar to that seen by Vahabi et al. who reported a statistically significant increase in cellular proliferation of human gingival fibroblasts (HGFs) after 24 hours.[17] However, unlike the Vahabi et al. study, which showed a reduction of cell viability of up to 60% after 72 hours, the present study showed sustained cellular viability and proliferation that was statistically similar to the control group for all subsequent test days. The difference in observations in the two studies is difficult to explain without a direct comparison between the two biomaterials
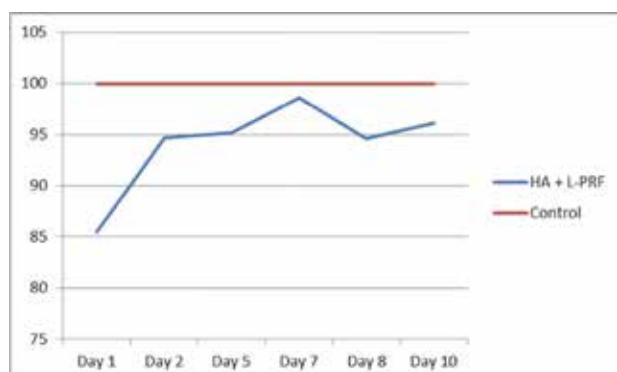

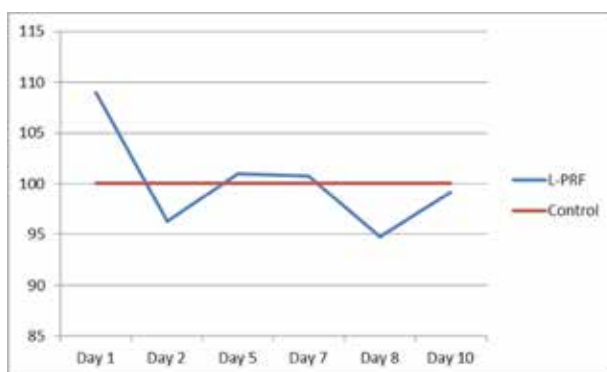**Figure 3:** HA + L-PRF vs Control (MTT assay).

Figure 4: L-PRF vs control (MTT assay).

associated with each investigation. Dohan Ehrenfest et al. showed that no significant differences were seen in cell viability and proliferation when measured using the MTT assay over 21 days for HGFs cultured with L-PRF 13. However, the MTT assay was only reported after day three, thereby possibly failing to record any earlier significant findings. Similar to the Dohan Ehrenfest et al investigation, the present study showed no statistically significant differences when L-PRF was compared to the control group at day 7, and for subsequent days thereafter.13 A recent study investigating the *in vitro* effect of a modified protocol to produce L-PRF, also showed no significant cell proliferation after 24 hours compared with the control group, suggesting that the protocol used to produce L-PRF in the current study may result in a L-PRF with a different biological effect compared with the abovementioned reports.[16] A previous investigation by Peck et al. showed that the L-PRF used in this study has a distinct fibrin architecture with most of the fibres being of a "larger" diameter.18 Whether this characteristic is significant in the biological behaviour of the biomaterial is still unclear.

Cross-linked HA
A limited number of studies have reported on the cell stimulating potential of the specific high molecular weight clHA used in the present study.[6,7] Previous research indicates that this formulation is biocompatible and is able to positively stimulate the proliferation of periodontal ligament cells *in vitro*.[6] These results were statistically significant when the biomaterial was diluted at ratios of 1:100 and 1:10. The authors can provide no conceivable reason why the diluted material evoked a higher proliferation rate than the undiluted material. We speculate, however, that this may possibly be due to the reduced viscosity of the material after dilution, thereby allowing better interaction with the growth medium and cell culture.

In the present study we showed that undiluted clHA resulted in a reduction of cellular proliferation for the first few days after cell culture. This was similar to what was observed by Fujioko- Kubayashee et al and colleagues. At day 5, cellular proliferation approximated that of the control group, and, by day 8, was significantly higher than that of the control group (p < 0.05). Therefore an augmented proliferative effect was noticed for the present study. Although HA has well-established water retention properties, the exact mechanism of HA and fibroblast interaction is unclear with "fibroblast stretching" cited as the most likely cause.19 In a recent study evaluating the effect of both clHA and non-cross linked HA on dermal fibroblasts, it was shown that the molecular structure, particularly the type and density of the cross-linkages, play a significant role in the ability of HA to stimulate fibroblast proliferation.19 Any biological effect of synthesized HA is product dependent and the results of each study should therefore be interpreted with this in mind. This might also explain the variations in clinical efficacy of dental related HA that are reported in the literature.

Hyaluronic Acid and L-PRF
Because HA has previously been used for tissue engineering and is commercially available for the management of several inflammatory-related conditions, the authors evaluated the cell viability and proliferative potential of a combination of clHA and L-PRF on fibroblasts. As L-PRF has an inherently high concentration of growth factors related to wound healing, it was assumed that the combination of the two biomaterials would stimulate cellular proliferation. The results from the current study indicate that the combined formulation was biocompatible, but had a varied effect on cell proliferation. Similar to clHA, the clHA+ L-PRF mixture showed a significant reduction in cell proliferation after 24 hours. Proliferation then increased over time, and on day 7, it peaked, showing results similar to those of the control group (p > 0.05). This proliferation was opposite to that seen for the L-PRF group. It is interesting to note that the clHA+L-PRF combination showed consistently lower cell proliferation when compared with the control group throughout the time period of the study. Although L-PRF on its own stimulated cell proliferation significantly after 24 hours, it seems the addition of clHA constrained this initial stimulatory effect. Whether this is related to the viscosity of the clHA or its concentration is critical to debate. It is known that diluted forms of clHA seem to better stimulate cell growth, and this might therefore explain the results seen in the present study 6.

CONCLUSION
L-PRF and clHA are unique biomaterials that are being used in tissue engineering. In the present study, we show that these materials on their own, or in combination, are biocompatible and stimulate cell growth. Interestingly, it appears that this specific method of L-PRF preparation results in a material that has a stimulatory effect that peaks within the first 24 hours. Cross-linked HA also stimulates growth positively, but unlike L-PRF this effect is prolonged over 8 days, implying a different, as yet unknown, mechanism of action. The combination of L-PRF and clHA seems to provide no further advantage to cellular growth when compared with using either of the materials on their own. Based on the above, we can speculate that although L-PRF results in an almost immediate stimulatory effect, that clHA, as a sole treatment choice, might be beneficial in the management of wounds that require more prolonged stimulation. Further research is required to determine the clinical implications of the above findings.

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Antibiotic release from leukocyte- and platelet-rich fibrin (L-PRF) – an observational study.

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ABSTRACT

Introduction

Leukocyte- and platelet-rich fibrin (L-PRF), an autologous derived platelet and leukocyte concentrate, was first introduced by Choukroun et al. in 2001 and is currently used in a wide range of medical procedures. Although various biological properties have been attributed to L-PRF, nevertheless, when tested for inherent antimicrobial activity, the biomaterial fails to demonstrate a clear and significant effect against a range of oral microbiota. Aims and objectives: To determine whether L-PRF prepared after a single oral dose of antibiotic had any significant antimicrobial effect over a 48 hour period. Methods: An in vitro laboratory study for which L-PRF was prepared from a single healthy volunteer who had previously ingested oral antibiotics. The resultant prepared L-PRF was tested for antimicrobial activity against Streptococcus mutans (ATCC 35668) using standard laboratory methods. Results: For all samples tested, measurable zones of inhibition were clearly visible after 24 hours, but were absent after 48 hours. Conclusions: L-PRF prepared after a single dose of oral antibiotic results in a measurable antimicrobial effect that is sustainable for 24 hours. Although L-PRF will remain structurally intact for a few days, it does not appear to influence the duration of the antimicrobial activity.

Introduction

Leukocyte- and platelet-rich fibrin (L-PRF), an autologous blood-derived biomaterial, was first introduced by Choukroun et al. in 2001 as a simple method of introducing autogenous growth factors to a wound site.[1] Since then, L-PRF has been utilized for a number of medical procedures, including the management of diabetic wounds, soft and hard tissue augmentation and dermatological lesions.[2-6] L-PRF is prepared using a single spin protocol that results in an easily manipulated biomaterial that can be applied directly to the site of surgery.[1] The structure of this biomatrix, which consists of a platelet and leukocyte concentrate interwoven within a fibrin mesh, is claimed to be one of the major factors that contribute to its clinical success. Previous studies indicate that the unique characteristic of fibrin make it an ideal drug delivery system that allows the distribution of an active

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ACRONYMS

L-PRF : Leukocyte- and platelet-rich fibrin

agent directly to the site required.[6] The fact that fibrin undergoes fibrinolysis over a period of time, has the potential of prolonging the drug release and may therefore influence the clinical outcome. Previous studies have combined antibiotics with fibrin (mostly fibrin sealants) for the management of various conditions such as osteomyelitis, endocarditis, and other "difficult to treat" infections.[7-11]

Although various biological properties have been attributed to L-PRF, nevertheless, when tested for inherent antimicrobial activity, the biomaterial failed to demonstrate a clear and significant antimicrobial effect against a range of oral microbiota.[12] We hypothesize that incorporating antibiotics into L-PRF may enhance its antimicrobial profile and that the structure of the L-PRF would allow for a prolonged release of the drug. The aim of this in-vitro pilot study was to determine whether L-PRF prepared after a single oral dose of antibiotic had any significant antimicrobial effect over a 48 hour period.

MATERIALS AND METHODS

The study was conducted at The University of the Western Cape, Cape Town, South Africa, in 2017. Ethical clearance was obtained from the University Research Ethics Committee (reference number: BM 16/3/31). Informed consent was obtained from the blood donor.

Preparation of the L-PRF

Thirty six milliliters of blood were obtained from a 24 year old healthy female volunteer who had undergone dental implant surgery. She had ingested a single dose of antibiotics, as surgical prophylaxis (Amoxicillin, 2 g orally), one hour prior to the surgical procedure. One hour after antibiotic ingestion, blood samples were collected in blood collecting tubes that contained clot activator, i.e., Vacuette® 9ml serum tubes with Z Serum Clot Activator (Greiner BioOne International AG, Germany). These were then immediately centrifuged at 400 x g for 12 minutes in a standard benchtop centrifuge (PLC-03, HiCare International, Taiwan) as previously described.[12] Some of the resultant L-PRF clots were then used during the surgical procedure, whilst the remaining clots were used for the current study. These L-PRF clots were then compressed using the PRF Box ™ (Process for PRF, Nice, France) to obtain uniform thickness of the sample specimens, two of which were selected and designated as Sample and Sample Two respectively.

Microbial Culture

The Oral and Dental Research Laboratory (Faculty of Dentistry, University of the Western Cape) sponsored samples of Streptococcus mutans (ATCC 35668, Quantum Biotechnologies, South Africa) for the study. The authenticity of the bacteria was confirmed via Gram stain, growth morphology on blood agar and API STREP 20 system (bioMerieux). Cultures were grown for 24 hours at 37°C. Two colonies were adjusted to 0.5 McFarland's standard (DensiCHEK Plus, bioMerieux) in sterile physiological saline. One millitre (1 ml) of the adjusted colonies was then spread plated on CASO agar (Merk Life Science GmbH, Germany).

Antimicrobial activity

The samples of prepared L-PRF were of comparable size and thickness due to the standardized protocol used in preparation. Each sample was placed in the centre of a bacteria-streaked agar plate and then incubated for 37°C in a standard laboratory incubation chamber for 24 hours. At that stage the plates were removed and the clear zones surrounding each L-PRF sample (zones of microbial inhibition), were repeatedly measured using a digital vernier caliper (Mastercraft, South Africa). In order to test the antimicrobial efficacy over the subsequent 24 hours, the samples were removed and placed in a fresh bacteria-impregnated agar plate. The plate was incubated for 24 hours as before and the inhibition zones again measured.

DATA ANALYSIS

Data from the test samples were captured using Microsoft Excel 2010 (Microsoft Corporation, Washington, USA) and statistically analyzed using one-way ANOVA and Tukey's test.

Table 1: Zones of inhibition measured in millimeters

24 Hours (Sample 1)	24 Hours (Sample 2)	48 Hours (Sample 1)	48 Hours (Sample 1)
33.97	29.41	0	0
41.57	42.70	0	0
32.20	31.22	0	0
29.53	44.03	0	0
32.90	31.99	0	0
32.91	47.15	0	0
39.65	47.06	0	0
40.75	31.82	0	0
35.11	42.82	0	0
41.33	31.60	0	0

RESULTS

The 24 and 48 hour results were recorded as the observable zones of inhibition in millimeters and are represented in Table 1. Zones of inhibition were noted for both L-PRF samples after 24 hours. According to statistical analysis using one-way ANOVA and Tukey HSD Test, no significant differences were noted for the sizes of inhibition zones when the two samples were compared after 24 and 48 hours, i.e., similar results were recorded at both 24 and 48 hours. No measurable inhibition zones were seen for either sample after 48 hours (Table 2), indicating that all antimicrobial activity had been lost at that time. A statistically significant difference was seen in the data between the two time points.

DISCUSSION AND CONCLUSIONS

Repeated systemic use of antibiotics to treat or minimize localized infection has its limitations, especially in areas with constrained blood supply. Furthermore, localized drug delivery systems often require specialized carriers to allow for the delivery of therapeutically sustainable doses of the active agent. In the present study we attempted to determine whether using an antibiotic- laden autologous blood-derived concentrate had any significant antimicrobial effect over a period of time. The results

Table 2: Tukey HSD results

Samples	Tukey Q stat	Tukey p-value	Tukey inference
S1 (24hrs) vs S2 (24hrs)	1.4702	0.7058178	insignificant
S1 (24hrs) vs S1 (48hrs)	26.6181	0.0010053	p<0.01
S1 (24hrs) vs S2 (48hrs)	26.6181	0.0010053	p<0.01
S2 (24hrs) vs S1 (48hrs)	28.0883	0.0010053	p<0.01
S2 (24hrs) vs S2 (48hrs)	28.0883	0.0010053	p<0.01
S1 (48hrs) vs s2 (48hrs)	0	0.8999947	insignificant

from the current study indicates that L-PRF prepared after a single dose of antibiotics provides measurable antimicrobial activity for at least 24 hours against specific oral microbiota. After 48 hours, the antimicrobial effect is markedly reduced, with no statistically relevant antimicrobial effect seen. This may suggest that although L-PRF has a unique structure, its ability to concentrate antibiotics and release them over time, may be limited. This has been seen in previous studies in which fibrin sealants directly supplemented with antibiotics showed a rapid antibiotic release over a short period of time (85% over 72 hours).[14-16] Woolverton et al. (2001) attributed this to rapid diffusion as a result of the antimicrobial molecules being small, ionic, and designed for oral and parenteral delivery.[6] However, antibiotics having a less soluble nature, unlike the current study, have been shown to exhibit a much longer sustained release from fibrin sealants.[17] Another reason for the rapid diffusion of antibiotics from L-PRF may be the limited binding capacity of the antibiotic used in the present study. Amoxicillin, when orally ingested, is 20% protein bound in the blood and about 60% of the drug is excreted in the urine within 6-8 hours.[18] The protein binding is mostly to albumin (the most abundant plasma protein) which has a specific binding site but a relatively low affinity for the antibiotic.[18] The drug has no known affinity to fibrin and this may explain the limited antimicrobial effect observed in the present study. It is therefore assumed that the drug was concentrated in the plasma component of the L-PRF and not directly bound to the fibrin or cells associated with the L-PRF matrix.

Unlike previous studies that have directly combined the antibiotic with the fibrin matrix, we attempted to incorporate antibiotics that were already in the blood (at peak plasma concentration) during the process of preparing the L-PRF matrix. This has the advantage of not requiring any additional materials or steps in incorporating the antibiotic into the L-PRF. Interestingly, certain clinical studies involving the use of L-PRF for dental surgical procedures have made use of oral antibiotic surgical prophylaxis prior to preparing L-PRF.[19] In these studies, no mention was made of the influence of antibiotic ingestion in the healing outcomes of the site treated with the L-PRF. The present study indicates that antibiotics are presumably incorporated into L-PRF after a single oral dose and may be active for at least 24 hours. We can therefore presume that the clinical studies that have administered antibiotics prior to surgery, have indeed incorporated antibiotics into the L-PRF, if the L-PRF was prepared at least one hour after antibiotic prophylaxis. A further investigation into the influence of pre-operative antibiotics as a factor influencing the clinical behaviour of L-PRF is therefore required.

CONCLUSION

L-PRF has restricted intrinsic antimicrobial activity. Within the limitations of the present study, we have shown that L-PRF prepared after a single dose of oral antibiotic, results in a measurable antimicriobial effect that is sustainable for 24 hours. Although L-PRF will remain structurally intact for a few days, this does not appear to influence the duration of the antimicrobial

activity. We therefore assume that the antibiotic is mostly concentrated in the plasma and is not directly bound to the structural components of the L-PRF matrix. Further research is required to determine the significance of these preliminary findings.

Conflict of Interest.
The authors declare no conflict of interest regarding the publication of this paper. This paper forms part of the requirements for partial fulfillment of the degree PhD.

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The use of leukocyte- and platelet-rich fibrin (L-PRF) to facilitate implant placement in bone-deficient sites: a report of two cases

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ABSTRACT

Successful dental implant treatment usually requires that the implant be placed in the ideal anatomic position, so that it will readily facilitate the placement of a functional and aesthetically acceptable restoration. However, this is not always possible, and in many cases augmentation procedures may be required to compensate for lost tissue structures. These interventions often require more complex surgery, as well as the use of graft material derived from animal sources. Leukocyte- and platelet-rich fibrin (L-PRF) is a newly developed platelet concentrate that has successfully been used in a number of surgical procedures to optimise wound healing. Several studies indicate that it may also have the ability to stimulate bone formation. In this article we present two cases where L-PRF was used to stimulate bone formation to facilitate ideal placement of implants.

Keywords: Leukocyte- and platelet-rich fibrin (L-PRF), bone augmentation, osseous regeneration

INTRODUCTION

The use of implant-supported restorations to replace missing teeth has become widely accepted as a viable treatment option in modern restorative dentistry. However, once teeth are lost due to pathology or trauma, the remaining alveolar bone anatomy is often compromised and there may be insufficient bone volume to allow placement of an implant

Figure 1: Pre-operative orthopantomograph showing the amount of bone available at the proposed implant sites in first quadrant (14, 15 area).

that would be functional. Frequently, aesthetics are also compromised. Several techniques have been developed to overcome this problem, including one or more combinations of guided bone regeneration, osteoconduction, osteo-induction, distraction osteogenesis, and revascularised bone grafts.[1] In this article we present two cases where a novel biomaterial, "leukocyte- and platelet rich fibrin (L-PRF)",[2] was used with the intention to enhance osseous regeneration and to enable the placement of dental implants in previously deficient bony sites.[3]

CASE 1

A 45-year-old female patient presented with complaints of "difficulty when chewing" on her right side. Both upper-right premolars had been extracted by her dentist due to failed en-
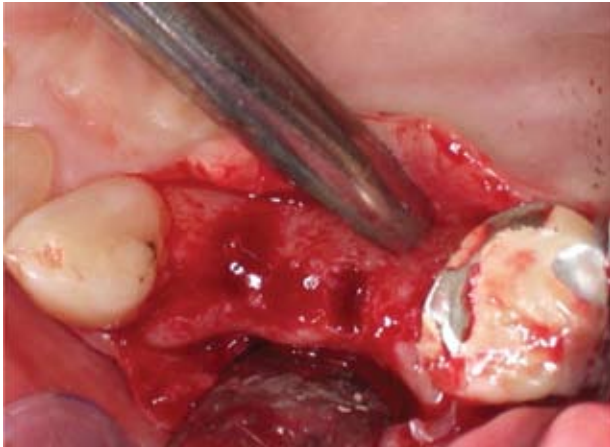
Figure 2: Osteotomy site preparation for the placement of two implants in 14, 15 area.

Figure 4: Immediate post-operative radiograph showing the distal implant in the maxillary sinus surrounded by L-PRF.

dodontic treatment and since then she had not been able to adapt to a removable partial denture. She therefore requested a "fixed" restorative option. Her medical history was unremarkable and she had no history of smoking or evidence of periodontal disease. It was decided that two implant-supported crowns in the region of teeth 14 and 15 would offer her the most stable, long-term restorative solution.

The clinical examination indicated a buccal bony defect in the area of 14 and 15, but the affected area was otherwise healthy and free from signs of pathology.

The pre-operative radiographic examination revealed insufficient bone height secondary to maxillary sinus pneumatisation in the area of extracted tooth 15, with only 2-3mm of vertical bone remaining (Figure 1). A sinus augmentation procedure was indicated to increase the bone available so there may be adequate support for the length of the intended implant.

Figure 3: Leukocyte- and platelet-rich fibrin (L-PRF) clot obtained from patient's blood.

The two implant sites were prepared simultaneously, as follows:

Surgical management of site 14 (upper right first premolar)
A midcrestal incision was made and a full-thickness mucoperiosteal flap was raised. No vertical releasing incisions were made. The bone in site 14 was judged to be sufficient for implant placement without any additional augmentation procedures, and therefore was prepared according to the standard protocol. During the procedure, intra-oral radiographs were repeatedly taken to confirm the correct depth and direction of the osteotomy site. Once the final width and depth had been reached, a 3.7mm by 10mm dental implant (Uniti®, Equinox, Holland) was inserted into the prepared site and torqued to 35Ncm.

Surgical management of site 15 (upper right second premolar)
As the residual vertical bone was less than 4mm in the 15 area, a 2mm pilot drill was used to prepare the osteotomy site to within 1mm of the sinus floor (Figure 2). A 3mm drill was then used to the same depth so as to allow for the insertion of osteotomes. Sixty millilitres of blood was then drawn from the antecubital vein of the patient and collected into six 10ml anti-coagulant-free blood collecting tubes (Vacuette® with Z Serum Clot Activator, Greiner Bio One International AG, Germany). These were then centrifuged at 400g for 12 minutes, using a standard laboratory table-top centrifuge (PLC-03®, Hi-care International, Taiwan). Immediately after being centrifuged, one L-PRF clot was separated from the underlying red blood cell layer and introduced into the 15 osteotomy site (Figure 3). The floor of the sinus was in-fractured using a 3mm osteotome (Southern Implants®, Irene, South Africa), and the lining was raised. Once the integrity of the sinus membrane was confirmed using the Valsalva manoeuvre, four more L-PRF clots were then introduced into the sinus with the osteotome to elevate the floor to the desired height. A 3.7mm by 13mm implant (Uniti®, Equinox, Holland) was then inserted, with the previously introduced L-PRF clots acting as a "protective layer" for the sinus lining.

After primary stability had been reached, titanium cover screws were placed on both implants and the remaining L-PRF was then applied to the lateral aspect of the alveolar ridge. The site was closed using 4-0 synthetic absorbable braided sutures (Clinisut®, Port Elizabeth, South Africa). An immediate post-operative radiograph was then taken that depicted the 10mm rise in sinus membrane height (Figure 4).

Post-operative healing was uneventful, and after two weeks the sutures were removed. The patient returned after six months having encountered no complications associated

Figure 5: Although taken at a slightly different angle, this six month post-operative radiograph reveals increased radio-opacity indicating the presence of new bone extending to the tip of the implant.

Figure 6: Cement-retained implant-supported PFM crowns (14, 15) in place.

with the initial surgery. At this time, the implants were surgically exposed and the healing abutments were placed. No movement or pain was associated with implant 15 and the radiographic examination revealed evidence of new bone formation extending to the tip of the implant (Figure 5). The implants were subsequently successfully restored with two cement-retained splinted porcelain-fused-to-metal (PFM) crowns (Figure 6).

CASE 2

A 48-year-old healthy male presented with complaints of a pain on the left side of his mouth that was associated with an unrestored dental implant (Mistral®, MIS implants, Tel Aviv, Israel) placed three months previously (Figure 7). Clinically, the patient experienced pain on percussion at the implant in the 27 area. Further clinical investigation revealed that the implant was mobile and had failed to osseo-integrate. It was decided to remove the failing implant and to subsequently

replace it with another, once adequate healing had taken place. Taking into account the previous history of implant failure together with the risk of potential bone resorption, it was decided that an alveolar ridge preservation (ARP) procedure was to be carried out immediately after removal of the failed implant at the same operative procedure.

Surgical procedure for simultaneous implant removal and ARP procedure

Under local anaesthesia, the implant in the area of 27 was removed and the resulting bony socket thoroughly curetted to remove all granulation tissue and fibrous debris. Blood was then drawn from the vein of the antecubital fossa and L-PRF prepared in a similar manner as described above. Three (3) L-PRF clots were then inserted into the 27 socket site and secured in place using 4-0 synthetic absorbable braided sutures (Clinisut®, Port Elizabeth, South Africa).

Implant placement

Healing was uneventful and three months after initial introduction of the L-PRF clot into the failed implant site, a follow-up radiograph revealed substantial new bone formation that was more radio-opaque compared with the immediate adjacent bone (Figure 8). The patient was then scheduled for implant surgery one week later to replace the initial failed implant. At surgical exposure of the 27 area, substantial new bone formation was evident with only minimal signs of the failed implant site still visible (Figure 9). A 4.2mm by 10mm dental implant (Screwvent®, Zimmer, California, USA) was then placed into the area without any complications being noted.

Figure 8: Radiograph indicating increased opacity at the failed implant site 3 months after the implant removal and placement of L-PRF clot in the area.

Figure 7: Initial presentation of patient with a painful, failed implant in 27 area.

Figure 9: Presence of new bone formation as seen clinically at the time of implant placement in 27 area.

CASE REPORT

DISCUSSION

Leukocyte- and platelet-rich fibrin (L-PRF), a second generation platelet-rich-plasma (PRP) concentrate, was first described by Choukroun in the early part of the 21st century.[2] It is considered a biomaterial that actively promotes wound healing in both hard and soft tissues.[3] It is prepared from the patient's own blood and requires no additional additives or specialised equipment for its preparation.[4-8] Blood is drawn from the patient directly into glass or silica coated plastic blood-collecting tubes and centrifuged at low speed to separate the platelets from the red blood cells (RBCs).[6] The absence of any anticoagulant during the centrifuge process allows for the unimpeded activation of platelets, thus triggering the coagulation cascade.[5] This results in the formation of a fibrin clot that contains a high concentration of leukocytes and platelets.[9] This L-PRF clot can then be moulded into a usable "membrane" that acts as a simple method of transferring high concentrations of platelets to any wound site. A three dimensional analysis of the L-PRF membrane indicates that it has a unique structure that traps platelets, leukocytes and cytokines in a mesh of fibrin strands. This imparts structural integrity to the biomaterial and allows it to be manipulated for use in variety of clinical procedures.[3]

The L-PRF membrane traps at least 95% of the platelets of the collected blood, allowing for the natural release of several growth factors (GFs) to the surgical area.[3,10,11] These GFs are released from the platelet granules, and include 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), platelet-derived-growth factor (PDGF) 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.[4,12] PDGF 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 also induces osteoconduction and promotes the production of hyaluronic acid, an essential component for wound contraction and remodelling. Although similar GFs may be released by platelet-rich plasma (PRP), the unique structure of L-PRF allows it to release these factors over a longer period of time, potentially optimising wound healing.[12,13] The presence of leukocytes (absent from PRP) also contributes to the release of TGF-beta and VEGF, further increasing the concentration of available GFs.[3,13]

L-PFR has been exploited in various clinical procedures including maxillofacial surgery, periodontal surgery, implantology and plastic surgery.[14,15] In a clinical study of maxillary sinus lift procedures in which L-PRF clots and L-PRF membranes were used as the only augmentation material, Mazor et al showed that L-PRF was capable of stimulating complete bone regeneration in the areas that initially had inadequate vertical bone height.[8] In a similar six-year follow-up study, Simonpeiri et al were able to demonstrate that using L-PRF as a sole grafting agent was a viable long-term option in sinus augmentation procedures.[16]

Case 1 demonstrated the use of L-PRF in osteotome mediated sinus augmentation procedures. By applying L-PRF to the osteotomy site, before fracturing the sinus floor, the biomaterial acts as a "protective cushion" that facilitates the safe upward displacement of the sinus membrane.[9] Studies indicate that using L-PRF in osteotome sinus floor elevation techniques, together with immediate implant placement, reduces the healing time between surgical placement and prosthetic restoration of the implants.[9]

L-PRF has been used to treat periodontal hard and soft tissue defects.[14,15] In vitro studies have confirmed that L-PRF selectively stimulates the growth of osteoblasts and gingival cells.[17] In a series of clinical trials conducted by Pradeep et al, it was shown that L-PRF could be used as a guided-tissue-regeneration (GTR) membrane to effect periodontal regeneration in three-wall bony defects and degree II furcation lesions.[14,15] Del Corso et al published several case reports showing the successful use of L-PRF membranes in the management of both single and multiple gingival recession defects.[18] Using multiple L-PRF membranes instead of connective tissue grafts, it was shown that the lost soft tissue could effectively be restored by layering the L-PRF membranes over each other, thereby increasing gingival height and thickness. The clinical results were maintained for at least one year.[18] Ramakrishnan et al confirmed this observation and showed that L-PRF could be used for root coverage procedures.[5]

L-PRF vs PRP (Where is the evidence?)

Since the initial development of PRP in the late 1990's, the platelet concentrate has undergone significant research, especially in the field of oral and maxillofacial surgery.[19] Although widely used in a variety of clinical fields, the ability of PRP to enhance wound healing has recently been questioned.[20] 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 with controls and suggested that no substantial evidence existed for its use in other dental procedures.[19] The heterogeneity in studies that have examined PRP is regarded as a major reason for the lack of conclusive evidence supporting its use.

L-PRF is a new form of platelet concentrate which has features that are distinctly unique when compared with PRP and therefore cannot be regarded as the same biomaterial.[4,6,10] However, as with PRP, there is a lack of long-term controlled trials that would endorse the use of L-PRF in routine surgical procedures. Recently, though, five-year follow-up reports have been published which demonstrate the clinical safety and efficacy of using this new biomaterial. Further reports are awaited.

CONCLUSIONS

In the cases presented above, the clinical application of L-PRF in bone regeneration procedures was demonstrated. These potential effects are directly related to the biological properties of the material, which allow it to act as a "membrane-laden leukocyte- and platelet-rich concentrate". Its preparation is inexpensive, requires no specialised equipment or the addition of animal derived products, and it could promise a new era in the field of wound healing. Further research is required to fully explore the broad potential of this biomaterial.

Declaration: No conflict of interest.

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Case Report

Alveolar Ridge Preservation Using Leukocyte and Platelet-Rich Fibrin: A Report of a Case

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In order for a dental implant to be restored optimally, it must be placed in an ideal anatomic position. However, this is not always possible, since physiological wound healing after tooth removal, often results in hard and soft tissue changes which ultimately compromises ideal implant placement. With the aim of minimising the need for tissue augmentation, several alveolar ridge preservation (ARP) techniques have been developed. These often require the use of grafting material and therefore increase the risk of disease transmission. Leukocyte and platelet-rich fibrin (L-PRF) is a newly developed platelet concentrate that is prepared from the patient's own blood. Clinical research has indicated that it improves wound healing and stimulates bone formation. We present a case where L-PRF was successfully used in an ARP procedure to facilitate implant placement in a compromised extraction socket.

1. Introduction

In order for a dental implant to be restored optimally, it must be placed in an ideal anatomic position. However, this is not always possible, since physiological wound healing following either tooth extraction, trauma, or pathology, often results in a deficiency of both hard and soft tissue. Unless augmentation procedures are carried out, placing an implant in these tissue-deficient sites would ultimately compromise the functional and aesthetic results [1]. Although several different augmentation procedures have been developed, many of them are associated with a number of disadvantages such as increased overall cost, the requirement for a second surgical site, and the use of animal derived products [2].

With the aim of minimizing the need for tissue augmentation, several authors have proposed techniques to preserve the anatomy of the alveolar ridge after tooth extraction. These procedures have collectively been termed alveolar ridge preservation (ARP) or socket preservation [2]. Several different ARP techniques exist, most of which include the use of a foreign graft materials. Because ARP is a relatively new procedure, no long-term studies regarding the technique have been published, and even though several case reports have been presented, there is no evidence to support the superiority of one technique over the other.

Recently, Choukroun introduced leukocyte and platelet-rich fibrin (L-PRF), a second-generation platelet concentrate that improves healing of the both hard and soft tissues [3]. We present a case where L-PRF was used in an ARP procedure to limit ridge resorption after tooth extraction, in order to maximise the tissue available for ideal implant placement.

2. Case Presentation

A 43-year-old healthy female presented for the restoration of her dentition in the upper right jaw. Upon clinical examination, it was noted that several posterior teeth were missing from the first quadrant and that the only remaining multirooted tooth (the upper right first molar) was severely periodontally compromised (Figure 1). The radiographic examination revealed the presence of unextracted roots in the areas immediately mesial and distal to the remaining molar (Figure 2). Based on the poor prognosis of the molar as well as the presence of the unextracted roots,
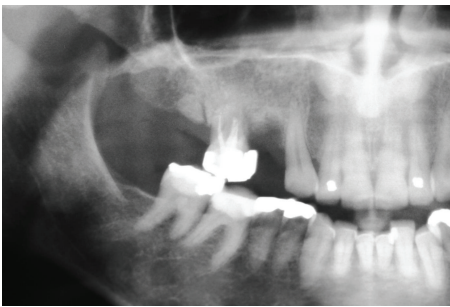
FIGURE 1: Initial presentation.

FIGURE 3: Extraction site immediately after tooth removal.

FIGURE 2: Radiograph shows hopeless upper molar with retained roots both mesial and distal to the tooth.

FIGURE 4: Formation of L-PRF.

a treatment plan that involved the extraction of the remaining tooth and roots, and subsequently replacing them with an implant-supported prosthesis, was deemed the best long-term restorative solution. In order to maximise the amount of available bone for implant placement, an ARP procedure was indicated at the time of extraction.

3. Initial Visit

After local anaesthesia had been obtained, the upper right molar together with the residual tooth roots were extracted atraumatically by using a 5 mm dental luxator (Dentsply Ltd., Surrey, United Kingdom). The remaining tooth sockets were curetted and all granulation tissue and socket debris were removed (Figure 3). At the same time, 30 mL of blood was drawn from the antecubital fossa of the patient into three separate blood collecting tubes (Vacuette with Z Serum Clot Activator, Greiner Bio One International AG, Germany). These were then immediately centrifuged at 400 g for 12 minutes, using a standard tabletop laboratory centrifuge (PLC-03, Hi-care International, Taiwan). Using this method, the blood in the tubes separated into three visible layers, that is, a red blood cell layer (RBC) that occupied the lower most part of the tube, a cell-free layer that occupied the uppermost part of the tube, and an L-PRF layer that was located between the two (Figure 4). For each tube, the L-PRF layer was removed, and compressed between saline soaked sterile gauze to form an "L-PRF membrane" (Figure 5). A total of three L-PRF membranes were formed and inserted into the
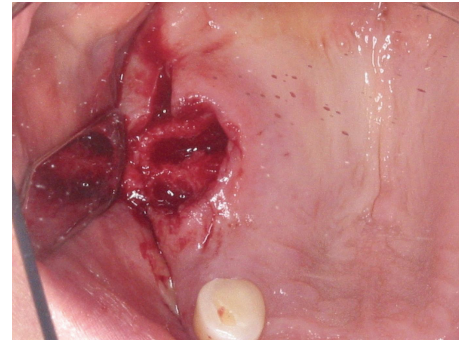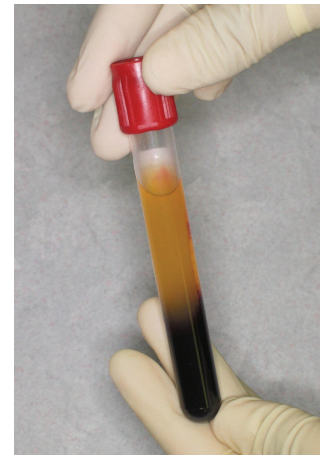
extraction socket site. These were then stabilised using 4–0 braided resorbable sutures (Clinisut, Port Elizabeth, South Africa) that were sutured over the wound site. Oral analgesics and a chlorhexidine 0.2% mouth rinse was prescribed during the healing period, and the patient was followed up two weeks later.

4. Follow-Up Visit

On the follow-up visit, the extraction site showed signs of healing with no evidence of residual inflammation. The site was free of infection and the L-PRF membrane was still clearly visible (Figure 6). Even though it remained exposed to the oral environment, there were no signs of membrane disintegration or infection. The patient also reported minimal pain during the postoperative period. Because of the positive response to treatment, she was scheduled for implant placement four weeks later.

5. Implant Placement

Implant placement was carried out 6 weeks after tooth extraction. A radiograph taken prior to implant placement confirmed new bone formation in the extraction area (Figure 7). Upon surgical flap reflection, the underlying
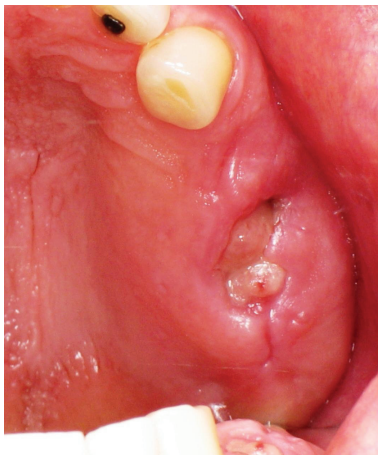
FIGURE 5: L-PRF membrane.

FIGURE 6: Extraction site healing 1 week after tooth removal (note the visibility of the L-PRF membrane).
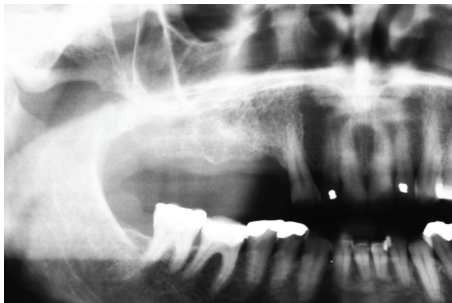

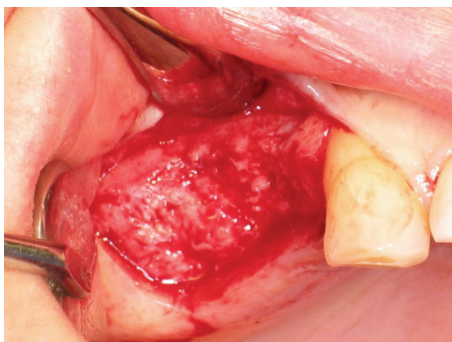FIGURE 7: Radiograph showing new bone formation.
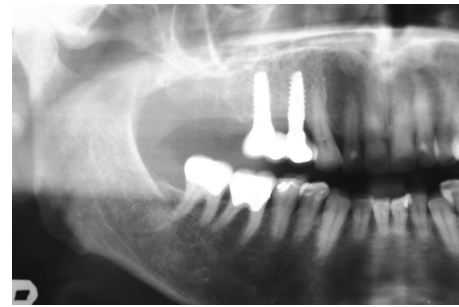

FIGURE 8: Flap reflection.

FIGURE 9: Radiograph showing stable peri-implant bone 3 months after restoration.

alveolar ridge was clearly visible. The ridge had retained its morphology with no signs of bone resorption or of the residual socket (Figure 8). At implant insertion, the quality of the newly formed bone was such that it allowed for the implant to be inserted at an insertion torque of more than 35 Ncm.

6. Prosthetic Management

Eight weeks after implant placement, the implant was restored with a cement retained crown and has since then remained in function without any complications.

At the 3 month follow-up after the restorative treatment had been completed, radiographic evidence of bone maturation was present at the peri-implant sites (Figure 9).

7. Discussion

The healing of an extraction socket is characterised by both internal and external changes that ultimately affect the shape of the alveolar ridge [2]. Studies indicate that during healing, bone does not regenerate to the level of bone crest or to the level of the neighbouring teeth, and therefore 100% socket fill does not occur. Using an animal model, Araujo and Lindhe showed that in the first 8 weeks following extraction, there is marked osteoclastic activity, resulting in the resorption of the facial and lingual bone walls, especially in the crestal region [4]. They also noted that bone resorption was greater on the facial wall and that any loss of ridge height was accompanied by a horizontal losson both facial and lingual walls of the extraction site.

Alveolar ridge preservation is a relatively new surgical procedure aimed at retaining maximum bone and soft tissue after a tooth has been removed [2]. By maintaining the original ridge morphology, there will be a minimal need for augmentation procedures thereby allowing the resultant restoration to be placed in an aesthetically and functionally ideal position.

During the last decade several different ARP techniques have been developed, most of which include the use of a graft material that is placed into the extraction socket [2]. This increases the treatment cost as well as increases the risk of disease transmission. Studies also indicate that in many cases, the graft material is not totally incorporated into the

newly formed bone and when compared to sites without graft material, they show less vital bone formation. In some cases ARP requires the use of collagen membranes. In these cases a 25% membrane exposure rate has been reported, and this directly affects the amount of bone fill that takes place within the socket [2].

Leukocyte and platelet-rich fibrin (L-PRF) was first described by Choukroun as cited by Dohan et al. 2006 [5]. It is considered a second-generation platelet concentrate and has been used in various surgical procedures in an attempt to enhance wound healing. It is prepared from the patient's own blood thereby eliminating the possibility of disease transmission or foreign body reactions.

The preparation technique of L-PRF is simple and requires no special equipment. Blood is drawn into standard glass/silica coated blood collection tubes and centrifuged at a predetermined speed to ensure cell separation. No anticoagulants are used during the procedure and natural coagulation can therefore take place. This unique preparation technique allows L-PRF to trap at least 95% of the platelets of the collected blood into a fibrin mesh [6]. The fibrin mesh can then be easily manipulated into a membrane that allows it to be transferred to any surgical site. Here, high concentrations of the collected platelets allow for the slow release of growth factors (GFs) from the platelet granules [7]. These GFs include 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), platelet-derived growth factor (PDGF), and transforming-growth factor-beta (TGF-beta). All of these play a role in replacing lost tissue, resurfacing of the wound, and restoring vascular integrity. Compared to other platelet concentrates, L-PRF releases these factors at a sustained rate over a longer period, thereby optimising wound healing [8]. Recently, L-PRF has also been shown to stimulate the growth of osteoblasts and periodontal ligament cells, both of which are significant for the regeneration of periodontal defects [6, 8–11].

Because of the *in vitro* efficacy of L-PRF, several clinical studies have been carried out to determine its clinical potential. Currently, L-PRF has been successfully tested in a number of procedures including maxillofacial surgery, periodontal surgery, and implantology [9]. Mazor et al. successfully used L-PRF as the only grafting material in a series of sinus augmentation procedures [10]. With this technique Mazor et al. were able to demonstrate that L-PRF could stimulate new bone formation in areas that were previously deficient of the amount of bone required for implant placement [10]. In a similar 6-year follow-up study, Simonpeiri et al. were able to demonstrate that using L-PRF as a sole grafting agent was a viable long-term option in sinus augmentation procedures [11].

L-PRF has also been used successfully to treat periodontal defects. *In vitro* studies have confirmed that L-PRF selectively stimulates the growth of osteoblasts and gingival cells [12]. In a series of clinical trials conducted by Pradeep and Sharma it was shown that L-PRF could be used as a guided-tissue-regeneration (GTR) membrane to affect periodontal regeneration in 3-wall bony defects and degree II furcation lesions [9, 13]. Del Corso et al. published several case reports showing the successful use of L-PRF membranes in the management of both single and multiple gingival recession defects [14]. The clinical results were maintained successfully for at least one year. Ramakrishnan et al. confirmed this observation and showed that L-PRF could be used for root coverage procedures [15].

8. Conclusion

In the above case report, we demonstrated the successful use of L-PRF in an ARP procedure. The biomaterial acts by releasing high-concentration growth factors to the wound site, thereby stimulating healing and new bone formation [16]. Unlike other ARP procedures, the use of L-PRF is a simple method that requires minimal cost and reduces the need for specialised grafting material. Because it is a completely autologous product, the risk of disease transmission and graft rejection is negated. Further long-term research is required to determine whether ARP procedures would benefit from the use of L-PRF.

Disclosure

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Request to include journal article as part of PhD Thesis

3 messages

Mogammad Peck <mpeck@uwc.ac.za>   Mon, Sep 3, 2018 at 11:40 AM
To: editorial@hindawi.com

To whom it may concern

I am busy completing a PhD by Publication at The University of the Western Cape (student number 9552379). I would humbly request that permission be granted for me to include the following article in dissertation.

"Peck MT, Marnewick J, Stephen L. Alveolar ridge preservation using leukocyte and platelet-rich fibrin: a report of a case. *Case Rep Dent*. 2011;2011:345048"

Thank you

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Hindawi <editorial@hindawi.com>   Tue, Sep 4, 2018 at 2:10 PM
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Best regards,

Amany

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Thank you for your prompt response

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Permission to include journal articles in dissertation

2 messages

Mogammad Peck <mpeck@uwc.ac.za>   Fri, Aug 24, 2018 at 10:43 AM
To: Bill Evans <bill.evans@wits.ac.za>

Dear Bill

Thank you for all the support recently with regards to the number of manuscripts that I have submitted for publication. As you might be aware of, I am in the midst of completing my PhD thesis via publication. As such I humbly request you permission to include the following articles in the written dissertation;

1. Peck MT, Hiss D, Stephen L. Factors affecting the preparation, constituents, and clinical efficacy of leukocyte- and platelet- rich fibrin (L-PRF). SADJ August 2016, 71 (7). 298-302
2. Peck MT, Marnewick J, Stephen L, Singh A, Patel N, Majeed A. The use of luekocyte- and platelet- rich fibrin (L-PRF) to facilitate implant placement in bone deficient sites: a report of two cases. SADJ March 2012, 67 (2). 54-59
3. Peck MT, Hiss D, Stephen L. The effect of preparation type on fibrin diameter of leukocyte- and platelet- rich fibrin (L-PRF). SADJ May 2018. 73 (4). 193-197.
4. Peck MT, Hiss D, Stephen L, Maboza E. Antibiotic release from leukocyte- and platelet- rich fibrin (L-PRF) - an observational study. SADJ May 2018. 73 (4).
5. Peck MT, Hiss D, Stephen L, Majeed A. Platelet- Rich Fibrin - The effect of storage time on platelet concentration. SADJ November 2015. 70 (10). 448-451.
6. Peck MT, Hiss D, Stephen L, Olivier. The effect of luekocyte- and platelet- rich fibrin (L-PRF) and cross linked hyaluronic acid on fibroblast viability and proliferation . SADJ. Accepted for publication

Thank you
Thabit

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Bill Evans <Bill.Evans@wits.ac.za>   Fri, Aug 24, 2018 at 11:48 AM
To: Mogammad Peck <mpeck@uwc.ac.za>
Cc: Noko Reagan Mojela <NMojela@sada.co.za>

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