Development of a new extraction method for platelet-rich plasma and partial purification of platelet-derived growth factor and transforming growth factor beta

by

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DECLARATION OF ORIGINALITY

The work described in this dissertation was carried out in the Department of Pharmacology of the University of Pretoria under the supervision of Dr A.D. Cromarty. I declare that this dissertation is my own original work submitted for the degree Master of Science. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.

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ABSTRACT

Platelet-rich plasma (PRP) is the cell free plasma, which has an enriched concentration of platelets and clotting factors with the ability to enhance the natural healing process. PRP is often used by physicians in an office setting to accelerate the healing of a variety of sports related injuries, chronic wounds and enhance skin rejuvenation. PRP mimics the wound healing cascade by enhancing the recruitment, proliferation and differentiation of cells involved in tissue regeneration. Although PRP is used to enhance healing, the efficacy thereof is debated as no clear-cut set of parameters is available that device manufacturers and protocols should follow. The lack of uniformity in the PRP preparation methods results in differing PRP volume, platelet contents and unavoidably platelet-derived growth factors. Therefore, the aim of this study was to develop a simple and rapid method for preparing autologous PRP in an office setting using a tabletop centrifuge for point-of-care use. The simplified preparation procedure involved a single centrifugation step of 18 ml of whole blood, which sufficiently enriched the platelet content in the PRP fraction. As activated platelets express and release growth factors and cytokines that mediate the different phases of the wound healing cascade, the extracted PRP fraction was activated with an ethanol, calcium chloride ($CaCl_2$) and platelet poor plasma (PPP) preparation in glass containers, without the collection of additional blood as required in some protocols. The activated PRP formed a fibrin clot, trapping the degranulating platelets and its released growth factors. The concentration of TGF-β_1 obtained from the fibrin clot was 45.49 ± 3.80 ng/ml, in range with the available literature. During the *in vitro* studies, the extracted PRP by the developed method was able to significantly induce cell proliferation in a dose dependent manner. Cells enumerated with the crystal violet assay indicated that the cells treaded with 5% or 10% PRP significantly increased the percentage of viable cells to 165-176% and 156-158%, when compared to the positive controls. Cells enumerated with the MTT-assay indicated that the cells treaded with 5% or 10% PRP increased the percentage of viable cells to 79-91% and 87-105% which is comparable to that of the positive control. Data from the cellular proliferation assays indicate that sufficient platelet-derived growth factors had been obtained with the preparation procedure. Furthermore, data from the *in vivo* studies indicated that the extracted PRP was able to augment soft tissue regeneration and bone formation. Treatment with the activated PRP resulted in symptom reduction and accelerated healing of various injuries. The simplified preparation and the use of the provided study product

packaged in a kit developed during this study will enable physicians to easily obtain autologous PRP, in an office setting for point-of-care use, with the ability to induce tissue regeneration.

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ABBREVIATIONS

℃	Degree Celsius
%	Percentage
5-HT	Serotonin
A	
α	Alpha
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
APS	Ammonium Persulfate
AT	Autologous Thrombin
B	
B	Beta
βTG	beta-thromboglobulin
Bromophenol Blue	(3',3",5',5"-tetrabromophenolsulfonphthalein
BSA	Bovine Serum Albumin
C	
C	Collagen
$C_{12}H_{22}CaO_{14}$	Calcium Gluconate
Ca^{2+}	Calcium Ion(s)
$CaCl_2$	Calcium Chloride
cAMP	Cyclic Adenosine Monophosphate
cm	Centimetre(s)
cm^2	Centimetres squared
CO_2	Carbon Dioxide
COX	Cyclooxygenase
CTGF	Connective Tissue Growth Factor(s)
D	
dl	Decilitre

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E	
EC	Endothelial Cell(s)
ECM proteins	Extracellular Matrix Proteins
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epithelial Growth Factor(s)
ELISA	Enzyme-Linked Immunosorbent Assay(s)
et al.	*et alia*
EU	Enzyme Unit(s)
F	
FCS	Foetal Calf Serum
FDA	U.S. Food and Drug Administration
FGF	Fibroblast Growth Factor(s)
G	
g	Gram(s)
G	Gauge
g	Gravitational Force
GAGs	Glycosaminoglycan(s)
GF	Growth Factor(s)
GFAP	Growth Factor Adhesion Protein(s)
GMP	Good Manufacturing Practices
GP	Glycoprotein(s)
GTPases	Guanine Triphosphatases
H	
h	Hour(s)
HCl	Hydrochloric Acid
HF	Haemostasis Factor(s)
HI FCS	Heat Inactivated FCS

H0	Null Hypothesis
I	
ICH Guideline for GCP	International Conference of Harmonisation Guideline for Good Clinical Practice
IGF	Insulin-Like Growth Factor(s)
IL	Interleukin(s)
ISO	International Standards Organization
K	
kDa	Kilodalton
KGF	Keratinocyte Growth Factor(s)
kHz	Kilohertz
L	
l	Litre(s)
LAL	Limulus Amebocyte Lysate
M	
M	Molar
MCC	Medicines Control Council
M-CSF	Macrophage Colony Stimulating Growth Factor(s)
MAPKs	Mitogen Activated Protein Kinase(s)
µg	Microgram(s)
mg	Milligram(s)
min	Minute(s)
µl	Microlitre(s)
ml	Millilitre(s)
µm	Micrometre(s)
mm	Millimetre(s)
MMPs	Matrix-Metalloprotease(s)
mRNA	Messenger RNA
MRI	Magnetic Resonance Imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide

N

N	Normality
n	Sample Size
$NaHCO_3$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
NF-kappa B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram(s)
nm	Nanometre(s)

O

O.D.	Optical Density

P

PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PDAF	Platelet-Derived Angiogenesis Factor(s)
PDGF	Platelet-Derived Growth Factor(s)
PDGFR	PDGF Receptor(s)
pH	Hydrogen Ion Concentration
PI3K	Phosphoinositide 3' Kinase
PMNLs	Polymorphonuclear Leukocyte(s)
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma

R

RBC	Red Blood Cell(s)
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rPDGF	Recombinant PDGF
rpm	Revolutions Per Minute
rTGF	Recombinant TGF

S

SAMA	South African Medical Association

SAMED	South African Medical Device Industry Association
SANS	South African National Standard
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of Mean
SMAD	Mothers against decapentaplegic, *Drosophila* homolog of
T	
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF	Transforming Growth Factor(s)
TGF-R	TGF Receptor(s)
TGS	Tris-Glycine-SDS
TNF	Tumour Necrosis Factor(s)
TSP	Thrombospondin
Tris	Tris (Hydroxymethyl)-Aminomethane
TXA_2	Thromboxane A_2
V	
V	Volt(s)
VAS	Visual Analogue Scale
VEGF	Vascular Endothelial Growth Factor(s)
vol:vol	Volume per Volume
vWF	von Willebrand Factor(s)
W & X	
W	Watt(s)
WB	Whole Blood
Xa	Activated Factor X

CHAPTER 1

1.1 LITERATURE REVIEW

1.1.1 INTRODUCTION

Platelet-rich plasma (PRP) has gained popular attention in the mainstream media due to the use of this product by high profile, professional athletes to enhance the natural healing process during an injury, as well as its use by aesthetic physicians to reverse the effect of ageing in celebrities.

PRP is the cell free plasma, which has an enriched concentration of platelets and clotting factors compared to the individual's whole blood (Eppley *et al.*, 2006, Rutkowski *et al.*, 2008, Wroblewski *et al.*, 2010). The primary motivation for use of PRP is to enhance the recruitment, proliferation and differentiation of cells involved in tissue regeneration (Foster *et al.*, 2009). The use of PRP to enhance certain physiological processes is not a new concept and the technique of using a patient's own blood components (autologous) to enhance healing has been used for many years (Knighton *et al.*, 1982, Knighton *et al.*, 1986, Wroblewski *et al.*, 2010).

The idea of using PRP for tissue regeneration was pioneered by Marx in 1998 when he combined PRP with autologous bone grafts to reconstruct mandibular defects. At present PRP is used to slow disease progression in osteoarthritis and accelerate recovery in injuries involving cartilage (Kon *et al.*, 2009, Kon *et al.*, 2010). As for soft tissue regeneration, initially only platelet poor plasma (PPP) was used during surgery as a fibrin derived sealant to enhance haemostasis (Eppley *et al.*, 2006, Knighton *et al.*, 1982). The use of PRP was thought to mimic the normal wound healing cascade and thus would have the benefit of a sealant combined with the regeneration potential of platelet derived factors, accelerating recovery in the patient (Knighton *et al.*, 1986, Man *et al.*, 2001). Today, PRP is also used for soft tissue augmentation such as facial rejuvenation therapy, for wrinkles and solar damaged skin, surgery free repair of sports injuries, such as ruptured Achilles tendons and tendonitis, chronic refractory plantar fasciitis, chondropathy and osteoarthritis, as well as cutaneous wound remodelling in diabetic ulcers (Foster *et al.*, 2009, Knighton *et al.*, 1986, Sánchez *et al.*, 2009, Zenker, 2010).

PRP acts as a delivery system for the variety of growth factors and cytokines contained within the alpha (α)-granules of the platelets (Fufa *et al*., 2008). Growth factors are polypeptides that play a vital role in normal wound healing (Lynch *et al*., 1989, Sporn *et al*., 1983). The concentration of growth factors that can be released in PRP is proportional to the number of platelets releasing their granules, and as the concentration of platelets in PRP is higher than in autologous plasma, higher than normal growth factor concentration can be delivered to the area where PRP is administered (Fufa *et al*., 2008, Randelli *et al*., 2008, Wroblewski *et al*., 2010). The higher than normal growth factor concentration thereby accelerates the "natural" healing of both soft and hard tissues (Fufa *et al*., 2008, Randelli *et al*., 2008, Wroblewski *et al*., 2010).

1.1.2 PLATELET BIOLOGY

Thrombocytes or platelets are small, anucleate cell fragments derived from the cytoplasm of megakaryocytes with a circulating number of 150 – 400 x 10^9 per litre (l) plasma in healthy individuals (Eppley *et al*., 2006, Everts *et al*., 2006b, Mehta and Watson, 2008). As platelets lack nuclei, they lack genomic DNA but do contain some megakaryocyte derived messenger RNA (mRNA) with the potential to produce proteins (Davì and Patrono, 2007). Although platelets do produce some proteins, most of the proteins found in platelet stores where produced and packaged by the megakaryocyte or endocytosed from the circulating plasma pool (Table 1.1) (Anitua *et al*., 2004, Rendu and Brohard-Bohn, 2001). With a limited life-span of around seven to ten days, platelets are quickly removed from the circulation by Kupffer cells once they are no longer functionally viable (Eppley *et al*., 2006, Everts *et al*., 2006b, Mehta and Watson, 2008, Rendu and Brohard-Bohn, 2001). Platelets have a complex membranous system made up of a dense tubular system and an open canalicular system (Rendu and Brohard-Bohn, 2001). The open canalicular system forms membrane invaginations connecting the cytosol and surrounding medium which increases the surface area for intake of stimulatory agonists and the subsequent release of wound healing factors (Rendu and Brohard-Bohn, 2001, Sister, 2013).

Platelets contain many organelles, structures and granules (Eppley *et al*., 2006, Everts *et al*., 2006b). Two types of granules, namely dense granules and the more prevalent, α-granules, each contain an array of different components (Eppley *et al*., 2006, Everts *et al*., 2006b). Each platelet can contain from 30 to 80 α-granules with each granule containing more than 60 bioactive proteins, including

growth factors, adhesive proteins and haemostasis factors (Table 1.1) (Eppley *et al.*, 2006, Everts *et al.*, 2006b, Mehta and Watson, 2008, Rožman and Bolta, 2007).

Table 1.1 Some major components found in platelet α-granules (Anitua *et al.*, 2004, Rožman and Bolta, 2007, Rendu and Brohard-Bohn, 2001, van den Dolder *et al.*, 2006)

Growth factors	Adhesive proteins	Haemostasis factors
Epidermal growth factor (EGF)	Fibronectin	Fibrinogen
Platelet derived growth factor (PDGF)	Vitronectin	Factor V
Transforming growth factor-α (TGF-α)	von Willebrand Factor (vWF)	Factor VII
Transforming growth factor-β (TGF-β)	Thrombospondin-1	Factor XI
Fibroblast growth factor (FGF)	Laminin-8	Factor XIII
Keratinocyte growth factor (KGF)		Kininogens
Vascular endothelial growth factor (VEGF)		Protein S
Connective tissue growth factor (CTGF)		Anti-thrombin
Granulocyte/macrophage colony stimulating growth factor (M-CSF)		Tissue factor pathway inhibitor (a single chain polypeptide which binds irreversibly to Xa)
Insulin-like growth factor-1 (IGF-1)		Plasminogen
Platelet-derived angiogenesis factor (PDAF)		Osteonectin
Tumour necrosis factor-α (TNF-α)		A_2 macroglobulin
Interleukin-1β (IL-1β)		
Interleukin-8 (IL-8)		
Hepatocyte growth factor		
Neutrophil chemotative protein		

The main function of platelets is the maintenance of haemostasis in conjunction with the fibrin system to form a thrombus or clot at the site of an injury (Figure 1) (Brass, 2009, Eppley *et al*., 2006, Everts *et al*., 2006b). Since circulating platelets are in a resting, non-thrombogenic state, they first need to be activated through adherance at the site of injury before a clot can be formed (Eppley *et al*., 2006, Everts *et al*., 2006b). During circulation, platelets are continually pushed against the blood vessel walls with no interaction taking place. When the vessel wall is damaged, von Willebrand Factor (vWF), released by the damaged vascular cells, coats the damaged area (Brass, 2009). The platelets attach to the wound site by binding to vWF via glycoprotein Ib and the exposed collagen via glycoprotein IV or Ia, forming a monolayer of platelets at the site of injury (Figure 1) (Brass, 2009, Davì and Patrono, 2007, Kroll *et al*., 1991, Rendu and Brohard-Bohn, 2001). This adhesion results in activation of the platelets resulting in platelet aggregation and the release of the intracellular granules.

After adherence to the injury site, the platelets become activated and aggregate (Brass, 2009, Eppley *et al*., 2006, Everts *et al*., 2006b, Katzung *et al*., 2009). Platelets are not only activated by binding to the exposed collagen, or vWF, but also by physiological agonists such as adenosine diphosphate (ADP), thromboxane A_2 (TXA_2) and thrombin (Figure 1) (Rožman and Bolta, 2007). Platelet activation is dependent on the balance between calcium ions (Ca^{2+}) and cyclic adenosine monophosphate (cAMP) levels in the cytosol (Fox *et al*., 1983, Rendu and Brohard-Bohn, 2001). Binding of a specific G-protein coupled surface receptor to its agonist leads to a decrease in cAMP in the cytosol and a release of Ca^{2+} from their stores in the dense tubular system (Davì and Patrono, 2007, Rendu and Brohard-Bohn, 2001). The release of stored Ca^{2+} activates several calcium-dependent proteases that aid in platelet activation, conformational changes and fibrin formation (Anitua *et al*., 2004, Fox *et al*., 1983, Rendu and Brohard-Bohn, 2001).

Once activated, the small discoid cells morph into spherical cells with protruding pseudopods. These “sticky” pseudopods aid in aggregation by forming bridges with plasma fibrin and glycoprotein IIb/IIIa between platelets (Figure 1) (Eppley *et al*., 2006, Everts *et al*., 2006b, Katzung *et al*., 2009, Marguerie *et al*., 1984). Platelet activation results in degranulation and thus the release of both the dense and α-granules approximately 10 min after coagulation has been initiated (Eppley *et al*., 2006). The dense granules provide ADP which further amplifies activation and recruits surrounding platelets to the wound site (Figure 1), whereas α-granules fuse with the open canalicular system and immediately

release the vast array of proteins into the surrounding medium (Davì and Patrono, 2007, Mehta and Watson, 2008). With strong platelet activation, tissue factor (also known as thromboplastin or factor III), a glycoprotein receptor for activated Factor VII (VIIa), is synthesised from the megakaryocyte derived mRNA (Davì and Patrono, 2007). Factor VIIa plays a role in the extrinsic pathway of the coagulation cascade where it converts Factor X to its active form (Xa) (Gailani and Renné, 2007). Factor Xa together with activated Factor V (Va) forms the prothombinase complex which cleaves prothrombin into thrombin in a Ca^{2+} dependent manner (Anitua *et al*., 2004, Gailani and Renné, 2007). Thrombin not only further amplifies activation of surrounding platelets but also cleaves fibrinogen to form fibrin, providing a stable insoluble clot (Figure 1) (Davì and Patrono, 2007, Gailani and Renné, 2007).

The platelet's function does not simply end once a clot is formed, as platelets are also the largest natural source of growth factors and initiate the wound healing stages that follow (Assoian *et al*., 1983, Mehta and Watson, 2008).

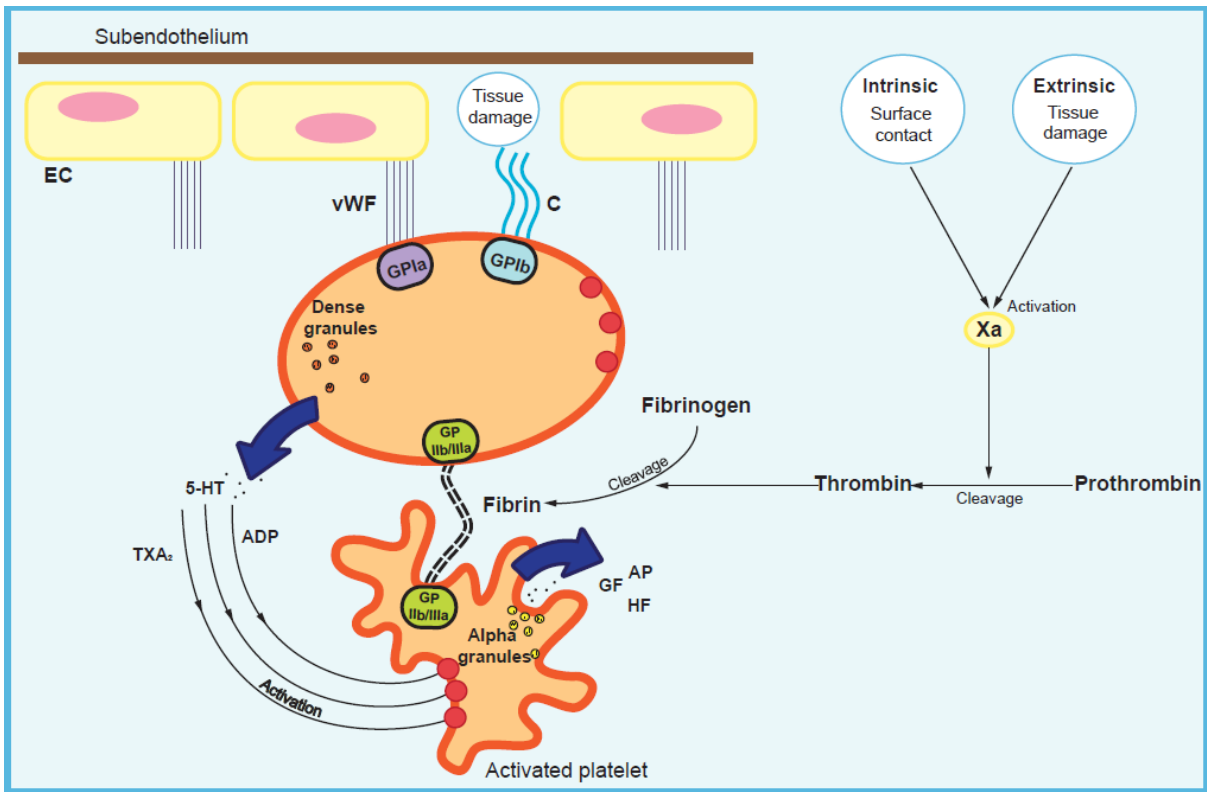

Figure 1 Schematic diagram of the activation of a platelet. Figure adapted for illustrative purposes from Brass, 2009, Davì and Patrono, 2007, Gailani and Renné, 2007, Katzung *et al*., 2009, Kroll *et al*., 1991, Rendu and Brohard-Bohn, 2001, Rožman and Bolta, 2007 ADP: adenosine diphosphate; AP: adhesion proteins; C: collagen; EC: endothelial cells; GPIa: glycoprotein Ia; GF: growth factors; GPIb: glycoprotein Ib; GP IIb/IIIa: glycoprotein IIb/IIIa; HF: haemostasis factors; TXA2: thromboxane A_2; vWF: von Willebrand factor; Xa: activated factor X; 5-HT: serotonin.

1.1.3 WOUND HEALING

In normal wound healing, platelets are the first cellular components to respond at the wound site (Rendu and Brohard-Bohn, 2001, Wroblewski *et al*., 2010). Platelet activation gives rise to the release of components necessary for the wound healing cascade (Rožman and Bolta, 2007). The wound healing cascade can be divided in to four, overlapping phases i.e. haemostasis, inflammation, proliferation and remodelling (Rožman and Bolta, 2007).

1.1.3.1 Haemostasis

Haemostasis is the first stage of wound healing and prevents further blood loss from the site of injury (Rožman and Bolta, 2007). Platelets, together with the plasma derived coagulation cascade facilitate haemostasis (Macri *et al*., 2007). The coagulation cascade has two pathways, the intrinsic - and extrinsic pathway, both involved in the formation of the prothrombinase complex that cleaves

prothrombin to thrombin (Figure 1) (Gailani and Renné, 2007). Thrombin, a serine protease, cleaves fibrinogen to fibrin which is a long fibrous molecule providing a stable insoluble clot (Eppley *et al*., 2006, Mehta and Watson, 2008).

The three dimensional fibrin clot, comprised of fibrin, fibronectin, vitronectin, vWF and thrombospondin, not only traps the degranulating platelets, but acts as a reservoir for the platelet released growth factors (Enoch and Price, 2004, Macri *et al*., 2007). Trapped platelets located in the fibrin network release more than 95% of their pre-synthesized growth factors within an hour after initiation of haemostasis (Eppley *et al*., 2006). The released growth factors initiate the wound healing cascade by attracting and activating fibroblasts, endothelial cells and macrophages and give rise to the second phase of wound healing namely the inflammatory response (Darby and Hewitson, 2007, Enoch and Price, 2004, Macri *et al*., 2007).

Although a high concentration of mitogenic factors are deposited by the platelets almost immediately post-injury, fibroblasts will only start migrating into the wound from the area around the wound about three days later (Macri *et al*., 2007). By trapping the growth factors in the clot it is possible to ensure that the growth factors will remain in the wound space and provide the needed mitogenic signals when the fibroblasts arrive at the wound site (Macri *et al*., 2007).

1.1.3.2 Inflammation

The initial haemostasis phase is followed by an inflammatory phase with the aim to remove any necrotic or damaged tissue in preparation for the proliferation phase (Everts *et al*., 2006b). Platelet degranulation activates the complement cascade which leads to the release of potent anaphalatoxins (Stadelmann *et al*., 1998). The anaphylatoxins promote the release of histamine from mast cells and basophils increasing the vascular permeability and attract neutrophils and monocytes to the site of injury (Clark, 2001, Stadelmann *et al*., 1998). Growth factor chemotaxis and increased vascular permeability ensures the infiltration of firstly neutrophils, followed by macrophages, and later lymphocytes to the wound site (Enoch and Price, 2004, Rožman and Bolta, 2007, Stadelmann *et al*., 1998). Once at the wound site, the neutrophils release nitric oxide, reactive oxygen species (ROS) and several lysosomal enzymes that destroy bacteria and clear the damaged tissue and inflammatory debris (Stojadinovic *et al*., 2008). Monocytes, attracted to the wound site by a variety of chemoattractants including TGF-β and PDGF, undergo a phenotypic change into macrophages once

at the wound site (Enoch and Price, 2004). Macrophages take over the role of primary protein secretors from the platelets and release additional pro-inflammatory cytokines and growth factors to continue the cascade of events involved in the healing process, recruiting fibroblasts and endothelial cells to the wound site (Everts *et al*., 2006b, Mehta and Watson, 2008). The infiltrated lymphocytes regulate the collagenases and may be involved in extracellular matrix remodelling (Enoch and Price, 2004).

1.1.3.3 Proliferation

The proliferative phase is governed by the proliferation of fibroblasts and the synthesis of predominantly new collagen (Haukipuro *et al*., 1991). Approximately three days post-injury (near the end of the inflammatory phase) fibroblasts, macrophages and blood vessels infiltrate the provisional matrix supplied by the fibrin clot from the wound edges (Clark, 2001, Rožman and Bolta, 2007). By the end of the first week, fibroblasts are the most prevalent cells at the wound site (Enoch and Price, 2004). Fibroblasts are attracted by a number of factors, most notably TGF-β and PDGF (Enoch and Price, 2004).

With the fibrin clot still intact, the fibrin and fibronectin in the clot provides a provisional scaffold for cell migration and adhesion of the fibroblasts, as these cells need to migrate into the wound bed from the wound edge (Rožman and Bolta, 2007). Adhesion and migration is facilitated by the expression of integrin receptors by the fibroblasts (under the influence of TGF-β and PDGF) which enables the fibroblast to bind to the scaffold (Martin, 1997).

With the dead cells and damaged extracellular matrix removed from the injured tissue, newly synthesized granulation tissue is produced by the fibroblasts to replace the fibrin clot and to fill the wound bed (Knighton *et al*., 1986, Stojadinovic *et al*., 2008). Growth factor stimulated fibroblasts produce an amorphous gel-like substance referred to as granulation tissue, which includes components such as glycosaminoglycans (GAGs), hyaluronate, fibronectin, and mostly Type I collagen followed by Type III collagen (Fitzpatrick and Rostan, 2003, Haukipuro *et al*., 1991, Rutkowski *et al*., 2008, Stojadinovic *et al*., 2008, Wroblewski *et al*., 2010). The fibroblasts gradually switch their major function to collagen production (Clark, 2001). During the proliferation phase, more tissue is produced than removed, partly due to the inhibition of matrix metalloproteases (MMP) (Stojadinovic *et al*., 2008). TGF-β stimulates the synthesis of tissue inhibitors of MMP (TIMMP). But,

not all MMP are inhibited as MMP are required to create a path for the fibroblasts to migrate into the dense fibrin clot (Clark, 2001). Some proteolytic enzymes escape inactivation by protease inhibitors by binding to the fibrin clot themselves and are thereby able to remove the fibrin clot. Enzymes such as plasmin, MMP-1, -2, -3 and -13 are able to cleave a path for cell migration and eventually remove the fibrin clot (Clark, 2001, Singer and Clark, 1999).

Granulation tissue is rudimentary and is later replaced by mature extracellular matrix components especially collagen Type I followed by Type III in soft tissue or collagen, hyaline cartilage and trabecular bone in hard tissue (Jeong Park *et al*., 2000, Stadelmann *et al*., 1998).

During the proliferation phase, TGF-β and PDGF among other growth factors promote angiogenesis, giving rise to a new capillary network infiltrating the fibrin clot and later the granulation tissue (Enoch and Price, 2004, Stadelmann *et al*., 1998, Werner and Grose, 2003).

1.1.3.4 Remodelling

The final stage of wound healing can take years to complete and involves remodelling the newly synthesised tissue to resemble the original tissue as closely as possible (Eppley *et al*., 2006).

In soft tissues, maturation of the extracellular matrix sees the removal of the rudimentary fibronectin and hyaluronic acid and replacement thereof by dense collagen bundles (Enoch and Price, 2004). The collagen is continuously remodelled by MMPs which are produced by fibroblast and macrophages, but as the remodelling phase nears its end, the MMPs are inhibited and collagen degradation decreases (Enoch and Price, 2004, Stadelmann *et al*., 1998). As the collagen matures and becomes older, more and more of these intramolecular and intermolecular cross-links are placed in the molecules. This important cross-linking step gives collagen its strength and stability over time (Diegelmann and Evans, 2004)

In hard tissues the hyaline cartilage and trabecular bone that replaced the granulation tissue during the proliferative phase, is resorbed by osteoclasts and replaced by compact bone (Jeong Park *et al*., 2000).

During the final stages of the remodelling phase, cell numbers, outgrowth of capillaries and blood flow at the wound site is decreased, with an overall reduction in metabolic activity of the wound (Eppley *et*

al., 2006). The acute wound is returned to its near normal anatomical structure, function and appearance (Enoch and Price, 2004).

1.1.4 GROWTH FACTORS

Growth factors are signalling polypeptides with important roles in the development, regeneration and restructuring of many tissues and play a role in inter- and intracellular commutation (Everts *et al*., 2006b, Fitzpatrick and Rostan, 2003, Rutkowski *et al*., 2008, Sánchez *et al*., 2009, Wroblewski *et al*., 2010). Platelets have large reserves of select growth factors stored in the granules and these can be released into the wound environment after activation (Table 1.1, Table 1.2) (Everts *et al*., 2006b, Rutkowski *et al*., 2008, Sánchez *et al*., 2009, Wroblewski *et al*., 2010). Of the growth factors released by the platelets, TGF-β and PDGF appear to be the most potent *in vivo* stimulators of proliferation, cellular differentiation, chemotaxis and apoptosis in cells involved in the wound healing cascade (Darby and Hewitson, 2007, Everts *et al*., 2006b, Rutkowski *et al*., 2008, Wroblewski *et al*., 2010).

1.1.4.1 Transforming growth factor beta (TGF-β)

Although TGF-β and TGF-α are classed under the same super family, these growth factors are entirely distinct peptides with their own receptor systems (Occleston *et al*., 2008, Sporn *et al*., 1987, Werner and Grose, 2003). TGF-β is a homodimeric peptide which consists of two identical disulfide liked β-subunits of 12.5 kiloDalton (kDa) (Sporn *et al*., 1987, Van den Eijnen-van Raaij *et al*., 1988). The TGF-β family consists of three isoforms of structurally related, multifunctional proteins with overlapping roles, namely TGF-β_1, TGF-β_2, and TGF-β_3 (Werner and Grose, 2003, Werz *et al*., 1996).

TGF-β is produced by a variety of cells, but blood platelets are the most concentrated source of TGF-β and in particular, TGF-β_1 (Van den Eijnen-van Raaij *et al*., 1988). TGF-β is synthesized as latent precursors by platelets and released in large amounts during degranulation (Border and Ruoslahti, 1992, Werner and Grose, 2003). Latent TGF-β is inactive and unable to bind to its receptors as it is bound to two other peptides to form large latent complex (Sporn *et al*., 1987, Werz *et al*., 1996). Activation is achieved by enzymatic cleavage of the large latent complex by proteases such as plasmin and MMPs as well as pH extremes of the surrounding medium (pH less than 3.5 and more than 12) and ROS, (Bierie and Moses, 2010, Kingsley, 1994, Pardali and Moustakas, 2007).

To mimic the activation process *in vitro*, TGF-β is activated by transient acidification, alkali or chaotropic agents such as ethanol, disrupting the non-covalent bonds between the inhibiting proteins of the large latent complex and TGF-β (Sporn *et al*., 1987, Werz *et al*., 1996).

Active TGF-β exerts its effect by binding to a heteromeric serine-threonine receptor complex on the cell membrane of cells such as fibroblasts, macrophages, epithelial cells, endothelial cells, mesenchymal cells, chondrocytes and osteoblast precursor cells (Mehta and Watson, 2008, Werner and Grose, 2003). The three TGF-β isoforms bind to three distinct membrane bound receptors to exert their biological effects (Occleston *et al*., 2008).

The first step in the signalling pathway involves the direct binding of TGF-β to TGF-β receptor Type 2 (TGF-βR2), an active serine-threonine receptor kinase (Occleston *et al*., 2008, Pardali and Moustakas, 2007). Binding leads to the recruitment of TGF-β receptor Type 1 (TGF-βR1) and the formation of a complex between TGF-βR2, TGF-βR1 and TGF-β (Doetschman *et al*., 2012, Occleston *et al*., 2008, Xu *et al*., 2010). TGF-βR1's serine residues are phosphorylated by TGF-βR2, which leads to signal propagation of SMAD dependent and SMAD independent pathways (Bierie and Moses, 2010). The SMAD proteins are homologs of Mothers Against Decapentaplegic from *Drosophila* and SMA from the *Caenorhabditis* (O'Kane and Ferguson, 1997).

The phosphorylation of the SMAD proteins leads to the downstream regulation of TGF-β induced gene transcription (Doetschman *et al*., 2012, Occleston *et al*., 2008, Pardali and Moustakas, 2007, Xu *et al*., 2010). Gene transcription is achieved by signal transduction that leads to the phosphorylation of two receptor regulated SMADs namely SMAD-2 and SMAD-3 (Pardali and Moustakas, 2007). SMAD-2 and SMAD-3 have high affinity for SMAD-4 with which it associates to form a functional trimeric protein complex (Pardali and Moustakas, 2007). The SMAD complex is translocated into the cell nucleus where it functions as a transcription factor for a variety of genes (Pardali and Moustakas, 2007, Xu *et al*., 2010).

The third type of receptor, TGF-βR3, has no cytoplasmic domain and alters signalling by presenting TGF-β to TGF-βR1 and TGF-βR2 (Occleston *et al*., 2008).

TGF-β not only regulates the SMAD pathway, but also activates the mitogen activated protein kinases (MAPKs), phosphoinositide 3' kinase (PI3K), small GTPases and the Ras superfamily (Pardali and Moustakas, 2007).

Some of TGF-β's effects on wound healing are indirect and mediated through an intermediate cell, such as macrophages, as these cells also produce and secrete latent TGF-β within the wound matrix, which is later activated, allowing for a continuous supply of TGF-β throughout the healing process (Sporn *et al*., 1987, Werner and Grose, 2003).

TGF-β's effects are both cell and concentration specific, as active TGF-β is mitogenic for fibroblasts and advanced tumour cell metastasis, but inhibits the proliferation of most other cells of mesenchymal origin, especially epithelial cells, mesenchymal cells, T-lymphocytes and B-lymphocytes and the early stages of oncogenesis (Table 1.2) (Pardali and Moustakas, 2007, Sing *et al*., 1988, Sporn *et al*., 1987, Werner and Grose, 2003, Xu *et al*., 2010).

During the inflammatory phase of wound healing, active TGF-β acts as a chemoattractant for neutrophils, monocytes, leukocytes and fibroblasts (Border and Ruoslahti, 1992, Werner and Grose, 2003). Active TGF-β modulates both the pro-inflammatory and anti-inflammatory responses by controlling the induction and synthesis of inflammatory cytokines by the recruited leukocytes (Border and Ruoslahti, 1992, Werz *et al*., 1996).

Throughout the inflammatory and proliferation phase of the wound healing cascade, TGF-β stimulates granulation tissue formation, followed by mature extracellular matrix production by fibroblasts and re-epithelialization of cutaneous wounds (Fitzpatrick and Rostan, 2003, Sporn *et al*., 1987, Werner and Grose, 2003). TGF-β prevents proteolytic degradation of newly formed matrix proteins, either by increasing the formation and secretions of protease inhibitors and / or decreasing the production of proteases themselves (O'Kane and Ferguson, 1997, Sporn *et al*., 1987).

Due to TGF-β induced extracellular matrix synthesis, the fibroblasts are able to anchor to the wound bed during the proliferation phase of the wound healing cascade, migrating and proliferating as needed (Werz *et al*., 1996).

In the remodelling phase TGF-β is able to stimulate the production of collagenases and other proteases, removing the excess material in the wound to mimic the original tissue (Fitzpatrick and Rostan, 2003).

In bone, TGF-β stimulates the proliferation of osteoblast precursor cells and bone collagen synthesis, whilst increasing osteoclast apoptosis and decreasing bone resorption (Mehta and Watson, 2008). A study by Brandes *et al*., (1991) demonstrated that administration of TGF-β significantly suppressed the acute and chronic phase of induced arthritis in rats. The authors credited the TGF-β induced inhibition of a) IL-1 induced chondrocyte protease activity and cartilage proteoglycan degradation, b) the formation of osteoclast-like cells and c) bone resorption to the suppression the disease (Brandes *et al*., 1991).

TGF-β is furthermore responsible for activation of endothelial cells for angiogenesis, chondroprogenitor cells for cartilage and in a somewhat contradictory manner induce proliferation in mesenchymal cells to increase the cell population in the wound, as TGF-β normally inhibits proliferation in these cells (Mehta and Watson, 2008).

It appears that TGF-β may have a cardio-protective function as it protects the cardiovascular system form the destructive actions of IL-1, maintains the rhythmic beating of myocytes *in vitro* and prevents the adhesiveness of neutrophils to the endothelium, which could evoke cellular damage (Sporn and Roberts, 1992).

TGF-β plays a predominant role in the formation of hypertrophic scars and keloid tissue as research has shown that TGF-β and its receptors are persistently expressed in hypertrophic scars and over expressed in keloid tissue with an overall increase in TGF-β activity in scar tissue (Werner and Grose, 2003).

1.1.4.2 TGF-β isoforms

The TGF-β isoforms are closely related with similar biological activities on the same receptor signalling system (Occleston *et al*., 2008). The predominant form of TGF-β is TGF-β_1, which is a hundred times more potent growth inhibitor compared to the other isoforms with a higher affinity toward the TGF-βR1 and TGF-βR2 (Occleston *et al*., 2008). In wound healing, rapid induction of TGF-

β_1 and TGF-β_2 is seen whereas in the many different cell types during wound repair (Werner and Grose, 2003). However, TGF-β_3 expression is low in an unwounded dermis and only increased 7 days post-wounding, whilst the concentration of TGF-β_1 decreases (O'Kane and Ferguson, 1997). TGF-β_1 deficient wounds have severely impaired late-stage wound healing (Werner and Grose, 2003). Table 1.2 lists the overlapping and unique biological activities of the three isoforms.

1.1.4.3 Platelet-derived growth factor

Although PDGF was first extracted and purified from human blood platelets in 1979, this molecule is synthesized and secreted from a variety of cells such as tumour cells, vascular endothelial cells, macrophages, smooth muscle cells and fibroblasts (Antoniades *et al*., 1979, Ross, 1989). PDGF, IL-1 and TGF-β, are able to induce PDGF gene expression in fibroblasts and smooth muscle cells inducing cell proliferation in an autocrine fashion (Bauer *et al*., 1985, Fitzpatrick and Rostan, 2003, Ross, 1989). Native PDGF has a molecular weight of 30 kDa and is comprised of two homo- or heterodimeric disulphide linked polypeptide chains (Heldin *et al*., 1985, Ross, 1989, Werner and Grose, 2003). It is only when bound by the disulphide chains that PDGF has mitogenic activity (Ross *et al*., 1986). The PDGF family includes PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD, although the dominant form of the growth factor found in platelets are PDGF-AB, followed by PDGF-BB and PDGF-AA (Ross, 1989, Werner and Grose, 2003).

This family of growth factors exert their effects by binding to three distinct transmembrane tyrosine kinase receptors, which are homo- or heterodimers of an α- and a β-chain (Werner and Grose, 2003). Each PDGF polypeptide chain can bind only to a specific receptor subunit, the A-polypeptide and C-polypeptide chains can only bind to the α-receptor subunit whereas the B-polypeptide and D-polypeptide chains can only bind the β-receptor subunit (Bonner, 2004, Ross, 1989). Therefore each PDGF molecule brings together two separate receptor subunits (αα, ββ or αβ) to create a stable dimeric association between the receptor subunits (Ross, 1989).

The PDGF receptors (PDGFR) have been found on cells from mesenchymal origin such as fibroblasts, smooth muscle cells, osteoblasts, osteoprogenitor cells, endothelial cells and glial cells and are involved in several biological processes such as cell proliferation, activation and chemotaxis (Heldin *et al*., 1985, Mehta and Watson, 2008, Ross, 1989). The number of the α- and β-subunits found on the cell membrane differ between the cells as fibroblasts for example, have at least 10-fold

more β-subunits on their cell membrane compared to α-subunits (Ross, 1989). PDGF-BB is therefore around 10-fold more mitogenic for normal human fibroblasts than PDGF-AA (Ross, 1989).

The PDGFR have an extracellular domain, capable of autophosphorylating several of their tyrosine cytoplasmic domains once PDGF has bound (Heldin *et al*., 1985, Ross, 1989, Waterfield *et al*., 1983). The PDGF-receptor complex mediates the binding of co-factors, activates signal transduction downstream leading to DNA synthesis, gene expression, protein synthesis, chemotaxis and increased cell migration (Heldin *et al*., 1985, Waterfield *et al*., 1983). Finally the PDGF-receptor complex is internalised from the cell surface via a clathrin-based system and degraded (Orth and McNiven, 2006, Ross, 1989).

PDGF plays a role in both normal and pathological cell development as it has mitogenic and a general trophic effect on cells (Heldin *et al*., 1985). PDGF plays a role in normal growth and development, formation of granulation tissue needed for wound repair and is involved in certain inflammatory conditions and tumour formation (Ross, 1989). Its trophic effects include amino acid transport, fluid endocytosis, cholesterol availability, prostaglandin synthesis and overall cell survival (Heldin *et al*., 1985).

PDGF is essential for proper wound healing as wounds without PDGF do not progress to complete healing (Werner and Grose, 2003). In wound healing, PDGF plays major roles in the inflammatory, proliferative as well as remodelling phase of the healing cascade (Bauer *et al*., 1985).

During the inflammatory phase, PDGF activated macrophages remove the damaged tissue in the wound whilst releasing the second source of growth factors to continue the repair process (Mehta and Watson, 2008). The macrophage released PDGF stimulates fibroblast proliferation and granulation tissue synthesis in the remodelling phase followed by induction of the myofibroblast phenotype, the production of mature extracellular matrix, collagenases and bone resorption in the remodelling phase (Bauer *et al*., 1985, Mehta and Watson, 2008, Werner and Grose, 2003). Its capacity to influence remodelling of the wound is attributed to its ability to bind non-specifically to extracellular matrix components, ensuring that PDGF is retained at the site of its release and therefore continuously available as a mitogen (Heldin *et al*., 1985, Roy *et al*., 1993). Bateman *et al*., 2005, demonstrated that

the adsorption of PDGF-BB to inorganic or collagen bone matrix increased osteoblast proliferation *in vitro.*

Low concentrations of PDGF is chemotactic for cells migrating into the wound such as neutrophils, monocytes and fibroblasts. Seppä *et al*., (1982) demonstrated that a PDGF concentration above 3 µg/ml reduced its chemotactic response. Furthermore many studies have indicated that high concentrations of PDGF were able to inhibit chemotaxis (Fufa *et al*., 2008, Seppä *et al*., 1982, Werner and Grose, 2003, Wroblewski *et al*., 2010). PDGF's chemotactic potential is due to its ability to stimulate prostaglandin synthesis in wound cells, attracting inflammatory and remodelling cells into the wound environment (Heldin *et al*., 1985). It is further capable of stimulating prostacyclin synthesis in smooth muscle cells, leading to vasodilation of blood vessels and inhibition of platelet aggregation (Heldin *et al*., 1985).

PDGF has been implicated in inflammatory conditions and is thought to be a major contributor to fibrotic diseases as both PDGF and its receptors are highly expressed in hypertrophic scars and keloids, a variety of pulmonary fibrotic diseases, hepatic and renal fibrosis as well as in scleroderma (Bonner, 2004, Ross, 1989, Werner and Grose, 2003). PDGF's roles in the progression of these diseases are mainly due to its potent mitogenic activity, its function as a chemoattractant and its ability to stimulate the production of ECM proteins (Bonner, 2004, Ross, 1989).

1.1.4.4 Roles of TGF-β and PDGF's in cancer

TGF-β is able to induce cell proliferation in certain cell populations, inhibiting cell division in others, while inducing apoptosis in some cells (Pardali and Moustakas, 2007). TGF-β induces cell cycle arrest at the G1 phase through SMAD mediated transcriptional regulation (Pardali and Moustakas, 2007).

Many human tumours have mutations targeting the TGF-βR, the SMAD proteins or misregulation of TGF-β leading to TGF-β being unable to act as a cytostatic agent or to induce apoptosis (Pardali and Moustakas, 2007). With the inability to induce apoptosis or cell cycle arrest combined with over-expression of the growth factor by the tumour itself, increased cancer cell proliferation takes place in an autocrine fashion (Pardali and Moustakas, 2007). In addition, TGF-β induces the production of other mitogenic factors such as PDGF from the adjacent cells (Bierie and Moses, 2010, Pardali and

Moustakas, 2007). The expressed TGF-β and PDGF in the tumour environment not only leads to increased cell survival, but induces angiogenesis of the surrounding vasculature, increases production of extracellular matrix proteins from the surrounding fibroblasts and inhibits the phagocytic effects of inflammatory cells and thereby contributing to the tumour's invasiveness (Bierie and Moses, 2010, Pardali and Moustakas, 2007).

To date there is no data indicating that exogenously applied growth factors can promote tumour initiation or growth (Fitzpatrick and Rostan, 2003).

Table 1.2 Comparison of the unique and overlapping biological activities of the TGF-β isoforms adapted from Occleston *et al.*, 2008, Sporn and Roberts, 1992 and http://www.phosphosite.org

TGF-β_1		**TGF-β_2**		**TGF-β_3**	
Gene regulation					
Positive regulation DNA-dependent transcription Activation of NF-kappa B transcription factor Transcription from RNA polymerase II promoter DNA binding	Negative regulation Transcription from RNA polymerase II promoter DNA binding	Unknown		Positive regulation DNA-dependent transcription DNA replication Transcription from RNA polymerase II promoter	Negative regulation DNA replication
Inflammation					
Positive regulation Inflammatory response Active induction of host immune response by virus IL-17 production Defense response to fungus	Negative regulation Immune response Phagocytosis	Positive regulation Immune response Neutrophil chemotaxis	Negative regulation Unknown	Unknown	Positive regulation Inflammatory response Active induction of host immune response by virus IL-17 production Defence response to fungus

TGF-β_1		**TGF-β_2**		**TGF-β_3**	
Signalling and metabolic pathways and cell cycle					
Positive regulation	Negative regulation	Positive regulation	Negative regulation	Positive regulation	Negative regulation

Cell division G1/S transition checkpoint Exit of mitosis Amino acid dephospholylation Histone deacetylation and acetylation Peptidyl-serine phosphorylation Protein import into nucleus Protein secretion TGF-β receptor signalling pathway SMAD protein nuclear translocation SMAD protein complex assembly MAPK activity Regulation of sodium ion transport Cellular protein metabolic process Induction of apoptosis	Cell cycle arrest Amino acid phosphorylation Phosphate metabolic process Protein export form nucleus	Cell-cell signalling Amino acid phospholylation Protein secretion TGF-β receptor signalling pathway Transforming growth factor β_2 production SMAD protein nuclear translocation Stress-activated MAPK cascade Phosphorinositide-3 kinase cascade	Unknown	Cell division Protein secretion TGF-β receptor signalling pathway SMAD protein nuclear translocation Activation of MAPK activity	TGF-β receptor signalling pathway
TGF-β_1		**TGF-β_2**		**TGF-β_3**	
Signalling and metabolic pathways and cell cycle					
Phosphorinositide-3 kinase activity ATP biosynthetic process		Activation of protein kinase-3 activity		 Response to progesterone	

Response to progresterone stimulus Response to estradiol synthesis Response Vitamin D Response to glucose stimulus	Sequestered calcium ion release into cytosol Epidermal growth factor receptor signalling pathway		Integrin biosynthetic process Cell adhesion mediated by integrin Intercellular junction assembly and maintenance Dopamine biosynthetic process Hair growth cessation (catagen)	synthesis Response to oestrogen stimulus Intercellular junction assembly and maintenance	
Miscellaneous					
Ageing Menstrual cycle phase		Menstrual cycle phase Heart contraction		Ageing Menstrual cycle phase	

TGF-β_1		**TGF-β_2**	**TGF-β_3**	
Wound healing				
Positive regulation Platelet activation Platelet degranulation Blood coagulation	Negative regulation	Positive regulation Wound healing Platelet activation Platelet degranulation Blood coagulation	Negative regulation Unknown	Positive regulation Wound healing Platelet activation Platelet degranulation Blood coagulation

Granulation tissue formation Collagen biosynthesis Chemotaxis Endothelial cell migration Organ regeneration	Endothelial cell migration	Collagen fibril organization Haematopoiesis Angiogenesis			Collagen biosynthesis
Embryogenesis					
Positive regulation Development of inner ear Lymph node development Endoderm development Odontogenesis Mammary gland development Ureteric bud development Salivary gland morphogenesis Gut development		Positive regulation Embryonic development Odontogenesis Salivary gland morphogenesis Embryonic gut development Heart morphogenesis Heart development Hair follicle development and morphogenesis Eye development Neuron development Generation of neurons		Positive regulation Development of inner ear Odontogenesis Palate development Mammary gland development Salivary gland morphogenesis Gut development Neurocranium morphogenesis Organ morphogenesis Alveolus development	
TGF-β_1		**TGF-β_2**		**TGF-β_3**	
Cell specific functions					
Positive regulation Cell death Cell proliferation	Negative regulation Cell proliferation Cell growth	Positive regulation Apoptosis Cell proliferation Cell growth Cell division Cell morphogenesis Cell migration	Negative regulation Cell proliferation Cell growth	Positive regulation Apoptosis	Negative regulation Cell proliferation

	Cell-cell adhesion	Cell cycle arrest			
Intercellular junction and assembly and maintenance					
Haemopoietic progenitor cell differentiation		Somatic stem cell division		Filopodium formation	
T cell homeostasis	T-cell differentiation				
Mononuclear cell proliferation					
		Epithelial to mesenchymal transition	Epithelial cell proliferation		
	Epithelial cell proliferation				
Smooth muscle cell differentiation	Fat cell differentiation				
Bone mineralization	Skeletal muscle development				
		Bone ossification			
Chondrocyte differentiation	Myoblast differentiation				
	Bone ossification	Glial cell migration			
Myeloid dendritic cell differentiation	Neuroblast proliferation	Cardioblast differentiation			
		Cardiac muscle cell proliferation		Bone mineralization	
Myelination					Neuron apoptosis

1.1.4.5 Growth factor synergism

It appears that growth factors do not function alone, but rather interact with a variety of other proteins and cytokines in synergy within the wound environment (Fitzpatrick and Rostan, 2003). Understanding the process of wound healing and the role of the different mediators raised the question of whether using selectively purified, or recombinantly produced specific growth factors involved in the healing cascade, would be beneficial for the healing process when used alone in the clinical setting. The use of PDGF-BB has been shown to stimulate the healing of chronic, full-thickness, lower-extremity diabetic neurotrophic ulcers and punch biopsies (Cohen and Eaglstein, 2001, Miller, 1999, Steed, 1995), but cost of production, transportation and storage of recombinant growth factors are expensive (Everts *et al*., 2006b, Fufa *et al*., 2008). Reviews regarding the effectiveness of a single growth factor in wound healing have been mixed (Singer and Clark, 1999) Singer reported that “the overall experience with growth factors and other mediators to accelerate wound healing have been discouraging. This is not surprising, considering that wound repair is a complex set of interactions among cytokines, formed blood elements, extracellular matrix and cells”.

In contrast to the complexity and cost of recombinant growth factor production, PRP can easily be isolated and is far less expensive (Saldalamacchia *et al*., 2004). It is far more cost effective to extract wound healing controlling growth factors from patients with very low risk of complications. An additional benefit is that multiple synergistically acting growth factors are present in the physiological ratio of concentrations required in the wound area during the wound healing cascade (Everts *et al*., 2006b).

1.1.5 IMPAIRED WOUND HEALING

Acute wounds heal in an orderly fashion although the end product is neither aesthetically or functionally perfect (Enoch and Price, 2004, Martin, 1997, Stadelmann *et al*., 1998). With impaired wound healing there is no orderly or sequential progression and structural integrity is absent as healing is disrupted at one or more points along the healing cascade (Enoch and Price, 2004, Stadelmann *et al*., 1998). Impaired healing may be due chemotherapy, or immunosuppressant treatment, metabolic disorders, ageing, vascular disease or radiation therapy (O'Kane and Ferguson, 1997).

The overproduction of extracellular matrix components is known as excessive healing and can lead to an altered structure and loss of function as is seen in fibrosis (Diegelmann and Evans, 2004). A decrease in the synthesis of extracellular matrix components or deficient healing leads to an incomplete, weak wound in a constant inflammatory state as which is seen in chronic wounds.

Altered biochemical and cellular environments, such as the lack of macrophages, as well as changes in the concentration of growth factors, cytokines and proteases could all account for the impaired healing (Enoch and Price, 2004). In chronic or severely infected wounds, neutrophils are not phagocytosed by the tissue macrophages which results in an increased release of ROS (damaging the surrounding tissue), proteases and pro-inflammatory cytokines (Martin, 1997, Stojadinovic *et al*., 2008). In these circumstances, the wound is maintained in a chronic inflammatory state.

Slow healing wounds usually produce an abundance of granulation tissue, but lack fibronectin as it is broken down by the increased levels of proteases (Macri *et al*., 2007, Stadelmann *et al*., 1998). Fibronectin is not only necessary for the migration of fibroblasts into the wound bed but also for binding and retention of growth factors released by the platelets (Macri *et al*., 2007).

Although chronic wounds are often rich in pro-inflammatory cytokines such as TGF-β, TNF-α and IL-8, any imbalance, lack or distribution of certain growth factors may explain the delayed or non-healing observed in non-healing wounds (Enoch and Price, 2004, Pietramaggiori *et al*., 2006). Studies have shown that high levels of TGF-β_1 are bound to the so called extracellular matrix “cuffs” around the blood vessels of venous ulcers, but very little growth factor in the wound bed itself (O'Kane and Ferguson, 1997). In contrast, normal wounds have a higher concentration of TGF-β_1 bound to the extracellular matrix and none to the blood vessel “cuffs”. O’Kane and Ferguson, 1997, mentioned that impaired wounds have a lower concentration of TGF-β_1 and TGF-β_2 expression and when these wounds were treated with TGF-β_1, it improved wound healing. Similar results were obtained with chronic wounds treated with PDGF or bFGF (Singer and Clark, 1999). Furthermore a lack of wound macrophages leads to a lack of macrophage-derived growth factors, which alters the synthesis of new tissue as macrophages replace the platelets as the main secretors of growth factors (Singer and Clark, 1999).

An imbalance in the concentration of wound proteases has been found to alter the normal wound healing cascade. Proteases such as neutrophil elastase and MMP-8 are capable of digesting extracellular matrix proteins and growth factors, respectively (Diegelmann and Evans, 2004). Excessive matrix degradation alters cell migration and prevents the progression of the wound healing cascade into proliferation phase, ultimately resulting in a weak, chronic wound (Clark, 2001, Diegelmann and Evans, 2004, Stojadinovic *et al.*, 2008). Chronic wounds, such as diabetic ulcers, are characterised by prolonged inflammation, impaired blood vessel formation, decreased collagen synthesis, an increase in the concentration of proteases, defective macrophage function and a decreased responsiveness to growth factors (Ashcroft *et al.*, 1995, Singer and Clark, 1999).

1.1.6 PLATELET-RICH PLASMA

1.1.6.1 Commercially available devices and in house protocols for PRP preparation

In practice, preparation of autologous PRP uses standard blood bank techniques such as plasmapheresis or centrifugation using commercially available kits, devices or in house protocols (Table 1.3) (Antoniades *et al.*, 1979, Fufa *et al.*, 2008, Knighton *et al.*, 1982, Knighton *et al.*, 1986, Luengo Gimeno *et al.*, 2006, Mazzucco *et al.*, 2008).

Normal blood bank techniques and plasmapheresis require large volumes of blood and the procedures are not available to the general practitioner. In house protocols require the use of large laboratory centrifuges, and is labour intensive as two centrifugation steps are needed with multiple transfer steps and laboratory equipment (Eppley *et al.*, 2006). The multiple steps required when using in house protocols may compromise sterility of the PRP which can lead to infection at the injection site (Eppley *et al.*, 2006).

To date there is, no clear-cut set of parameters that device manufacturers or in house protocols should follow, allowing for variation in whole blood and PRP volume, platelet concentration and whether the platelets are activated for point-of-care protocols or devices (Table 1.3) (De Somer *et al.*, 2006, Eppley *et al.*, 2006).

The use of commercially available point-of-care kits and devices in combination with a tabletop centrifuge is a more appealing option as this allows for easy preparation in a doctor's consulting rooms or in an operating theatre, which require smaller blood volumes and where the prepared PRP

is used immediately. Smaller systems collect up to 60 ml of whole blood and usually have a PRP yield of around 10% by volume and a platelet yield of two to eight fold the baseline platelet concentration (Eppley *et al*., 2006). The volume of PRP prepared in this way is sufficient to treat an injury or skin rejuvenation as two to 6 ml PRP is normally injected into the desired area (Foster *et al*., 2009, Zenker, 2010). To preserve the platelet membrane's integrity, smaller kits and devices require that the whole blood is combined with a citrate dextrose anti-coagulant before centrifugation to prevent platelet activation and clot formation before the PRP fraction is collected (Eppley *et al*., 2006).

The commercial kits and devices available in South Africa include the RegenKit® (RegenLab, Le Mont-sur-Lausanne, CH), MyCells® Autologous Platelet Preparation kit (Kaylight Corporation, CH) and GPS®III Gravitational Platelet Separation System (Biomet, Warsaw, US). All of these devices are approved by the U.S. Food and Drug Association (FDA) only for the extraction of PRP at point of care, as none of these devices have been evaluated for any clinical indications by the U.S. FDA (FDA, 2012, Biomet, [n.d]).

With the RegenKit® approximately 20 ml of whole blood is collected in specialised blood collection tubes preloaded with citrate anti-coagulant and a density gradient medium which separates the whole blood into PRP and PPP by centrifugation (RegenLab, 2011). The PRP is collected from the tube under sterile conditions and injected into the desired area. The GPS®III Gravitational Platelet Separation System's protocol for the collection of PRP requires additional steps. Approximately 50-60 ml whole blood is drawn from the patient in a syringe, citrate anti-coagulant added to the whole blood, the anti-coagulated blood loaded into a disposable, centrifuge-specific separation tube where it is centrifuged, the PRP collected and injected into the desired site (Biomet, [n.d.]). The MyCells® Autologous Platelet Preparation kit combines gravitational platelet separation and specialised blood collection tubes with a platelet filtration system to concentrate and isolate the platelets (Theodor, [n.d.]). Depending on the volume of PRP required 10 - 20 ml whole blood is collected in blood collection tubes. The tubes are centrifuged and PRP collected with separation filter and injected at the desired site.

Table 1.3 Overview of the protocols of the available in house protocols, kits and devices for obtaining PRP

In house protocols, commercial devices or kits				
Author or manufacturer	**Required volume**	**Method for producing PRP**	**PRP volume**	**Activator**
ActivAT™ (Sorin Group, Mirandola, IT)	WB N/A. 12 ml PPP	PPP activated with ethanol, glass beads and $CaCl_2$ to produce AT. AT combined with PRP 1:10 ratio (vol:vol)	5 - 55 ml	AT, $CaCl_2$, ethanol, glass
(Anitua *et al.*, 2004)	10 ml WB	WB centrifuged at 460 *g*, 8 min at room temperature. PRP collected just above RBC pellet. PRP combined with $CaCl_2$ in glass tubes and incubated at 37ºC.	0.5 ml	$CaCl_2$, glass
(Creaney *et al.*, 2011)	8.5 ml WB	WB centrifuged at 2000 *g*, for 15 min, buffy coat collected.	1.5 ml	None
Electro Medics 500 (Medtronic, Minneapolis, US)	450 - 500 ml WB	With the aid of a gradient density cell separator, WB centrifuged at 5600 rpm, PPP discarded. PRP and RBC centrifuged at 2400 rpm, PRP collected. PRP combined with $CaCl_2$ and thrombin.	70 ml	$CaCl_2$, thrombin
GPS®III Gravitational Platelet Separation System (Biomet, Warsaw, US)	50 - 60 ml WB	WB centrifuged at 3200 rpm, 15 min, PPP discarded. PRP collected.	10 ml	None
(Kon *et al.*, 2009, Kon *et al.*, 2010)	150 ml WB	WB centrifuged at 1800 rpm, 15 min, plasma collected. Plasma centrifuged at 3500 rpm, 10 min, PRP collected. PRP activated with $CaCl_2$.	20 ml	$CaCl_2$
(Knighton *et al.*, 1982)	10 - 35 ml WB	WB centrifuged at 900 rpm, 20 min, plasma collected. Plasma centrifuged at 2500 rpm, 20 min, platelet pellet collected. Platelets resuspended in buffer solution and released with thrombin. PPP combined with $CaCl_2$. PRP and PPP combined.	N/A	$CaCl_2$, thrombin,
In house protocols, commercial devices or kits				

Author or manufacturer	Required volume	Method for producing PRP	PRP volume	Activator
(Knighton *et al.*, 1986)	60 ml WB	WB centrifuged at 135 *g*, 20 min at 4ºC, plasma collected. Plasma centrifuged at 750 *g*, 10 min at 4ºC, platelet collected and washed with buffer solution. Platelets released with thrombin. Spent platelets removed with centrifugation 950 *g*, 5 min at 4ºC.	10 ml	Thrombin
(Krašna *et al.*, 2007)	9 ml WB	WB centrifuged at 272 *g*, 7 min and plasma collected. Plasma centrifuged at 1288 *g*, 7 min, platelet pellet collected. Platelet pellet combined with calcified thrombin in a 1:1 ratio (vol:vol).	1.5 ml	$CaCl_2$, thrombin
Magellan™ (Medtronic, Minneapolis, US)	WB for PRP N/A; 3 ml WB	WB combined with glass fiber and $CaCl_2$ to produce AT. AT combined with PRP 1:4 ratio (vol:vol).	12.5 ml	AT, $CaCl_2$, ethanol, glass
(Mazzucco *et al.*, 2004)	N/A	Platelet resuspended in buffer solution. PPP activated with $C_{12}H_{22}CaO_{14}$ in a 5:1 ratio, volume to volume, incubated at 37ºC and centrifuged to produce AT. Resuspended platelets, AT and $C_{12}H_{22}CaO_{14}$ combined in a 8:2:1 ratio (vol:vol:vol).	N/A	AT, $C_{12}H_{22}CaO_{14}$
MyCells® Autologous Platelet Preparation kit (Kaylight Corp, CH)	10 - 20 ml WB	WB centrifuged at 1450 - 2010 *g*, 10 min in a specialised tube containing a separator gel, PPP discarded, tube vortexed. Filter inserted into vortexed tube and PRP collected.	2.5 ml	None
(Na *et al.*, 2011)	10 ml WB	WB centrifuged at 160 *g*, 10 min, plasma collected. Plasma centrifuged 400 *g*, 10 min, PRP collected. PRP activated with $CaCl_2$.	1.5 ml	$CaCl_2$
Plateltex® (Plateletx SRO, Vlasska, CZ)	50 ml WB	WB centrifuged at 160 *g*, and plasma collected and transferred to graduated tubes. PRP collected activated with $C_{12}H_{22}CaO_{14}$ and batroxobin. PPP combined with $C_{12}H_{22}CaO_{14}$ for fibrin gel	6 ml	Batroxobin, $C_{12}H_{22}CaO_{14}$
In house protocols, commercial devices or kits				
Author or manufacturer	**Required volume**	**Method for producing PRP**	**PRP volume**	**Activator**

RegenKit® (RegenLab, Le Mont-sur-Lausanne, CH)	20 ml WB	WB centrifuged and PRP collected.	5 ml	None
(Sánchez *et al.*, 2007)	N/A	WB centrifuged at 460 *g*, 8 min, fraction above RBC pellet collected (PRP). PRP activated with $CaCl_2$.	1 ml	$CaCl_2$
(Sánchez *et al.*, 2009)	80 ml WB	WB centrifuged at 460 *g*, 8 min, fraction above RBC pellet collected (PRP). PRP transferred to a glass bowl and activated with $CaCl_2$.	2 ml	$CaCl_2$, glass
Selphyl® (Aesthetic Factors, Wayne, US)	9 ml WB	WB centrifuged at 1100 rpm, (the rotational force was not mentioned) 6 min in a specialised tube containing a separator gel, plasma collected. Plasma transferred to tube containing $CaCl_2$.	4 ml	$CaCl_2$
Thrombin Assessing Device (Thermogenesis, Rancho Cordova, US)	10 ml PPP	PPP combined with ethanol, ceramic beads and $CaCl_2$ to produce AT. AT combined with PRP 1:3 ratio (vol:vol).	24 ml	AT, $CaCl_2$, ethanol, glass

Literature from the late 1980s to early 1990s refers to plasma-derived products as fibrin glue, sealant or gel if the product mainly prepared from PPP. In later years, when platelets were thought to be a beneficial additive, literature often refers to the plasma derived products as PRP, platelet-rich fibrin matrix, platelet-gel, platelet-clot, platelet lysate or plasma rich in growth factors or wound healing factors. The plasma products are produced from a pure platelet concentrate or pellet or combination of PRP and PPP. The platelet yield and volume vary considerably.AT: “autologous thrombin”; $CaCl_2$: calcium chloride; calcium gluconate: $C_{12}H_{22}CaO_{14}$; PPP: platelet poor plasma; PRP: platelet-rich plasma; RBC: red blood cells; vol: volume; WB: whole blood

1.1.6.2 To activate or not to activate the extracted platelets

Most growth factors are stored in a latent form in the platelet granules, which are released after activation and degranulation of the platelets. It is therefore assumed that PRP will only contain active growth factors once the platelets have been activated by thrombin (Everts *et al.*, 2006b). It has been suggested that administered PRP containing non-activated growth factors may be in contact with the wounded tissue but unable to provide the necessary growth signals (Everts *et al.*, 2006b). For this reason some protocols and devices (although none of the devices available in South Africa) combine the collected PRP with calcium chloride ($CaCl_2$) or calcium gluconate ($C_{12}H_{22}CaO_{14}$) with or without non-autologous thrombin (i.e. bovine) (Table 1.3) (Anitua *et al.*, 2004, Eppley *et al.*, 2006, Knighton *et al.*, 1986, Krašna *et al.*, 2007, Mazzucco *et al.*, 2008, Randelli *et al.*, 2008, Wroblewski *et al.*, 2010).

The Ca^{2+} provided by the $CaCl_2$ or $C_{12}H_{22}CaO_{14}$ replenishes the Ca^{2+} bound by the citrate anti-coagulant that was added to prevent coagulation and platelet activation, whereas the thrombin further mimics the final stages of the coagulation cascade (Eppley *et al.*, 2006). By initiating platelet activation the content of the α-granules are released and a fibrin clot can be formed. Just as in normal wounding the newly formed clot is able to act as a growth factor reservoir for the released growth factors at the desired site (Eppley *et al.*, 2006).

Using thrombin from a foreign source is worrisome as the PRP cannot be classified as autologous as it is able to act as an antigen with the possibility of immunological reactions including hypersensitivity reactions in some individuals (Anitua *et al.*, 2004, De Somer *et al.*, 2006). Some protocols require the preparation of autologous thrombin before isolation of the required treatment PRP (Knighton *et al.*, 1986). To prepare autologous thrombin, conversion of prothrombin into thrombin can be achieved with the use of Ca^{2+}, ethanol, and a negatively charged surface such as glass (Anitua *et al.*, 2004, De Somer *et al.*, 2006, Everts *et al.*, 2006b, Knighton *et al.*, 1986). However, this process requires drawing of additional whole blood, a longer preparation time and added procedures, all delaying the preparation of PRP and the possibility to compromise sterility (Everts *et al.*, 2006b).

1.1.6.3 Optimal platelet and growth factor concentration

To induce tissue regeneration the optimal growth factor concentrations are needed. As the growth factor concentration is linearly related to the platelet number, the optimal platelet concentration in the

PRP becomes critical (Eppley *et al*., 2006, Everts *et al*., 2006b). Research suggests that the inconsistency experienced with PRP administration may be due to variations in the platelet concentration in the isolated PRP preparations. Some manufacturers relate a quality PRP product to the volume of platelets extracted. In reality, the platelet concentration is mostly related to the volume of whole blood drawn for the preparation of the PRP and the protocol used to obtain the PRP. Marx initially demonstrated that PRP with a platelet concentration around 3 - 4 fold the baseline platelet concentration was able to induce bone regeneration in mandibular defects (Marx *et al*., 1998). Marx later stated that "working definition" of PRP is a PRP volume with a platelet count of 100 x 10^{10} platelets/l (Marx, 2001). As the average human platelet count ranges from 150 – 400 x 10^9 platelets/l, this would result in a two-and-a-half to 6 fold increase in platelet concentration above baseline. Anitua and co-workers, on the other hand, stated that platelet count in excess of 300 x 10^9 platelets/l would be able to induce tissue regeneration (Anitua *et al*., 2004). A PRP volume with a high platelet concentrate may not result in tissue healing; in fact the opposite may occur, as Creaney *et al*., (2011) found that normal concentrations of platelets appeared to be more efficacious as an increased concentration thereof in patients treated with elbow tendinopathies who failed conservative treatment (Creaney *et al*., 2011, Everts *et al*., 2006b). Weibrich *et al*., (2004) determined the effect of varying concentrations of platelets in the PRP volume on peri-implant bone regeneration. The authors inserted titanium screws in the right and left distal femur of 20 New Zealand white rabbits. One of the implant sites of each rabbit was treated with a low, intermediate or high platelet concentration PRP before closure. The authors noted that a intermediate platelet concentration of two to eight fold the basal platelet count was able to substantially increase bone regeneration (Weibrich *et al*., 2004). The authors noted that a high platelet concentration of 6 - 11 times the basal platelet concentration appeared to have an inhibitory effect on the osteoblasts as TGF-β, one of the most abundant growth factors found in PRP, is known to have an anti-mitogenic effect at high concentrations.

1.1.6.4 Contraindications to the use of PRP

As PRP therapy is based on the ability of the platelets to release growth factors and other mediators that lead to enhanced regeneration of the desired area, patients with a low platelet count or who are on anti-platelet therapy are typically excluded from treatment (Foster *et al*., 2009, Zenker, 2010).

Patients treated for sports related soft tissue injuries will generally only be considered for PRP treatment once conservative treatment and physiotherapy has failed or as an alternative to corticosteroid injection therapy (Foster *et al*., 2009). PRP treatment will only be considered for intra-articular injections in early degenerative diseases or as a prevention of a surgical intervention (Foster *et al*., 2009, Kon *et al*., 2010, Kon *et al*., 2012, Tinsley *et al*., 2012).

1.1.6.5 Clinical studies and applications

PRP is used to treat notoriously difficult to heal injuries or wounds where limited blood perfusion decreases the platelet content and thus growth factor concentration at the wound or injury site.

Bone reconstruction, particularly in the field of dentistry, led the way in the use of PRP in tissue regeneration (Marx *et al*., 1998). This was followed by a plethora of studies that describe the use of PRP in sports related soft tissue injuries, generally with positive results. PRP therapy is typically used in acute and chronic tendinopathies and chronic refractory plantar fasciitis to accelerate healing and reduce pain, and in osteoarthritis and rotator cuff repairs where conservative treatment has failed, to prevent surgical intervention or PRP combined with surgery to accelerate healing (Chahal *et al*., 2012, Foster *et al*., 2009, Kon *et al*., 2009, Kon *et al*., 2010, Kon *et al*., 2012, Sánchez *et al*., 2012, Tinsley *et al*., 2012).

Kon *et al*., (2009) treated 20 male athletes with chronic patellar tendinosis with PRP therapy who had failed to respond to conservative therapy. The patients were followed up 6 months after the injections and the authors noted statistically significant improvement in the patients' pain scores and ability to return to full-tendon loading activity. Kon *et al*., (2010) later did a similar study where they treated patients with chronic osteoarthritis (pain and swelling of the knee for more than 4 months) with a Kellgren score of 0 - IV with three PRP intra-articular injections. The authors noted that their preliminary results indicate that the treatment with PRP injections reduced knee pain and improved knee function and quality of life in younger patients who did not have severe articular degeneration.

Knighton *et al*., 1986, treated 49 patients with chronic non-healing cutaneous ulcers with a PRP-gel applied topically to the wound site. The authors noted that the wounds had new granulation tissue formation and accelerated epithelisation with complete healing achieved around 11 weeks after treatment, although they had no control patients in the study (Knighton *et al*., 1986). Kazakos *et al*.,

(2009) investigated the use of a PRP-gel on acute traumatic wounds. The authors treated one group with a PRP-gel applied topically to the wound site once a week and the other with standard conventional treatment (Kazakos *et al*., 2009). The clinical end-point was the time required to bring about adequate tissue regeneration in order to undergo reconstructive plastic surgery. The authors noted that the acute wounds treated with the PRP-gel healed faster and patients were able to undergo plastic reconstructive surgery in nearly half the time when compared to the standard care group. Furthermore, the authors also noted that the PRP-gel treated wounds required smaller skin grafts to cover the wound area in comparison to the standard treatment group.

Fewer studies are available that describe the use of PRP to treat the ageing skin and wound healing, although the use of PRP as so-called “Dracula injections” have received popular media attention (Cho *et al*., 2011, Na *et al*., 2011, Sclafani, 2010, Zenker, 2010). Zenker, (2010) performed a skin rejuvenation trial in a population aged from 35 - 60 years of age. She noted that younger individuals (< 35 years of age) responded better to PRP treatment and only required booster treatments every 12 - 24 months. As the age in the population increased the time to follow up treatment decreased and patients 60 years and older responded less to treatment and required more frequent booster injections.

Sclafani, (2010) used PRP as a single treatment filler and noted that it was well tolerated and can produce a significant correction of deep nasolabial folds without development of excessive fibrosis or the need for an injection of a foreign substance such as fillers (Sclafani, 2010). Cho *et al*., (2010), treated nude mice with UV radiation induced wrinkles and showed that the PRP-injected group had statistically significant reduction in wrinkles compared to the saline-injected group (Cho *et al*., 2011). Na *et al*., (2011) applied PRP after the skin was resurfaced with a fractional carbon dioxide laser. The authors suggested that application of autologous PRP could be an effective method for enhancing wound healing, reducing transient unwanted adverse effects, and improving skin tightening after skin resurfacing (Na *et al*., 2011). These suggestions were confirmed by Shin *et al*., (2012). The authors found that when PRP was applied topically after fractional laser therapy, it lead to improved skin elasticity, a lower erythema index and an increase in collagen density when compared to fractional laser alone.(Shin *et al*., 2012)

In a study to investigate the efficacy of a single injection of PRP for the correction of deep nasolabial folds, none of the patients noted any fibrosis, irregularities or restricted movement (Sclafani, 2010)

Most of the studies that demonstrated positive results were however uncontrolled. These studies also differ on the timing and volume of PRP administration, the platelet concentration used and how the PRP was obtained, the number of PRP applications per patient as well as the study end-points to determine the benefit of the treatment. Even meta-analysis of the available studies on PRP in soft tissue augmentation and bone regeneration had varying opinions on the effectiveness of PRP in healing and regeneration (Bae *et al*., 2010, Plachokovan *et al*., 2008, Sheth *et al*., 2012).

It is clear from the different protocols, kits and devices that no uniformity of the PRP products exists as not only the volume of blood collected differs, but also the platelet extraction techniques, the centrifugal force used and the time of centrifuging (Table 1.3). An optimal platelet concentration, PRP volume and extraction technique should be established to ensure standardisation in the PRP product.

1.2. AIM AND OBJECTIVES

1.2.1 AIM

The aim of this study was to develop a simple and rapid method for preparing autologous PRP, in an office setting using a tabletop centrifuge for point-of-care use.

The PRP should be enriched to a platelet concentration that provides sufficient autologous TGF-β and PDGF capable of enhancing wound healing, without the use of non-autologous thrombin.

1.2.2 HYPOTHESIS

H0: Platelet-derived growth factor can be obtained in therapeutic concentrations using a thrombin free extraction method of PRP

H0: Transforming growth factor beta can be obtained in therapeutic concentrations using a thrombin free extraction method of PRP.

1.2.3 ALTERNATIVE HYPOTHESIS

A thrombin free extraction method of platelet rich plasma does not yield therapeutically usable concentrations of growth factors.

1.2.4 OBJECTIVES

The primary objective of this study is to:

- Quantify the concentrations of PDGF-AB and TGF-β_1, which can be extracted from less than 20 ml freshly drawn blood samples from healthy human subjects.

The secondary objectives of this study are to:

- Determine the concentrations of recombinant PDGF-AB and recombinant TGF-β_1 (alone and in combination) required to increase cell proliferation in a primary fibroblast culture, compared to PDGF-AB and TGF-β obtained from PRP using a thrombin free extraction method.

1.2.5 OUTCOME MEASURES

1.2.5.1 Primary outcome measures

- Simplify and reduce the number of isolation steps while maximizing yield of platelets in the preparation of PRP

- Quantify the PDGF-AB and TGF-β_1 extracted from whole blood drawn from healthy volunteers using commercial Enzyme-Linked ImmunoSorbent Assays (ELISA)

1.2.5.2 Secondary outcome measures

- To determine the minimum growth factor concentration *in vitro* at which cell proliferation takes place, in order to establish the clinical usability of the new extraction method

CHAPTER 2

2.1 MATERIALS AND METHODS

2.1.1 MATERIALS

2.1.1.1 Media and reagents

PRP preparation

10% Calcium chloride

A mass of 10 g calcium chloride (BDH Chemicals Ltd., Poole, UK) was dissolved in 100 ml deionised water.

Calcium containing platelet poor plasma (PPP) solution

A volume of 100 µl 10% calcium chloride solution was added to 500 µl PPP.

5% Ethanol

A volume of 5 ml absolute ethanol (Merck, Darmstadt, DE) was made up to 100 ml using deionised water.

20% Ethanol

A volume of 20 ml absolute ethanol was made up to 100 ml using deionised water.

Citrate dextrose anticoagulant (0.15 M citrate, 2% glucose pH 4.2)

To prepare the citrate dextrose anticoagulant, a mass of 8 g of citric acid monohydrate (Sigma-Aldrich, St. Louis, US), 22 g of anhydrous dextrose (Sigma-Aldrich, St. Louis, US) and 24 g of sodium citrate dihydrate (Sigma-Aldrich, St. Louis, US) was dissolved in 1 l of deionised water. The solution was mixed well with the aid of a magnetic stirrer. The solution was filter sterilised by passing though a 0.22 µm filter (Merck Millipore, Darmstadt, DE).

Phosphate buffered saline solution (PBS, pH 7.4)

A mass of 9.23 g BBL™ FTA Hemagglutination buffer (Becton, Dickinson and Company, Franklin Lakes, US) was dissolved in 1 l of deionised water. The PBS solution was sterilised by autoclaving (HICLAVE, Hirayama Manufacturing Corp., Kasukabe-Shi, Japan), the solution for 20 min at 121°C.

Bradford assay

Bradford reagent

A mass of 100 mg of Coomassie Brilliant Blue G250 was dissolved in 50 ml of 96% ethanol (Merck, Darmstadt, DE). A volume of 100 ml of 85% phosphoric acid (Merck, Darmstadt, DE) was added to the Coomassie Blue solution. The volume of the solution was made up to 1 l with deionised water. The Bradford reagent was filtered through a Whatman no. 1 filter paper (Whatman International Ltd., Maidstone, UK) and then stored in a 1 l Schott bottle (covered with foil to protect the reagent from light) at room temperature.

BSA standards

A stock solution was prepared by dissolving 10 mg BSA powder in 10 ml of PBS; the solution was gently agitated to avoid foaming. From the stock solution, further dilutions (to achieve final concentrations of 200 mg/ml, 300 mg/ml, 400 mg/ml, 500 mg/ml, 600 mg/ml, 700 mg/ml, 800 mg/ml and 900 µg/ml) were prepared. The dilutions were prepared by adding 200 µl, 300 µl, 400 µl, 500 µl, 600 µl, 700 µl, 800 µl or 900 µl of the stock solution to 800 µl, 700 µl, 600 µl, 500 µl, 400 µl, 300 µl, 200 µl or 100 µl PBS. The blank containing no BSA was included in the calibration curve.

SDS-PAGE

30% Acrylamide stock solution (3.4% cross linked)

A mass of 145 g acrylamide (Merck, Darmstadt, DE) and 5 g *N, N'*-methylenebisacrylamide (Merck, Darmstadt, DE) cross linker was dissolved in 300 ml deionised water. The solution was warmed on a hotplate (Fried Electric Ltd., Haifa, IS) to no more than 37°C to facilitate dissolving. The final volume

was adjusted to 500 ml by the addition of deionised water. The solution was aliquoted into 50 ml polypropylene tubes (covered with foil to protect the reagent from light).

10% Ammonium persulfate (APS)

A mass of 1 g APS (Promega, Madison, US) was dissolved in 10 ml deionised water and stored at 4℃ until use.

1% Bromophenol blue

A mass of 100 mg Bromophenol blue (3',3",5',5"-tetrabromophenolsulfonphthalein) powder (Sigma-Aldrich, St. Louis, US) was dissolved in 10 ml deionised water.

De-stain solution

A volume of 50 ml methanol (Minema, Jhb, ZA) and 10 ml glacial acetic acid (Merck, Darmstadt, DE) was dissolved in 40 ml deionised water.

0.25% Coomassie Blue reagent

A mass of 250 mg of Coomassie Brilliant Blue G250 (Polyscience, Inc., Warrington, US) was dissolved in 100 ml de-stain solution.

1 M or 1N HCl

A volume of 4.8 ml 32% HCl (Merck, Darmstadt, DE) was added to 40 ml then diluted to 50.0 ml with deionised water.

0.5 M Tris pH 6.8

A mass of 30.275 g Tris (hydroxymethyl)-aminomethane (Merck, Darmstadt, DE) was dissolved in 400 ml deionised water with the aid of a magnetic stirrer. The pH was adjusted to 6.8 by adding 1 M HCl drop-wise to the solution. The final volume of the solution was adjusted to 500 ml by the addition of deionised water.

1.5 M Tris pH 8.8

A mass of 90.825 g Tris (hydroxymethyl)-aminomethane was dissolved in 400 ml deionised water with the aid of a magnetic stirrer. The pH was adjusted to 8.8 by adding 1 M NaOH drop wise to the solution. The final volume of the solution was adjusted to 500 ml by the addition of deionised water.

20% Sodium dodecyl sulphate (SDS) stock solution

A mass of 20 g SDS (BDH Chemicals Ltd., Poole, UK) was dissolved in 100 ml deionised water. The solution was mixed gently but well with the aid of a magnetic stirrer and warmed on a hotplate to facilitate dissolving. To prepare 10% SDS solution, 5 ml of 20% SDS solution was diluted with 5 ml deionised water.

5% Stacking gel

A volume of 250 µl 0.5 M Tris pH 6.8, 330 µl of a 30% acrylamide stock solution, 20 µl of a 10% SDS stock solution and 20 µl 10% APS (Sigma-Aldrich, St. Louis, US) was dissolved in 1.4 ml deionised water. Just before casting 2 µl N,N,N',N'-Tetramethylethylenediamine (TEMED) was added and rapidly mixed before casting on top of the set resolving gel with a well forming comb in place.

12% Resolving gel

A volume of 1.3 ml 1.5 M Tris pH 8.8, 2 ml 30% acrylamide stock, 50 µl 10% SDS and 50 µl APS was dissolved in 1.6 ml deionised water. Just before casting the gel 2 µl TEMED was added and quickly mixed well before casting the gel into the prepared mini-gel cassette (10 x 8 cm and 1.5 mm thick).

Laemmli sample buffer (5 times concentrate)

A volume of 2.0 ml 0.5 M Tris (hydroxymethyl)-aminomethane pH 6.8, 3.2 ml glycerol (Sigma-Aldrich, St. Louis, US), 1.6 ml 20% SDS, 0.8 ml β-mercaptoethanol (Sigma-Aldrich, St. Louis, US) and 100 µl 1% bromophenol blue was dissolved in 6.8 ml deionised water. The Laemmli sample buffer was aliquoted in 1.5 ml polypropylene microtubes and stored at -20℃ until use.

TGS buffer (10x concentrate)

A mass of 30 g Tris (hydroxymethyl)-aminomethane, 144 g glycine and 10 g SDS was dissolved in 1 l of deionised water. The buffer was stored at 4℃ until use. Before use, the buffer was diluted 10 fold in deionised water.

Cell viability assays

10% Acetic acid solution

A volume of 10 ml glacial acetic acid was dissolved in 70 ml of deionised water mixed and made up to 100 ml.

Cell counting fluid

A volume of 1 ml 0.1% crystal violet solution and 2 ml glacial acetic acid (Merck, Darmstadt, DE) was dissolved in 97 ml deionised water. The solution was mixed well with the aid of a magnetic stirrer and stored at 4^{o}C until used.

0.1% Crystal violet solution

A mass of 100 mg crystal violet powder (Merck, Darmstadt, DE) was dissolved in 100 ml of deionised water produced in house using a reverse osmosis and ion exchange system (ELGA Labwater, Bucks, UK). The solution was mixed well with the aid of a magnetic stirrer. Once the powder had completely dissolved, the solution was filtered through a Minisart® Sartorius 0.22 µm syringe filter.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) stock solution

A mass of 200 mg MTT-powder (Sigma-Aldrich, St. Louis, US) was dissolved in 40 ml PBS solution in a 50 ml polypropylene tube (Greiner Bio-One, Kremsmuenster, AT) covered with foil. The MTT-solution was filter sterilized using a Minisart® Sartorius 0.22 µm filter and stored away from light at 4^{o}C until used.

Dulbecco's Modified Eagle Medium (DMEM)

DMEM was prepared according to the manufacturer's instructions. Briefly, a mass of 13.4 g DMEM powder, supplemented with L-glutamine (D5648-10x1L, Sigma-Aldrich, St. Louis, US) was dissolved in 1 l sterile water with the aid of a magnetic stirrer. To ensure complete solubilisation of the DMEM powder, the pH of the solution was adjusted to 4.0 with 1 M HCl. Thereafter, 3.7 g of sodium bicarbonate ($NaHCO_3$) was added to 1 l of the medium. The pH was re-adjusted to 7.1 by dropwise addition of 1 M NaOH. The medium was filter sterilized through a Minisart® Sartorius 0.22 µm filter and divided into 500 ml aliquots. A volume of 5 ml was removed from each 500 ml aliquot and the solution was supplemented with 5 ml of BM Cyclin to contain a final concentration of 1% penicillin/ streptomycin solution (Roche Diagnostics, Indianapolis, US).

Working solutions contained 0%, 0.1%, 0.5%, 1% or 10% sterile heat inactivated foetal calf serum (HI FCS - PAA Laboratories Inc. Ontario, GC). To prepare medium supplemented with 10% HI FCS, 50 ml of the 500 ml aliquot was removed and the remaining solution supplemented with 50 ml HI FCS. From the medium supplemented with 10% HI FCS, the 1%, 0.5% and 0.1% solutions were prepared by diluting the 10% solution in a ratio of 1:9, 1:19 and 1:99 in serum free DMEM. The medium was stored at 4^{o}C until use.

70% Ethanol

A volume of 73 ml 96% ethanol (Merck, Darmstadt, DE) was made up to 100 ml with deionised water.

10% Formalin solution

A volume of 100 ml of a 36.5 - 38% formalin solution (Sigma-Aldrich, St. Louis, US) was diluted to 370 ml using deionised water.

4 mM HCl containing 0.1% Bovine serum albumin (BSA)

A volume of 40 µl 32% HCl was dissolved in 100 ml of deionised water. A mass of 100 mg powder BSA (Sigma-Aldrich, St. Louis, US) was dissolved in 100 ml of the 4 mM HCl solution.

Recombinant Platelet-derived growth factor-AB (rPDGF-AB)

rPDGF-AB (Sigma-Aldrich, St. Louis, US) stock solution was prepared by reconstituting the total contents of the purchased vial (10 µg) in 10 ml of 0.22 µm filtered 4 mM HCl containing 0.1% BSA. Working solutions were prepared, ranging from 10 ng/ml to 2000 ng/ml by diluting the stock solution with 0.22 µm filtered 4 mM HCl containing 0.1% BSA. Aliquots containing 500 µL of the reconstituted rPDGF-AB were stored in 1.5 ml polypropylene microtubes (J-Plast, Roodepoort, ZA) at -20° until used.

Recombinant Transforming growth factor beta-1 (rTGF-β_1)

rTGF-β_1 (Sigma-Aldrich, St. Louis, US) stock solution was prepared by reconstituting the total contents of the purchased vial (2 µg) in 2 ml of 0.22 µm filtered deionised water.

Working solutions were prepared ranging from 10 ng/ml to 2000 ng/ml by diluting the stock solution with sterile PBS containing 2 mg/ml BSA. Aliquots containing 500 µL of the reconstituted rTGF-β_1 were stored in 1.5 ml polypropylene microtubes at -20°C until used.

1 N Sodium hydroxide (NaOH)

A mass of 2g NaOH (Bio-Zone, Van Riebeeck Park, ZA) was dissolved in 50 ml deionised water.

Tris-HCl buffer (pH 7.5/ 0.15 M NaCl/ 0.1% glucose)

A mass of 2.06 g Tris(hydroxymethyl)-amminomethane (Merck, Darmstadt, DE), 8.77 g sodium chloride and 1 g D-glucose was added to 900 ml deionised water. The solution was well mixed with the aid of a magnetic stirrer. The pH was adjusted to pH 7.5 by addition of either 1M HCl or NaOH. The volume was adjusted to 1 l with deionised water.

Tris-citrate dextrose buffer solution

To prepare the Tris-citrate buffer solution, a volume of 90 ml of Tris-HCl buffer was added to a volume of 10 ml citrate dextrose buffer. The solution was mixed well with the aid of a magnetic stirrer and filter sterilised through a 0.22 um filter.

Trypsin /Versene solution

The Trypsin /Versene solution (Highveld Biological (PTY) Ltd., Sandton, ZA) contained 0.25% Trypsin, 0.05% EDTA solution in a calcium and magnesium free Dulbecco buffer.

0.025% Trypsin / Versene solution

A volume of 5 ml Trypsin / Versene solution was dissolved in 45 ml PBS solution.

Cell cultures

Chicken embryo fibroblasts

The chicken embryo fibroblast cells were primary cultures of cells derived from 5 – 7 day old chicken embryos. Procedures for the isolation of chicken embryo fibroblasts are described in Methods section. The cells were maintained in DMEM supplemented with 10% HI FCS.

Normal human dermal fibroblasts

Human dermal fibroblasts were kindly donated by Southern Biotech (PTY) Ltd. (Centurion, ZA). The cells were maintained in DMEM supplemented with 10% HI FCS.

Vials containing $CaCl_2$ and ethanol for platelet activation

10% Calcium chloride solution

A mass of 13.25 g pharmaceutical grade calcium chloride dihydrate (Merck, Darmstadt, DE) was dissolved in a volume of 100 ml purified water for injection (Altis Biologics Pty Ltd, Pretoria, ZA).

5% Ethanol

A volume of 26 ml pharmaceutical grade 96% ethanol (Merck, Darmstadt, DE) was made up to a volume of 500 ml with purified water for injection.

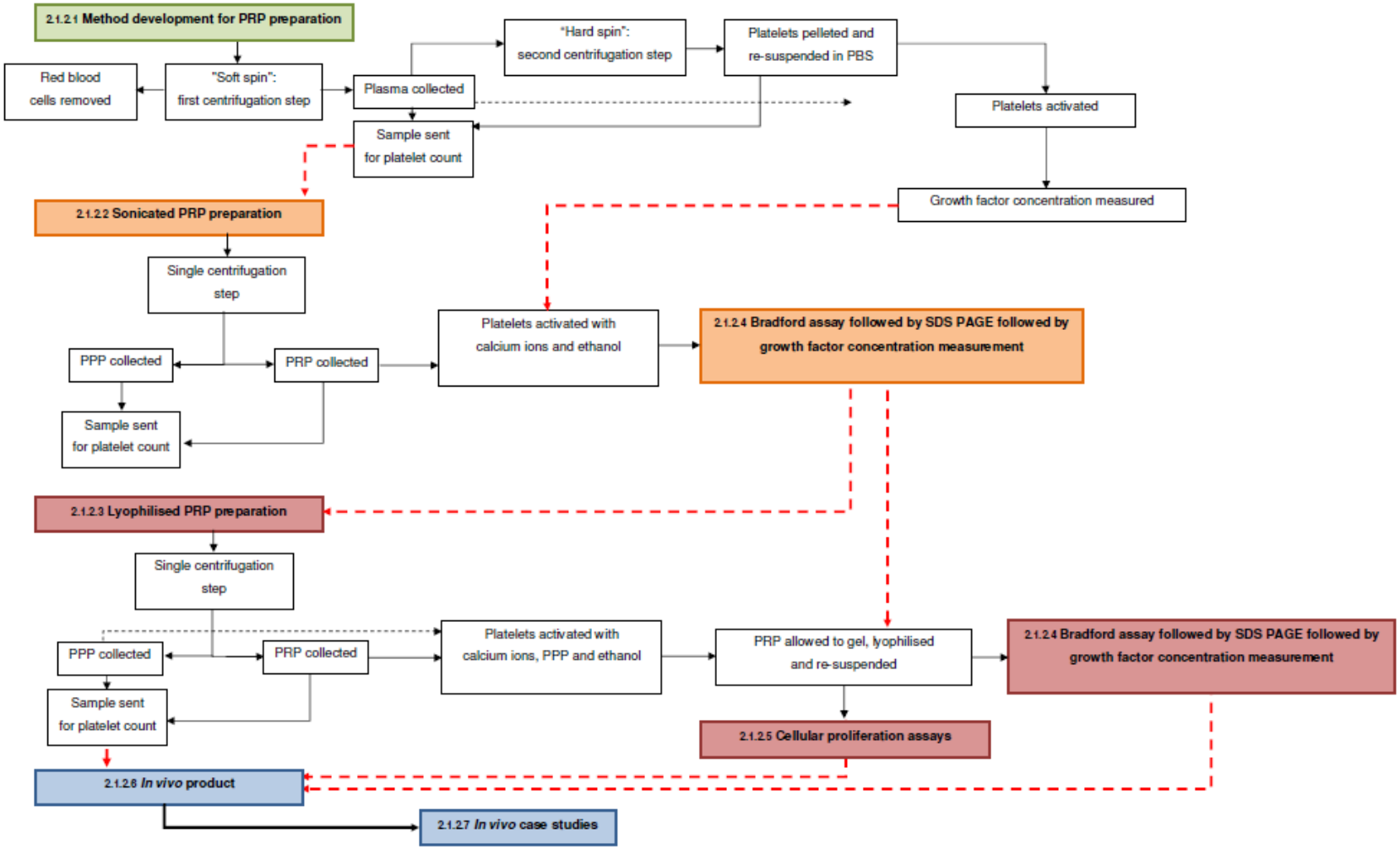

Figure 2.1 PRP preparation method optimisation algorithm. Initial method development was followed by two method optimisation experiments and a final commercial product. The alterations in the two optimisation experiments and final product were based on the results obtained in the previous experiment's results as indicated by the red line.

2.1.2 METHODS

2.1.2.1 Method development for PRP preparation

All procedures were carried out under sterile conditions. To produce a standardised PRP product, the platelet count, PRP volume and extraction technique should be taken into consideration. Literature describing in house protocols were compared from which the initial method for the PRP preparation was developed. Traditionally in house protocols require two centrifugation steps, firstly a “soft-spin” to separate the blood cells from the plasma and secondly a “hard-spin” to separate the plasma from the platelets.

Three hundred millilitres of venous blood was drawn from each of three volunteers into blood bags under strict aseptic conditions. The blood collection bags were pre-loaded with 30 ml citrate dextrose anticoagulant to avoid coagulation. Red blood cells were removed by centrifuging whole blood at 200 *g* at 4°C for 10 min (Sigma 3K15, Harz, DE) in 15 ml polypropylene centrifuge tubes (Geiner Bio-One, Kremsmuenster, AT) and approximately 100 ml plasma was collected in two 50 ml tubes. A 500 µl sample of the plasma was sent to AMPATH National Laboratories (Drs du Buisson, Kramer, Swart, Bouwer Inc., Centurion, ZA) for platelet counting. A 5 ml sample of this plasma was retained and frozen at -70°C (Figure 2.1).

The remaining plasma was divided into 6, 15 ml tubes and centrifuged again at 3200 *g* at 4°C for 15 min (Allegra™ X-22, Beckman Coulter, Brea, US) to pellet the platelets. The supernatant was removed and the platelet pellets washed by re-suspending each pellet in PBS. The pellets were gently agitated by slow inversion 15 times to avoid stimulation or damage of the platelets. The washed platelets were centrifuged again at 3200 *g* at 4°C for 10 min (Antoniades *et al*., 1979, Knighton *et al*., 1982, Knighton *et al*., 1986, Luengo Gimeno *et al*., 2006). The washed pellets were re-suspended in 2 ml of PBS each. A sample of 500 µl of the re-suspended platelets was assayed for platelet count by flow cytometry, based on the principles of light scattering (Figure 2.1). At AMPATH National Laboratories, the ADVIA Hematology System (Siemens AG, Erlangen, DE) is used for obtaining platelet counts.

Aliquots of 1.5 ml were prepared from the remaining re-suspended platelet pellets and the platelet rich plasma and stored at -70°C until use. Repeated freezing and thawing is not recommended due the possibility of protein denaturation, although this action aids in the lysis of the platelets (Bowen-Pope *et al*., 1984, Fernandez *et al*., 1982).

Growth factor quantitation during method development

For quantitation of human PDGF-AB, three of the 1.5 ml aliquots of the re-suspended platelets were thawed on ice for each of the three volunteers. Platelets were re-suspended in the PBS solution to a total volume of 2.5 ml, 2.8 ml or 4.2 ml, with careful agitation to obtain approximately $1x10^9$ platelets/ml. The volume of buffer solution added depended on the platelet count for each sample. Five hundred microlitres of the platelet suspension was added to 500 µl 20% ethanol or to 500 µl calcium and PPP solution. The suspension was gently agitated multiple times and allowed to stand on ice for 15 min. The calcium and PPP solution (autologous thrombin) was prepared by adding 10% $CaCl_2$ to PPP in a 1:5 ratio, volume to volume.

The suspension was centrifuged at 16300*g* (Labnet Spectrafuge 24D, Labnet Laboratories Inc., Edison, US) for 15 min, the supernatant collected and frozen at -70^0C. The calculated number of 5.0 $x10^8$ platelets were used per quantitation assay (Antoniades *et al*., 1979, Knighton *et al*., 1982, Knighton *et al*., 1986, Luengo Gimeno *et al*., 2006).

PDGF-AB

PDGF-specific ELISA (Quantikine® Human PDGF-AB Immunoassay, R&D Systems, Minneapolis, US) was used for the quantitative determination of human PDGF-AB concentrations released from activated platelets. Three samples per volunteer where used in the PDGF-AB specific ELISA.

This assay used the sandwich enzyme immunoassay technique, where a mouse monoclonal antibody specific for PDGF-BB was pre-coated on the microtiter plate by the manufacturer. The immobilised antibody would bind PDGF-AB or PDGF-BB present in the standard or samples. An enzyme-linked polyclonal antibody against PDGF-AA conjugated to horseradish peroxidase is then added to the wells, followed by a substrate solution. Colour development is proportional to the amount of PDGF-AB

bound in the initial step. The colour development is stopped by the addition of 2 N sulphuric acid (stop solution) and the intensity of the colour is measured.

Citrate treated plasma has not been validated by the manufacturer for use with this PDGF-specific ELISA kit. However, Evert *et al.*, 2006 compared the PDGF-AB concentration with the PDGF-specific ELISA Quantikine® Human PDGF-AB Immunoassay from the PRP prepared from three devices all of which used citrate anticoagulant in the preparation of PRP and found no impact on the final PDGF-AB concentration. The final citrate anticoagulant volume was negligible and would therefore not affect the ELISA.

All reagents were prepared according to the manufacturer's instructions and samples, standards and controls were assayed in duplicate. The assay procedure followed the manufacturer's protocol.

Briefly, 100 µl the standard, control (Calibrator Diluent RD6-11) or sample was added per well. The samples included: neat (5.0 x 10^8 platelets/ml), 10 fold dilution, 50 fold dilution and 100 fold dilution of the samples with Calibrator Diluent RD6-11 to a platelet concentration of 5.0 x 10^7 platelets/ml,1.0 x 10^7 platelets/ml or 5.0 x 10^6 platelets/ml per sample respectively. Standards and samples were pipetted into the wells and any PDGF-BB present was bound to the immobilised antibody. After washing away any unbound substances, an enzyme-conjugated monoclonal antibody was added to the wells. Following a wash to remove any unbound antibody, a substrate solution was added to the wells and colour developed in proportion to the amount of PDGF-AB captured in the initial step. The colour development was stopped by addition of a stop solution and the intensity of the colour is measured. The optical density (O.D.) was determined using a microplate reader (ELx 800 UV Universal microplate reader, BioTech instruments Inc., Vermont, US) set to a wavelength of 450 nm and reference wavelength of 570 nm. The concentrations of the PDGF-AB standards were plotted against the corresponding absorbance to give a standard curve used to determine the total PDGF-AB in the unknown samples.

The method that yielded the highest PDGF concentrations per extract was used for further experimentation.

2.1.2.2 Sonicated PRP preparation

Sixteen milliliters venous blood was drawn using two 8 ml BD Vacutainer® CPT™ Cell Preparation tubes (Becton, Dickinson and Company, Franklin Lakes, US) from 30 volunteers under strict aseptic conditions. According to the manufacturer's protocol, the BD Vacutainer® CPT™ Cell Preparation tubes were gently inverted 8 times after the blood was drawn to ensure correct mixing of the anticoagulant and centrifuged at 1800 *g* for 20 min at room temperature within 2 hours of blood collection. After centrifugation, the mononuclear cells and platelets were visible as a whitish layer at the bottom of the plasma layer (Figure 2.2). This layer is further referred to as the PRP layer. The mononuclear cells were not removed from the PRP fraction, as this would require additional steps with the possible risks of contamination. The concentration of mononuclear cells in the samples were not measured, but according to the manufacturer approximately $7.02 \times 10^6 \pm 21.44 \times 10^6$ cells is recovered per 8 ml of whole blood (Becton Dickinson and Company, 2009).

BD Vacutainer® CPT™ tube

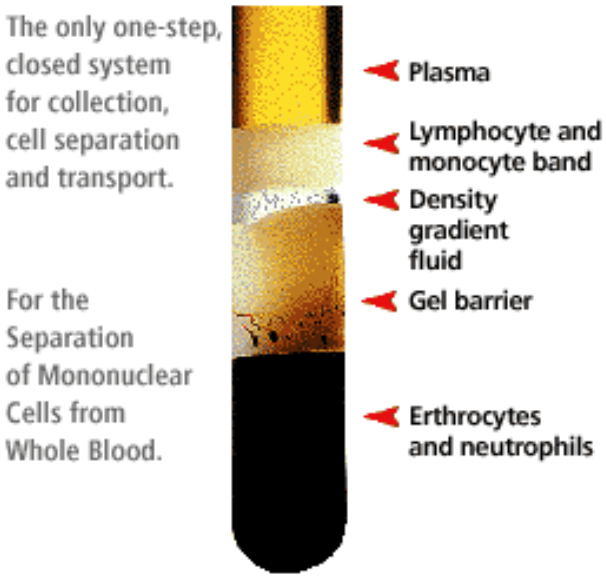

Figure 2.2 Layering of formed elements in the BD Vacutainer® CPT™ Cell Preparation tubes. From Becton Dickinson and Company, 2009. After centrifugation the plasma was collected and labelled as platelet poor plasma (PPP), thereafter the mononuclear cells and platelets were collected and labelled as platelet-rich plasma (PRP).

The following was conducted under sterile conditions. Approximately two thirds of the upper layer of plasma was collected with a sterile 3 ml plastic pasture pipette (J-Plast, Roodepoort, ZA) without disturbing the cell abundant lower plasma layer and labelled as PPP. The remaining cell containing plasma layer was collected with a sterile 3 ml plastic pasteur pipette and transferred into 15 ml polypropylene centrifuge tubes. A 500 µl sample of the PRP and PPP layers from each sample were assessed for platelet count. The volumes of each of the harvested PRP and PPP fractions were recorded.

A volume of 1.5 ml 10% calcium chloride solution was added to the PPP followed by the addition of 0.5 ml 5% ethanol to the PRP. Both suspensions were gently agitated and allowed to stand for 5 min at room temperature. A volume of 1 ml PPP was added to the PRP suspension and left to stand until a fibrin clot had formed.

As the formed platelet gel acts as a reservoir for the released growth factors, an attempt to disrupt the fibrin gel and release the available growth factors by ultrasonication (Bransonic 52, Krackeler Scientific Inc., Albany, US) for 5 min at 40 kHz and 120 W followed by centrifugation at 16000 *g* for 5 min at room temperature was done. The supernatant was collected and stored at -70℃.

2.1.2.3 Lyophilised PRP preparation

The concentration of growth factor obtained from the fibrin clot supernatants after ultrasonic treatment were extremely low (see results) which necessitated a different approach to obtain free growth factors. Therefore, the abovementioned PRP preparation procedure was repeated as before with slight alterations with the final clot lyophilised (Figure 2.1).

An additional 3 ml BD Vacutainer® K2E tube (purple EDTA tube) was also collected to assess the platelet count in the whole blood from each volunteer before the PRP preparation procedure. To determine the platelet count in whole blood AMPATH National Laboratories requires an additional EDTA tube to be collected from the patient. Platelet counts on the whole blood, the PRP and the PPP samples were performed. The volumes of the harvested PRP and PPP were recorded to calculate the percentage recovery.

To further optimise the PRP preparation procedure and establish which components would be available in the commercially available kit, the all components were added to 10 ml sterile glass multidose vials (Anchor Rand, Tulisa Park, ZA).

The total volume of PPP (approximately 7 ml, see results) was added to a sterile glass vial that already containing a volume of 1.5 ml sterile 10% calcium chloride. Transfer of the PPP was carried out with a sterile 3 ml, unlubricated polypropylene syringe (New Promex Corp., Bergvlei, ZA) and 21 G needle (Becton Dickinson and Company, Franklin Lakes, US). The PPP suspension was gently agitated and left at room temperature. The needle on the syringe was re-capped to maintain sterility. The total volume of PRP (approximately 3 - 6 ml, see results) was added to a sterile 10 ml glass vial containing 0.5 ml 5% ethanol. The PRP was transferred with the same sterile 3 ml, uncoated polypropylene syringe and 21 G needle. The PRP was gently agitated and allowed to stand at room

temperature for 5 min. A volume of 1 ml PPP suspension was added to the PRP suspension and gently agitated and left at room temperature until a fibrin gel had formed (Everts *et al.*, 2006a).

The vial seal was broken and the fibrin gels were transferred to 15 ml polypropylene containers and frozen at -70°C and lyophilised in a Freezone® 6 lyophilisation system (LABCONCO, Kansas City, US). Once the platelet gels were completely lyophilised, the gel powder was re-suspended, to the corresponding PRP volume obtained for that particular sample, in sterile PBS. The tubes were repeatedly inverted to dissolve the dry gel powder. The dissolved gel powder was divided into 1.5 ml aliquots and stored at -70°C.

2.1.2.4 Qualitative and quantitative analysis of protein content for the sonicated and lyophilised PRP preparations

Bradford assay

Protein concentration of the total protein recovered by the optimised preparation method was determined by the protein quantification method outlined by Bradford (1976), using a 96-well round bottom cell culture plates.

The samples were diluted 100 fold in PBS. One hundred microliters of PBS, BSA standards or the diluted samples were transferred into 10 ml polypropylene test tubes. Five millilitres of the Bradford assay solution was added to each of the polypropylene test tubes. The solutions were gently agitated with a pipette to avoid foaming of the proteins and incubated at room temperature for 2 min. Two hundred microlitres of the prepared blank, each of the samples and the BSA standards were added to a round bottom 96-well plate in triplicate and the absorbance measured at 595 nm on a FLUORstar OPTIMA (BMG Labtech, Offenburg, DE).

The concentrations of the BSA standards were plotted against the corresponding absorbance to give a standard curve used to determine the total protein in the unknown samples.

SDS-PAGE

The extracted growth factor preparations were assayed by electrophoresis on 12% SDS-PAGE gels and analysed after staining with Coomassie Brilliant Blue. Molecular weight standards from Fermentas (Inqaba Biotechnological Industries (Pty) Ltd., Pta, ZA) were used for mass calibration.

The extracted samples were prepared according to the method described by Laemmli (1970) and BIORAD instruction manual for Mini-PROTEAN 3 for SDS-PAGE analysis. Briefly, the samples were diluted 2 fold or 10 fold in deionised water. The samples and standards were further diluted 5 fold in Laemmli sample buffer and boiled for 4 min at 95℃.

The 12% resolving gel was prepared and cast into the SDS-PAGE apparatus (SE 245 Dual Gel Caster Hoefer, Inc., San Francisco, US), overlaid with 5% ethanol and allowed to set. Once the 12% resolving gel was set, the top surface was dried, quickly washed with stacking gel solution before the 5% stacking gel was cast onto the 12% resolving gel, the sample well comb inserted and left to set. Twenty micro litres of the standard or samples were loaded into individual wells created by the comb. The TGS buffer solution was diluted and added to the electrophoretic unit (SE 260 Mini-vertical Gel Electrophoresis Unit Hoefer, Inc., San Francisco, US) and the electrophoretic analysis performed at 100 V for 2 hours (Hoefer power supply PS 300-B Hoefer, Inc., San Francisco, US). Thereafter, the gel was stained in a solution of 0.25% Coomassie Blue overnight and followed by de-staining for 2 hours (Laemmli 1970; BIORAD; Promega).

TGF-β_1 has a molecular mass of 25 kDa. Once reduced by the reducing buffer, two co-migrating subunits were expected to be seen on the polyacrylamide gel at a molecular mass of 13 kDa (Desjardins *et al*. 1985).

PDGF-AB has a molecular mass of 30 kDa. Each molecule comprises of two covalently linked subunits, once reduced by the reducing buffer, the two subunits were expected to be seen on the polyacrylamide gel at a molecular mass of 16kDa for the A chain and 14kDda for the B chain (Ross. 1989).

Growth factor quantitation

Quantitative determination of human PDGF and TGF-β_1 was conducted using ELISA kits specifically for determining human growth factors the, using 1.5 ml of the -70°C frozen platel*et ali*quots after thawing on ice. With careful agitation the platelets were re-suspended.

Platelet suspensions were standardised when using the sonicated or lyophilised PRP preparations. The initial growth factor quantitation described above was intended to determine the concentration of growth factor obtained after the platelets had been treated with different platelet or growth factor activators to determine which solution would be best suited for the job. To compare the PRP preparation method to the available literature the platelet suspensions were not diluted to a standard concentration, as the platelet count varied between samples the amount of platelet-derived growth factor obtained would vary from sample to sample. The quantitative PDGF-specific ELISA was performed as described above.

TGF-β_1

A quantitative TGF-β_1-specific ELISA kit for Human / Mouse (TGF-β_1 ELISA kit, eBiosciences, San Diego, US and Quantikine® Human TGF-β_1 Immunoassay, R&D Systems, Minneapolis, US) were used for the measurement of activated human TGF-β_1 concentrations released from activated platelets without sonication, supernatants collected from the sonicated fibrin gels and the reconstituted lyophilised fibrin gels.

This assay used the sandwich enzyme immunoassay technique, where a mouse monoclonal antibody specific for TGF-β_1 was pre-coated on the microtiter plate by the manufacturer. The immobilised antibody binds TGF-β_1 present in the standard or samples. An enzyme-linked polyclonal antibody against TGF-β_1 conjugated to horseradish peroxidase is then added to the wells, followed by a substrate solution. Colour develops in proportion to the amount of TGF-β_1 bound in the initial step. The colour development is stopped by the addition of a diluted HCl solution (stop solution) and the intensity of the colour is measured.

All reagents were prepared according to the manufacturer's protocol. All samples, standards and controls were assayed in duplicate.

Briefly, the samples were diluted 10-fold before acid treatment. To activate the samples, 20 µl 1 N HCl was added to 100 µl of sample. The samples were incubated for 10 min at room temperature and neutralized with 20 µl 1 N NaOH. Fifty microlitres Assay Diluent RD1-73 was added to each well, followed by 50 µl of standard, background control (PBS), or activated sample per well. The microtitre plate was gently tapped to mix the contents and incubated for 2 hours at room temperature. Thereafter each well was aspirated and washed for a total of four washes by filling each well with a total of 400 µl of Wash Buffer per wash per well. After the last wash, any remaining Wash Buffer was removed and the microtitre plate blotted on a clean paper towel. One hundred microlitres of TGF-β_1 Conjugate was added to each well and incubated for 2 hours at room temperature. The microtitre plate was washed again to remove any excess TGF-β_1 Conjugate. One hundred microlitres of Substrate Solution was added to each well, incubated for 30 min at room temperature whereafter 100 µl of Stop Solution was added to each well. The microtitre plate was gently tapped to ensure thorough mixing. The optical density was determined using a microplate reader set to a wavelength of 450 nm and reference wavelength of 570 nm. The concentrations of the TGF-β_1 standards were plotted against the corresponding absorbance to give a standard curve used to determine the total TGF-β_1 in the unknown samples.

2.1.2.5 Cellular proliferation assays

Isolation of chicken embryo fibroblasts

All work was carried out under the guidelines of SANS 10386:2008: "The care and use of animals for scientific purposes". The Animal Use and Care Committee of the University of Pretoria approved the Research Protocol (H22/06 – The use of chicken embryo fibroblast cultures in toxicity assays (2006)). Chick embryo fibroblasts were harvested using an adapted method of Knox *et al*., (1979). Fertilized chicken eggs, obtained from Eagle's Pride Hatcheries (Montana Park, ZA) were used to obtain a primary fibroblast culture, by euthanizing 7 day post fertilization embryos.

Briefly, the 7 day old incubated chicken eggs were sterilized externally by spraying the eggs with 70% ethanol. The top of the egg shell was gently cracked and removed with a pair of sterile forceps. The allantoic membrane was opened and the embryo removed from the egg with another pair of sterile forceps, decapitated and placed in a sterile Petri dish (Becton Dickinson and Company, Franklin Lakes, US). The embryo was finely minced using opposing scalpels, rinsed with pre-warmed RPMI supplemented with 10% HI FCS and placed in a 50 ml polypropylene tube. (The pre-warmed medium was prepared by placing the 50 ml polypropylene container containing the medium in a 37°C humidified incubator gassed with 5% CO_2 - 95% air (C150, Binder GmbH, Tuttlingen, DE), an hour before the chicken embryos were euthanized.

Once the suspension had settled, the medium was removed and the pieces again washed with pre-warmed medium and the medium removed. The minced pieces were incubated for 15 min at 37°C with 10 ml 0.025% trypsin on a plate shaker (K-model VRN-200, Gemmy Industrial Corp., Taipei, TW).

The supernatant containing the detached cells were discarded and the trypsinisation repeated. The supernatant containing the detached cells after the second digestion step was collected, into a sterile 15 ml polypropylene centrifuge tube, the tube was filled with fresh medium containing 10% HI FCS and the cells pelleted by centrifugation at 200 *g* for 5 min. The pooled cells were re-suspended in 1 ml medium and transferred into a 75 cm^2 culture flask (Greiner Bio-One, Kremsmuenster, AT). An additional 5 ml DMEM supplemented with 10% HI FCS was added to the culture flask. The procedure above was repeated until enough flasks were prepared. The cells were incubated at 37°C in a humidified incubator gassed with 5% CO_2 - 95% air. After 24 h, the cultures were rinsed with fresh DMEM supplemented with 10% HI FCS to remove unattached cells and an additional 5 ml medium added. After the cultured cells reached confluence viable embryonic chicken fibroblasts were sub-cultured in DMEM supplemented with 10% HI FCS and incubated at 37°C in a humidified incubator gassed with 5% CO_2 -95% air.

Harvesting of cells

Cells that reached satisfactory growth were ready to be harvested. The culture medium from a 75 cm^2 tissue culture flask was aspirated and the cell layer rinsed with 5 ml Trypsin/Versene solution. The cells were harvested by adding 5 ml Trypsin/Versene solution to the 75 cm^2 culture flask and incubated for 5 - 10 min until the cells detached from the bottom of the flask.

Further trypsination of the cells were inhibited by the addition of 5 ml of the appropriate serum supplemented medium to the flask. The cell suspension was aspirated into 15 ml polypropylene centrifuge tubes, and the tubes filled with serum free medium. The tubes, containing 15 ml of the cell suspension, were centrifuged at 200 *g* for 5 min. The supernatant was discarded and the cell suspension was re-suspended in either a volume of 1 ml serum free medium, medium supplemented with 0.1% FCS, medium supplemented with 0.5% FCS, medium supplemented with 1% FCS or medium supplemented with 10% FCS.

Cell counting

A volume of 50 µl of the 1 ml cell suspension was added to a volume of 450 µl cell counting fluid. Approximately 20 µl of the cell suspension containing the counting fluid was added to the counting chambers on a hemocytometer (Reichert, NY, US). The cells were counted with the aid of a light microscope (Reichert-Yung Microstar 110, NY, US) at a magnification of 400 x.

The cell suspension was diluted in the 15 ml polypropylene centrifuge tubes to 5.0 x 10^4 cells/ml based on the cell count of the alliquot.

Cell proliferation procedure

Round bottom 96-well sterile tissue culture plates (Nalgene Nunc International, Penfield, US) were prepared by adding a volume of 80 µl of the appropriate medium to the wells. Thereafter a volume of 100 µl of the previously prepared cell suspension was added to the wells. The tissue culture plates were incubated for 1 hour at 37°C in a 5% CO_2 atmosphere in a closed container with sterile deionised water before the addition of a volume of 20 µl of the platelet extract or growth factors. A

concentration range of 1 - 100 ng/ml was prepared for the recombinant growth factors and 0 - 10% of the lyophilised PRP preparation. Chicken embryo fibroblasts were treated with rPDGF-AB or rTGF-β_1. Normal dermal fibroblasts were treated with the lyophilised PRP preparation from volunteers.

The negative control groups received a volume of 20 µl of the appropriate serum free medium or in the case of the recombinant growth factors, the appropriate reconstitution buffer used to prepare the recombinant growth factors. The positive control wells received a volume of 20 µl of the HI FCS to give a final volume of 200 µl per well. Thereafter the cell cultures were incubated for 24 or 72 hours at 37°C in a 5% CO_2 atmosphere in a closed, but not sealed container with sterile deionised water. At the termination of the incubation period, the viable cells were enumerated using either the MTT or crystal violet assay.

Enumeration of viable cells

MTT-assay

MTT, a yellow water-soluble substrate is used to measure viability, proliferation and activation of cells (Mosmann, 1983). The MTT-assay is based on the ability of the mitochondrial dehydrogenase enzymes in living cells to reduce the yellow water-soluble MTT substrate into a water-insoluble dark purple formazan product (Vega-Avila and Pugsley, 2011). The amount of formazan produced in the assay is directly related to the number of viable cells (Vega-Avila and Pugsley, 2011).

The MTT-assay can be used to distinguish between cell proliferation and activation as it is able to detect cells which are not dividing but are still metabolically active (Vega-Avila and Pugsley, 2011).

After the cell incubation periods, a volume of 20 µl of the MTT solution was added to each well of the tissue culture plate. The cell cultures were re-incubated for 4 hours at 37°C in a 5% CO_2 atmosphere. At the termination of this incubation period, the tissue culture plates were centrifuged at 700 *g* for 10 min. The supernatant was carefully aspirated from the wells to avoid the loss of sedimented cells. The wells were washed with a volume of 150 µl PBS per well to remove any remaining coloured culture media. The tissue culture plates were centrifuged at 700 *g* for 10 min and the supernatant again aspirated without disturbing the pellet.

The tissue culture plates were left to dry in the dark where after 100 µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, US) was added to each well to solubilise the purple formazan crystals. The tissue culture plates were gently shaken until the formazan crystals were completely dissolved. The tissue culture plates were protected from light. The O.D. of each well was determined using a microplate reader set to 570 nm and using a reference wavelength of 630 nm.

Crystal violet assay

Crystal violet, also known as Gentian violet, is a triphenylmethane dye able to stain DNA (Bonnekoh *et al*., 1989). The Crystal violet assay provides quantitative information about the relative density of the cells as the amount of dye taken-up and the intensity of purple colour produced are proportional to the number of cells (Vega-Avila and Pugsley, 2011).

At the termination of the incubation period, the medium was carefully aspirated from the wells to avoid disturbing the cell layer. To ensure all the medium has been removed, the tissue culture plates were blotted upside down on a clean paper towel. A volume of 100 µl of the 10% formalin solution was added to each well of the tissue culture plate. The tissue culture plates were incubated for 1 hour at room temperature. Thereafter the formalin was aspirated from each well and the tissue culture plate blotted against a clean paper towel to ensure the complete removal of the formalin. The tissue culture plates were left to dry. Once dry, 100 µl of 0.1% crystal violet solution was added to each well and incubated for 30 min. After the incubation period, the 0.1% crystal violet solution was aspirated from each well and the tissue culture plate gently rinsed under running tap water until the water was clear. The plates were left to dry, after which a volume of 100 µl 10% acetic acid was added to each well to solubilise the purple stain. The tissue culture plates were gently shaken until the purple stain was completely dissolved. The tissue culture plates were protected from light during the shaking. The O.D. of each well was determined using a microplate reader set to 570 nm. Three wells served as blanks, these wells did not contain cell cultures, but were fixed and stained with 0.1% crystal violet the same as the experimental wells.

2.1.2.6 Preparation of vials containing $CaCl_2$ and ethanol for *in vivo* use

The following was conducted in a GMP accredited, clean, aseptic room at Altis Biologics under the supervision of Dr Duneas and the responsible pharmacist. Before production started the particle and microbiological plate count was determined by Vivid Air CC (Irene, ZA) and confirmed that the facility complies with the necessary ISO standards. During the preparation of the products, all basic clean room regulations were followed and any items that may compromise the integrity of the clean room removed. A clean room garment, overshoes, sterilised gloves, hairnet, facemask and hood were worn at all times.

In a cleaned and sterilised washbasin, all glassware and metal spatulas to be used in the facility were washed and disinfected by completing the following steps three times. The glassware and metal spatulas were firstly washed in diluted soapy water, followed by rinsing the equipment in hot tap water, then cold tap water and finally deionised water. The glass equipment was transferred into the preparation room and rinsed with deionised water three times. Thereafter the glassware and metal spatulas were placed in an ultrasonic bath for 1 min and rinsed with purified water. The glassware and metal spatulas were placed on stainless steel trays, covered with foil and dry heat sterilised at 250°C for 45 min. Once cooled, the glassware and metal spatulas were placed in the preparation room. All plastic or plastic covered items were sterilised by spraying with 70% ethanol before being moved to the preparation room. The stoppers and metal caps (Anchor Rand, Tulisa Park, ZA) for the 10 ml multidose vials were sterilised by autoclaving them in a glass beaker with water covered with foil for 20 min at 121°C.

The final products were prepared by filtering 1.5 ml of the 10% calcium chloride solution or 0.5 ml of the 5% ethanol solution with a Minisart® Sartorius 0.22 µm syringe filter into a 10 ml sterile multidose vial, the stopper and metal cap placed on the vial and closed with a crimping device.

The sealed vials were sterilised by autoclaving in a glass beaker with water covered with foil for 20 min at 121°C. A total of 60 calcium chloride and 60 ethanol vials were prepared. Three samples from each type of vial together with two BD Vacutainer® CPT™ Cell Preparation tubes were set aside for endotoxin testing.

Endotoxin testing

A qualitative test for Gram negative bacterial endotoxin, Limulus Amebocyte Lysate (LAL) PYROGENT™ plus Single Test Vials (LONZA, Basel, CH), was used to ensure the final commercial calcium chloride vials, ethanol vials and BD Vacutainer® CPT™ Cell Preparation tubes were pyrogen free.

This assay is intended to be used as an *in vitro* end-product test for human parenteral drugs, biological products and medical devices using the gel-clot method. The assay is based on the US FDA guidelines for testing human parenteral drugs, biological products and medical devices. Endotoxins are detected as follow: the Gram-negative bacteria endotoxin catalyzes the activation of a proenzyme in the LAL. The activated enzyme (coagulase) hydrolyzes specific bonds within a clotting protein (coagulen) also present in the LAL. Once hydrolyzed, the coagulen self associates and forms a gelatinous clot. The samples are visually inspected for the presence of a gelatinous clot.

All reagents were prepared according to the manufacturer's protocol and samples, standards and controls were assayed in duplicate. The assay procedure was followed according to the manufacturer's instructions. All reagents were allowed to equilibrate to room temperature before use. The vials containing LAL served as the test. All reagents used were endotoxin free. The endotoxin standard was prepared by reconstituting the lyophilised endotoxin with 10 ml LAL Reagent Water to a concentration of 1.0 EU/ml. The standards were prepared by 6 serial two fold dilutions to 0.03 EU/ml.

Briefly, 250 µl of the negative control (LAL Reagent Water), positive controls or samples were added to the LAL vials. The contents of the vials were gently mixed and immediately incubated in a water bath for 1 min at 37ºC. At the end of the incubation period each vial was carefully removed and inverted 180º to determine whether a firm gel, that remains intact shortly, had formed.

2.1.2.7 *In vivo* case studies, a pilot study

To confirm the clinical effect of PRP prepared using the extraction procedure, a case study registry to investigate the efficacy of activated PRP for various cosmetic and sports related indications was opened. The Faculty of Health Sciences Research Ethics Committee of the University of Pretoria

(Research Protocol 78/2007) as well as the South African Medical Association (SAMA) approved the investigation of PRP. The study was conducted in accordance with the approved protocol, the ICH Guideline for GCP, and the Declaration of Helsinki. According to the South African Medical Device Industry Association (SAMED) “randomised, double blinded clinical trials as for pharmaceutical products are not generally conducted for medical devices” and “applications for clinical trials should be reviewed by an ethics committee and follow approved protocols” (Pearce, 2011).

Physicians were asked to include patients, which they felt, would benefit from PRP treatment for their condition, were able to fully understand the purpose of the trial, his/her role as a participant in the study and were able to attend at least one follow up visit to their physician.

Patients were included in the study if:

- The patient requested a skin rejuvenation procedure
- The patient suffered from tendonitis and PRP treatment(s) would be a suitable alternative to conventional therapy
- The patient suffered from osteoarthritis, had failed conventional therapy and may benefit from PRP treatment(s)
- The patient suffered from chronic non-healing wounds and PRP treatment would be a suitable alternative to conventional therapy
- The patient was identified with a non-union of bone, had failed conventional therapy and may benefit from PRP treatment

Patients were excluded from the study if:

- The patient would not be able to attend a follow-up visit(s) after a treatment
- The patient was on antiplatelet therapy or anticoagulant therapy
- The patient had a low platelet count (less than 50 x 10^9 l)
- The patient had a low hemoglobin level (less than 10 g/dl)
- The patient had an active tumour or metastatic disease

- Concomitant use of aspirin or other cyclooxygenase (COX) inhibitors 7 days prior to treatment with PRP, chemotherapy or steroid therapy
- The patient had chronic liver pathology
- The patient was pregnant or breastfeeding

Volunteers, willing to consent to participate in this study were informed of the nature, extent, design and conduct of the study and their consent was obtained in writing prior to inclusion to the study. Volunteers were given the opportunity to ask questions and were informed that they may withdraw from the study at any time, for any reason.

Patients were excluded if they were on anti-platelet therapy, had a low platelet count or haematocrit.

Study product

Physicians were supplied with two 8 ml BD Vacutainer® CPT^{TM} Cell Preparation tubes, one 21 G multisampler needle, needle holder and 21 G needle (Becton, Dickinson and Company, Franklin Lakes, US), one sterile 3 ml, uncoated polypropylene syringe, one 10 ml sterile glass multidose vial containing 1.5 ml 10% $CaCl_2$ and 10 ml sterile glass multidose vial containing 0.5 ml 5% ethanol.

On the day of the treatment

Physicians were asked to complete a *case study report form* (Appendix C) containing the patient identification number, where they had to:

a) Give a detailed patient diagnosis (for which indication the patient was being treated)

b) Describe in detail how the PRP would be administered

c) Mark the severity of the patient's condition or injury according to their diagnosis on a visual analogue scale (VAS). VAS on the *case study report form*, ranged from 0 (healthy or normal with no loss of function) to 10 (extremely severe with complete loss of function). A VAS is a horizontal line, 10 cm in length, anchored by the words *"Healthy/normal"* on the left hand side and *"Extremely severe"* on

the right hand side. The VAS score is determined by measuring in centimetres from the left hand end of the line to the point that the physican marks (Figure 2.3a).

If possible, physicians were asked to take a photograph, MRI or X-ray of the treatment area before commencement of treatment.

In the same *case study report form*, the patient was asked to indicate on a VAS the severity of their condition or injury as perceived by them.

On the day of the follow-up visit(s)

In the same *case study report form* used on the day of treatment, physicians were asked to describe their impression of the outcome of the PRP treatment by

a) A written description

b) Mark the severity of the patient's condition or injury according to their diagnosis on a VAS ranging from 0 (healthy or normal with no loss of function) to 10 (extremely severe with complete loss of function).

c) Rate their satisfaction with the product on a scale from 0 (not at all satisfied) to 4 (highly satisfied) (Figure 2.3b)

If possible, physicians were asked to take a follow-up photograph, MRI or X-ray of the treatment area

In the same *case study report form*, the patient was asked to indicate on a VAS the severity of their condition or injury as perceived by them as well as to rate their satisfaction with the product on a scale from 0 (not at all satisfied) to 4 (highly satisfied).

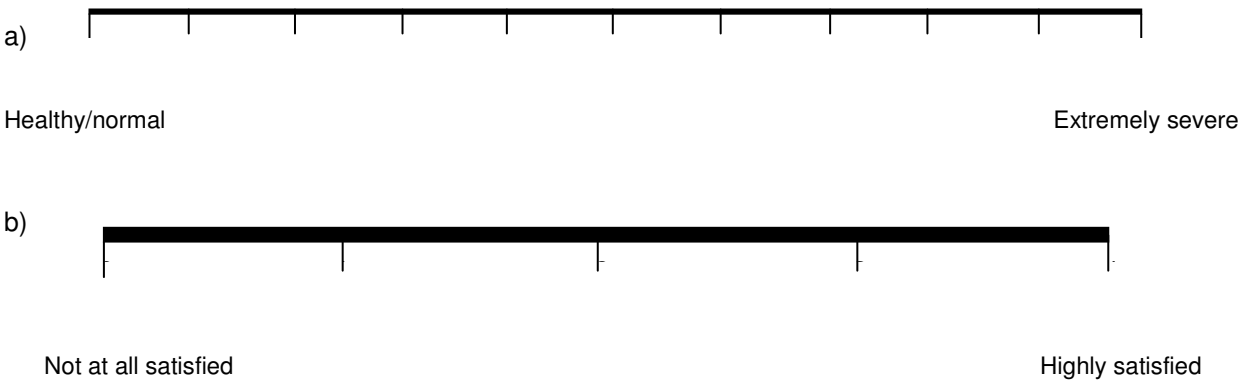

Figure 2.3 Physicians and patients were asked to a) indicate the severity of the condition or injury to be treated with PRP on a Visual Analogue Scale (VAS) from 0 to 10, 0 being healthy or normal or 10 extremely severe. As well as b) their satisfaction with the product from 0 to 4, 0 being not satisfied and 4 highly satisfied.

2.1.2.8 Expression of results

Platelet concentration of PRP preparations

The mean platelet concentration and its 95% confidence interval were determined and summarized in Table 3.1. Results were expressed as the mean platelet count times a factor of a 10^9 per litre ± standard error of mean (SEM). Platelet counts were analysed by AMPATH National Laboratories. The Wilcoxon Signed Rank Test or the one-way analysis of variance (ANOVA) was performed using GraphPad Prism version 4.1 for Windows, GraphPad Software, San Diego California US, www.graphpad.com. The difference was considered statistically significant at $p \leq 0.05$.

PRP collection efficiency

The collection efficiency expressed as a percentage was calculated with the following equation:

$$\textit{Collection efficiency}\ (\%) = \frac{(\textit{Volume of PRP} * \textit{PRP platelet count})}{(\textit{Volume of Whole blood} * \textit{Whole blood platelet count})}$$

The collection efficiency is summarized in Table 3.1.

Total protein quantitation

The mean concentration and its 95% confidence interval were determined. Results were expressed as the mean total protein concentration ± SEM in Table 3.2. Student's t-test was performed using GraphPad Prism version 4.1 for Windows, GraphPad Software, San Diego California US, www.graphpad.com. The difference was considered statistically significant at $p \leq 0.05$.

Growth factor quantitation

The mean concentration and its 95% confidence interval were determined. Results were expressed as the mean growth factor concentration ± SEM in Table 3.3. At least two O.D. readings were obtained per variable as suggested by the manufacturer. Wilcoxon Signed Rank Test was performed

using GraphPad Prism version 4.1 for Windows, GraphPad Software, San Diego California US, www.graphpad.com. The difference was considered statistically significant at $p \leq 0.05$.

Cellular proliferation assays

MTT-assay

Results were expressed as the mean percentage of viable cells ± standard error of mean (SEM) in comparison to either the positive of negative control. Percentage cell viability was determined by the following equation:

$$\%\,Viability = (Mean\ O.D.\ experimental\ well\ /\ mean\ O.D.\ of\ control\ wells) * 100$$

Outliers were detected and excluded from the analysis using Grubb's test. Both parametric and non-parametric tests were performed. Each experiment was done in triplicate with at least 3 O.D readings per variable in each experiment. ANOVA and Kruskal-Wallis non-parametric test were performed using GraphPad Prism version 4.1 for Windows, GraphPad Software, San Diego California US, www.graphpad.com. The difference was considered statistically significant at $p \leq 0.05$.

Crystal violet-assay

The mean O.D. from the blank wells was subtracted from the O.D. of the experimental- and control wells. Results were expressed as the mean percentage of viable cells ± standard error of mean (SEM) in comparison to either the positive or negative control. Percentage cell viability was determined by the following equation:

$$\%\,Viability = (Mean\ O.D.\ experimental\ well\ /\ mean\ O.D.\ of\ control\ wells) * 100$$

Outliers were detected and excluded from the analysis using Grubb's test. Both parametric and non-parametric tests were performed. Each experiment was done in triplicate with at least 3 O.D readings per variable in each experiment. One-way ANOVA and Kruskal-Wallis non-parametric test were

performed using GraphPad Prism version 4.1 for Windows, GraphPad Software, San Diego California US, www.graphpad.com.

***In vivo* case studies**

The percentage symptom improvement from baseline was calculated with the following equation:

$$\%\,Symptom\ improvement\ = \frac{(Pretreatment\ score - Posttreatment\ score)}{Pretreatment\ score} * 100$$

The satisfaction score was presented in a table.

CHAPTER 3

3.1 RESULTS

3.1.1 PRP PREPARATION

3.1.1.1 Platelet counts and PRP volume

Traditionally, PRP preparation methods require two centrifugation steps, a “soft spin” to separate the red blood cells from the plasma and a “hard spin” to pellet the platelets.

Three hundred millilitres of venous blood was drawn from three volunteers under strict aseptic conditions. The whole blood was first centrifuged at 200 *g* for 10 min at 4ºC (“soft spin”), the plasma collected (PRP) and centrifuged at 3200 *g* for 10 min at 4ºC (“hard spin”). The supernatant was discarded and the pellet resuspended in PBS.

From Table 3.1 the average number of platelets harvested from three volunteers after a “soft spin” of 200 *g* was 667 x 10^9/l and after the “hard spin” was 3982 x 10^9/l. The data indicates that after the first centrifugation step, the platelet concentration is already enriched 1.7 - 4.4 fold when compared to the literature average platelet count in whole blood. The second or “hard spin” further pellets the platelets 6.0 fold compared to the PRP platelet count and 10.0 - 26.5 fold compared to the literature average platelet count of whole blood.

Table 3.1 showed that only a single centrifugation step was needed when a large volume of whole blood was collected from the patient. The platelets are sufficiently concentrated after the first centrifugation step or “soft spin” and produced a PRP product with a platelet count between two to four times the baseline platelet counts, from 300 ml of whole blood (Figure 2.1).

For a practical approach and optimised PRP preparation, a smaller volume of blood needs to be drawn from a patient in a clinical setting. The in house protocols and commercially available kits or devices collect between 8.5 - 500 ml of whole blood, whereas the commercial kits and devices available in South Africa collect between 20 - 60 ml of whole blood from patients in a clinical setting to deliver a PRP volume of 2 - 6 ml.

Table 3.1: Mean platelet count (x 10^9/ l) and volume (ml) obtained from the prepared platelet-rich plasma (PRP) and platelet poor plasma (PPP) during the method development and optimisation phases

		Number of platelets (x 10^9 ± SEM)				Volume (ml)				Collection efficiency
		Whole blood	**PRP**	**Pellet**	**PPP**	**Whole blood**	**PRP**	**Pellet**	**PPP**	**PRP**
Method development	**Literature**	150 - 400	350 - 3200 (2 to 8 fold)	-	-	8.5 - 500	0.5 - 70	Pellet resuspended to PRP volume	-	5 - 300%
	Method development (*n*=3)	-	667 ± 11	3982 ± 647		300	100	Resuspended in 1.5 ml aliquots (6 aliquots)	-	81%
Method optimisation	**Sonicated** (*n=27)*	-	680 ± 131	N/A	17 ± 3	16	5.4 ± 1.0	N/A	7.4 ± 1.4	83%
	Lyophilised (*n*=29)	313 ± 58	657 ± 29	N/A	14 ± 1	16	3.8 ± 0.7	N/A	7.0 ± 1.3	57%

Table 3.1 indicates the results for optimisation of PRP preparation. Sixteen milliliters venous blood was drawn from 30 volunteers under strict aseptic conditions in two 8 ml BD Vacutainer® CPT™ Cell Preparation tubes and was centrifuged at 1800 *g* for 20 min at room temperature within 2 hours of blood collection.

The BD Vacutainer® CPT™ Cell Preparation tubes with sodium citrate were evaluated for their ability to concentrate the platelets to two to four times the baseline platelet count. These tubes were selected as possible kit components as they contain sodium citrate (the desired PRP anticoagulant) and blood separation media, designed to separate the mononuclear cells from the whole blood after a single centrifugation step (Becton Dickinson and Company, 2009). The separation occurs during centrifugation when the gel portion of the medium moves to form a barrier, separating the mononuclear cells, platelets and plasma from the red blood cells and granulocytes (Becton Dickinson and Company, 2009).

After centrifugation, approximately two thirds of the plasma was collected with a sterile 3 ml plastic pasture pipette without disturbing the cell abundant lower plasma layer and labelled as PPP. The remaining cell containing plasma layer was collected with a sterile 3 ml plastic pasture pipette and transferred into 15 ml polypropylene centrifuge tubes. A 500 µl sample of PRP and PPP from each sample was assessed for a platelet count. The volumes of each of the harvested PRP and PPP fractions were recorded.

Table 3.1 indicates that the BD Vacutainer® CPT™ Cell Preparation tubes were able to yield similar platelet concentrations when compared to the yields of the platelet count after a “soft spin” from 300 ml of whole blood. The platelet concentration in the PRP fraction was enriched 1.7 - 4.5 fold in the sonicated method and 1.6 - 4.6 fold in the lyophilised method when compared to the literature average platelet count in whole blood. When comparing the PRP to the PPP fraction, the PRP fraction had been significantly enriched with platelets in comparison to the PPP fraction. No significant difference was observed between the platelet counts of the PRP fractions from Table 3.1. The volume collected from whole blood, PRP and PPP as well as the collection efficiency was compared to the literature.

3.1.12 Bradford assay

The total protein recovered from the different optimal PRP preparation methods was determined by the protein quantification method outlined by Bradford (1976). The method made use of 96-well round bottom plates for the final measurement. Two hundred microliters of the prepared blank, samples and BSA standards were added in triplicate and the absorbance was measured at 595 nm. The linear regression equation was used to calculate the quantity of protein obtained from each sample of the PRP preparations. No major difference in total protein concentration was observed between total protein recovered from the first and second preparation of samples using the optimised method (Table 3.2). The protein concentrations were standardised to ensure equal loading for SDS-PAGE analysis (3.1.3).

Table 3.2 The total concentration of protein obtained during the sonicated and lyophilised PRP preparations determined by the Bradford protein assay

Total protein concentration (mg/ml ± SEM)	
Sonicated PRP preparation *n* = 27	**Lyophilised PRP preparation** *n* = 29
22.6 ± 1.5	25.2 ± 7.6

3.1.1.3 SDS-PAGE

The PRP preparations were monitored by polypeptide analysis using Coomassie Brilliant Blue staining of 12% SDS-PAGE. The PRP preparation separation profile was compared to a Fermentas molecular weight marker. Low levels of high molecular weight proteins were indicated by Coomassie staining with apparent molecular masses of 116 kDa, masses between 66.2 - 116 kda, 45 kDa and 25 kDa. Albumin, with a molecular mass of 67 kDa, corresponded to the 66.2 kDa band of the molecular weight marker (Anderson and Anderson, 1977, Tirumalai *et al*., 2003).

PDGF-AB has a molecular mass of 30 kDa. Each molecule comprises of two covalently linked subunits, once reduced by the reducing buffer, the two subunits are expected to migrate through the polyacrylamide gel at an apparent molecular mass of 16 kDa for the A chain and 14 kDa for the B chain. The bands at position A and B on Figure 3.1 indicates the probable sites of the A chain and B chain respectively. The TGF-β_1 has a molecular mass of 25 kDa and the reduced subunits are expected to co-migrate through the polyacrylamide gel at an apparent molecular mass of 13 kDa. The band at position C on Figure 3.1 indicates the probable site of the TGF-β_1 subunits. The bands are faint and may indicate a low concentration of the growth factors present in the platelet-derived growth factor suspension. As SDS-PAGE is only a qualitative indication of the possible presence of PDGF-AB and TGF-β_1, enzyme-linked immunological assays were also preformed to detect the presence and concentration of PDGF-AB and TGF-β_1.

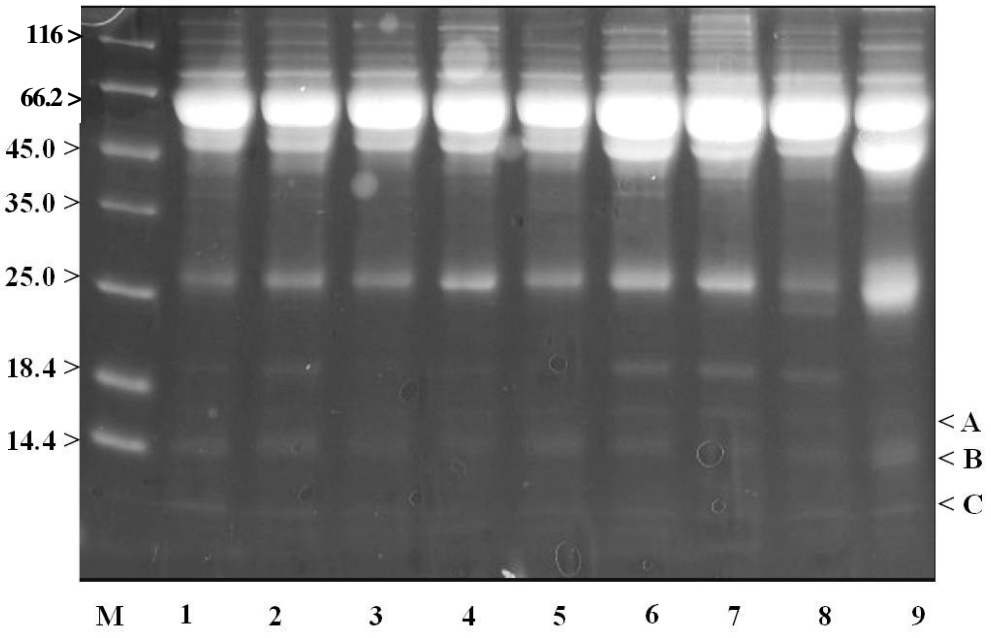

Figure 3.1 Analysis of the possible presence of the desired growth factors in the extracted growth factor suspension by SDS-PAGE on a 12% gel. M represents the protein molecular weight marker in the first lane. The protein sizes of the marker are indicated on the left hand side (in kDa) of 12% resolving gel. Lanes 1 - 9 represents the PRP samples from 9 volunteers. A, B and C indicate the expected molecular mass of the subunits of PDGF-AB and TGF-β_1 at 16 kDa, 14 kDa and 13kDa respectively.

3.1.1.4 Growth factor quantitation

The growth factor concentration was determined for the method development phase and for the optimised preparation procedures (sonicated and lyophilised PRP preparations). The quantity of growth factor extracted from each sample of the PRP preparations was determined from the standard curve, transformed to a log scale. Table 3.3 shows no significant difference between the observed concentrations of PDGF-AB extracted with ethanol, or the $CaCl_2$ and PPP solution used during the method optimisation phase. From the data summarised in Table 3.3, the $CaCl_2$ and platelet poor plasma solution was combined with ethanol to be used as an acceptable platelet activation or lysis reagent and further used during the optimisation of the PRP preparation.

During the first phase of the PRP preparation optimisation (sonicated PRP preparation), PRP was allowed to incubate at room temperature until a fibrin gel had formed after the PRP was collected and activated using the lysis reagent (combination of $CaCl_2$, PPP and ethanol). The fibrin gels were sonicated for 5 min, followed by centrifugation at 16000 *g* for 5 min at room temperature in an attempt to disrupt the gels and obtain the growth factors. The supernatant was collected and frozen at -70ºC until the growth factor concentrations was determined. The PDGF-AB and TGF-β_1 concentration was determined for the collected supernatant. The concentrations of PDGF-AB and TGF-β_1 obtained from the sonicated PRP preparation were low.

The PRP preparation optimisation phase was repeated with slight alterations. Again, the PRP was allowed to incubate at room temperature until a fibrin gel had formed after the PRP was collected and activated. The fibrin gels were transferred to 15 ml polypropylene containers, frozen at -70℃, lyophilised and re-suspended in sterile PBS of the corresponding PRP volume. The concentration of TGF-β_1 determined from the re-suspended lyophilised fibrin gels were 45.49 ± 3.80 ng/ml, results were summarized in Table 3.3. The concentration of TGF-β_1 from the lyophilised fibrin gels were comparable to the available literature.

Table 3.3 The concentration of growth factors obtained from different PRP preparations were determined using ELISA

	Protocol	PDGF-AB (ng/ml ± SEM)	TGF-β_1 (ng/ml ± SEM)	Platelet concentration
Method development and optimisation	Ethanol	18.4 ± 9.8	N/A	10.0 - 26.5 fold (Ave 18 fold)
	$CaCl_2$ and PPP solution	33.9 ± 23.3	N/A	10.0 - 26.5 fold (Ave 18 fold)
	Sonicated PRP preparation	0.50 ± 0.88[1]	$1.0 \times 10^{-1} \pm 0.8 \times 10^{-1}$	1.7 - 4.5 fold (Ave 3.1 fold)
	Lyophilised PRP preparation	N/A	45.5 ± 3.8	1.6 - 4.6 fold (Ave 3.1 fold)
Literature	Plateltex (Mazzucco *et al.*, 2008)	60	0.8	N/A
	Equine (Carter *et al.*, 2003)	0.7 ± 0.1	7.5 ± 1.3	N/A
	Rat (Yamaguchi *et al.*, 2012)		H-PRP: 101 ± 24 L-PRP: 210.5 ± 37.2	N/A
	PCCS (PRF) (He *et al.*, 2009)	18	50	5.13
	Curasan (He *et al.*, 2009)	90	100	4.85

Initially platelets were activated with ethanol and a combination of $CaCl_2$ and PPP. During optimisation of the extraction procedure, platelets were activated and the platelet-rich plasma was allowed to form a fibrin gel. In an attempt to disrupt the fibrin gels, the gels were sonicated or lyophilised. Table 3.3 compares the concentration of growth factors obtained from the different preparations in relation to platelet concentration, collection efficiency and the available literature.

3.2.1 CELLULAR PROLIFERATION ASSAYS

3.2.1.1 Effect of PRP on fibroblasts

To investigate whether the PRP obtained from the optimised lyophilised PRP preparation was able to stimulate cell proliferation, normal dermal fibroblasts were treated with PRP obtained from three volunteers. The normal dermal fibroblasts were incubated 24 h or 72 h in medium supplemented with 10% HI FCS (positive control) or a dose range of 0 - 10% PRP. At the termination of the incubation period, the viable cells were enumerated using either the crystal violet or MTT-assay.

The effect of PRP obtained from the optimised lyophilised PRP preparation is shown in Figure 3.2, 3.3 and 3.4 respectively.

Crystal violet assay

From Figure 3.2.1, 3.2.2 and 3.2.3 the PRP treated cells stimulated the proliferation of fibroblasts in a dose dependant manner in comparison to the negative control (serum free medium), an increase in the percentage PRP had increased the percentage viable cells. When compared to the positive control (medium supplemented with 10% HI FCS), the cells treated with 5 or 10% PRP significantly ($p \leq 0.05$) increased the percentage viable cells to 165% and 156% in Figure 3.2.1 and to 176% and 158% in Figure 3.2.3 after 24 h of incubation, respectively.

From Figure 3.2.1, 3.2.2 and 3.2.3, 5% PRP showed the strongest effect on cellular proliferation, followed by 10% PRP after 24 h incubation. A 5 - 10% PRP preparation would therefore contain 2.3 - 4.6 ng/ ml TGF-β_1 (Table 3.3).

From Figure 3.3.1, 3.3.2 and 3.3.3 a similar dose-response relationship is seen after 72 h of incubation in comparison to 24 h. When compared to the positive control (medium supplemented with 10% HI FCS), the cells treated with 5 or 10% PRP increased the percentage viable cells to 149% and 124% in Figure 3.3.1, 121% in Figure 3.3.3 and 117% and 114% in Figure 3.3.3 after 72 h of incubation, respectively. Although an increase in proliferation is seen after 72 h in comparison to the positive control, it is not significant or as pronounced as after 24 h. Cells treated with 0.01% or 0.10%

PRP had a significant ($p \leq 0.05$) decrease in percentage viable cells when compared to the positive control.

MTT-assay

From Figure 3.4.1, 3.4.2 and 3.4.3 the PRP treated cells stimulated the proliferation of fibroblasts in a dose dependant manner in comparison to the negative control (serum free medium), an increase in the percentage PRP had increased the percentage metabolising cells. When compared to the positive control (medium supplemented with 10% HI FCS), the cells treated with 0 - 1% PRP had a significant ($p \leq 0.05$) decrease in the percentage metabolising cells. The percentage metabolising cells in the 5% and 10% PRP groups increased to 79% and 105% in Figure 3.4.1, 90% and 97% in Figure 3.4.2 and 91% and 87% in Figure 3.4.3 after 72 h of incubation, respectively. The increase in percentage metabolising cells in the 5% and 10% treated groups is comparable to that of the positive control.

Crystal violet assay: The effect of PRP on normal dermal fibroblasts after 24 h incubation period.

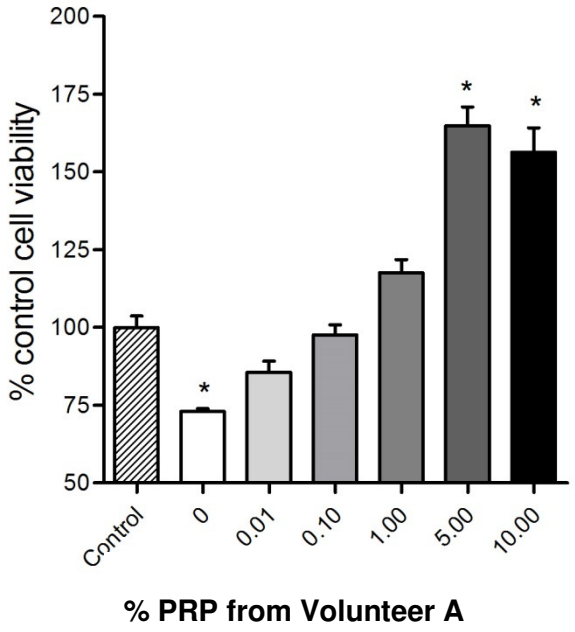

Figure 3.2.1 Effects of PRP obtained from volunteer A on normal dermal fibroblasts[1].

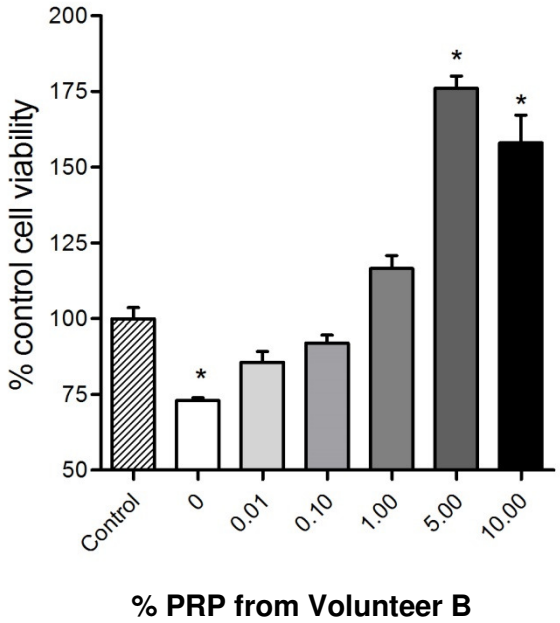

Figure 3.2.2 Effects of PRP obtained from volunteer B on normal dermal fibroblasts[1].

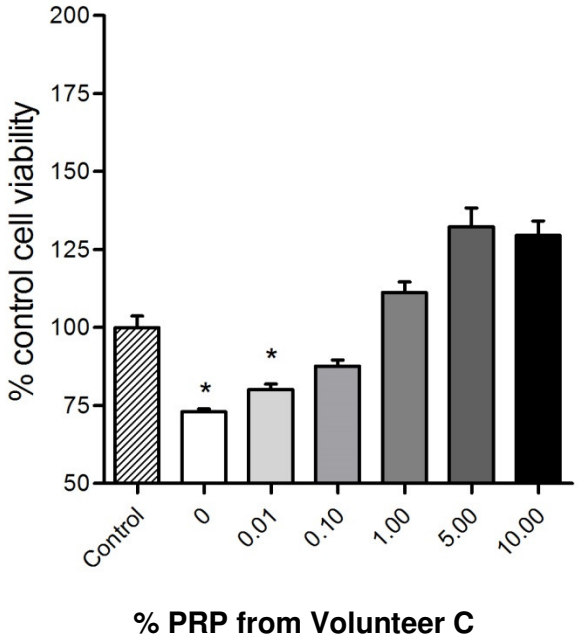

Figure 3.2.3 Effects of PRP obtained from volunteer C on normal dermal fibroblasts[1].

* $p \leq 0.05$ when compared to the positive control

1. Each endpoint represents the mean of at least three different experiments ± SEM

Crystal violet assay: The effect of PRP on normal dermal fibroblasts after 72 h incubation period.

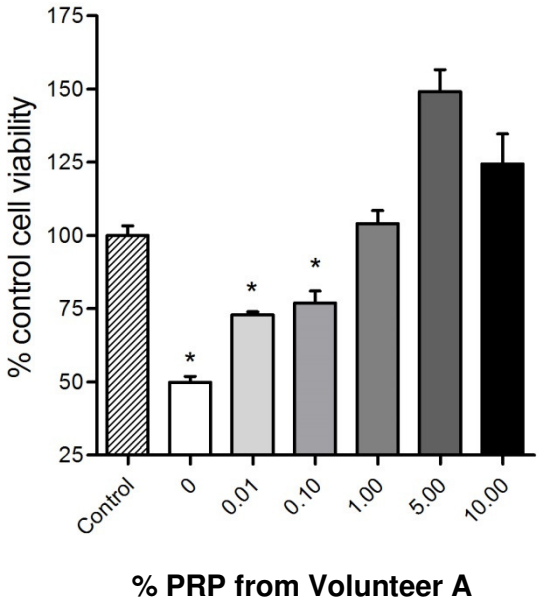

Figure 3.3.1 Effect of PRP obtained from volunteer A on normal dermal fibroblasts.[2]

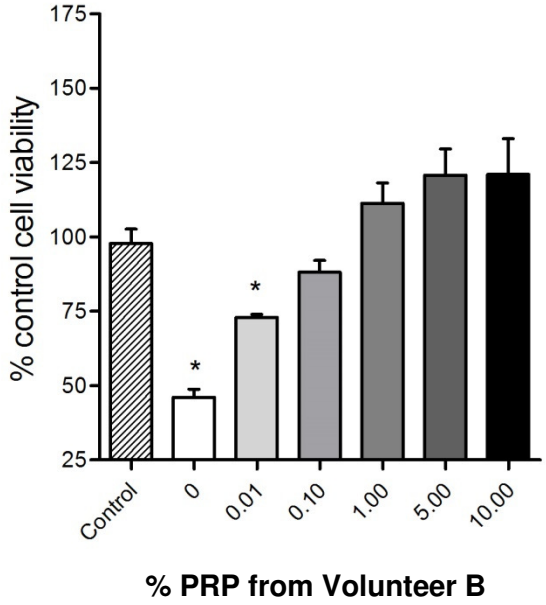

Figure 3.3.2 Effect of PRP obtained from volunteer B on normal dermal fibroblasts.[2]

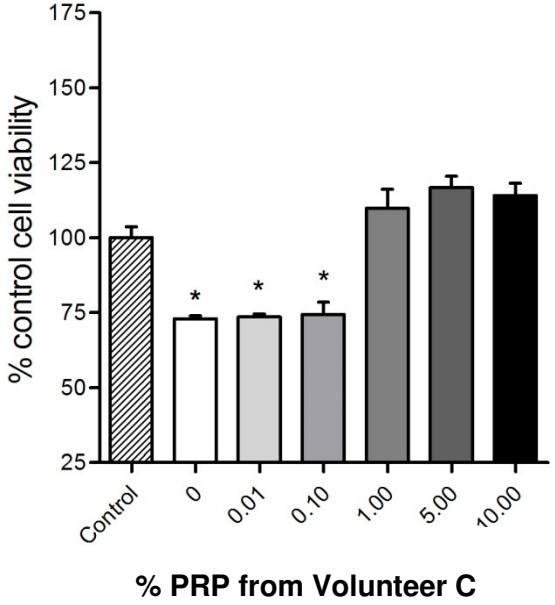

Figure 3.3.3 Effect of PRP obtained from volunteer C on normal dermal fibroblasts.[2]

* $p \leq 0.05$ when compared to the positive control

2. Each endpoint represents the mean of at least three different experiments ± SEM

MTT-assay: The effect of PRP on normal dermal fibroblasts after 72 h incubation period.

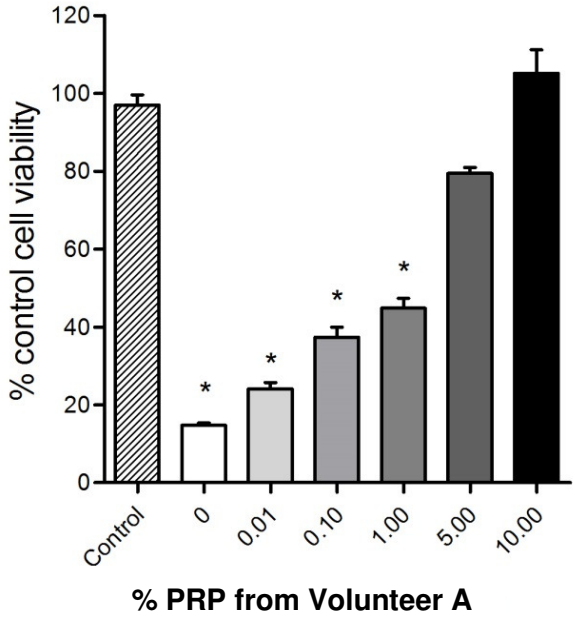

Figure 3.4.1 Effect of PRP obtained from volunteer A on normal dermal fibroblasts.[3]

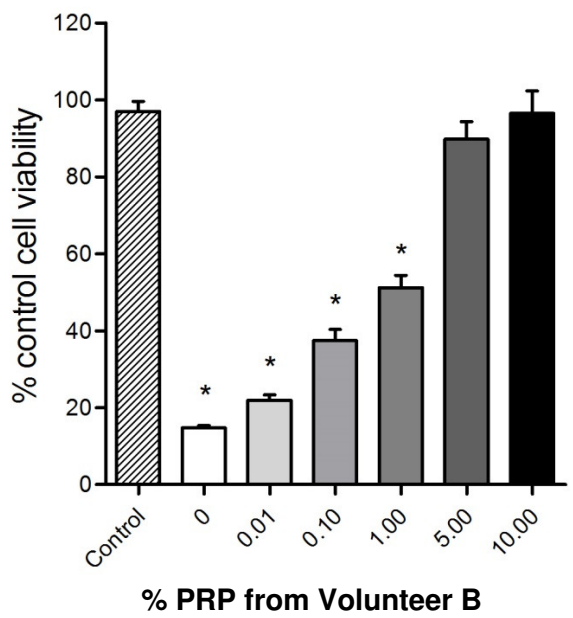

Figure 3.4.2 Effect of PRP obtained from volunteer B on normal dermal fibroblasts.[3]

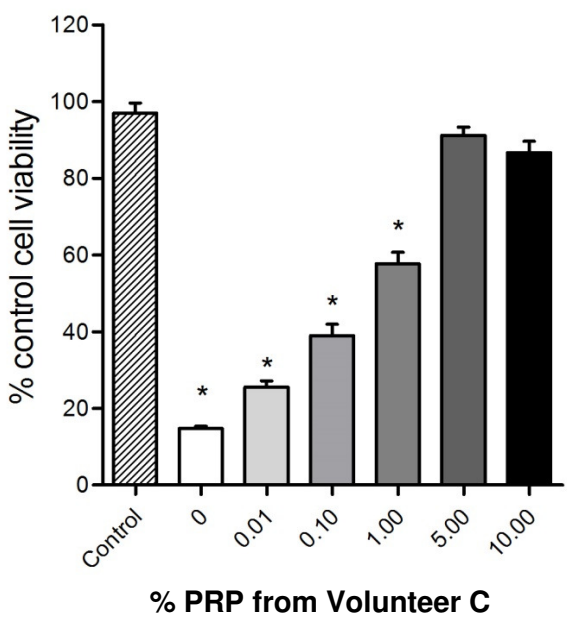

Figure 3.4.3 Effect of PRP obtained from volunteer C on normal dermal fibroblasts.[3]

* $p \leq 0.05$ when compared to the positive control

3. Each endpoint represents the mean of at least three different experiments ± SEM

3.2.1.2 Effect of single growth factors on fibroblasts

To investigate whether a single growth factor would be able to induce cell viability and proliferation at a concentration comparable to that obtained from PRP, chicken embryo fibroblasts where incubated for 72 h with rPDGF-AB or rTGF-β_1 in serum free medium, medium supplemented with 0.5% HI FCS or medium supplemented with 1.0% HI FCS. HI FCS provides the optimal environment for cell proliferation as it contains various biologically active proteins and nutrients needed for cell growth and survival. The experiment was designed in an attempt to limit the influence of potential growth factors in HI FCS as far as possible. The effect of the recombinant growth factors is shown in Figures 3.5.1 and 3.5.2.

From Figures 3.5.1 and 3.5.2 no significant difference was observed between cells treated with 0%, 0.5% or 1.0% HI FCS (Control). No significant difference was observed in the cells treated with rPDGF-AB or TGF-β_1 after 72 h of incubation.

MTT-assay: The effect of recombinant growth factors on chicken embryo fibroblasts after 72 h incubation period.

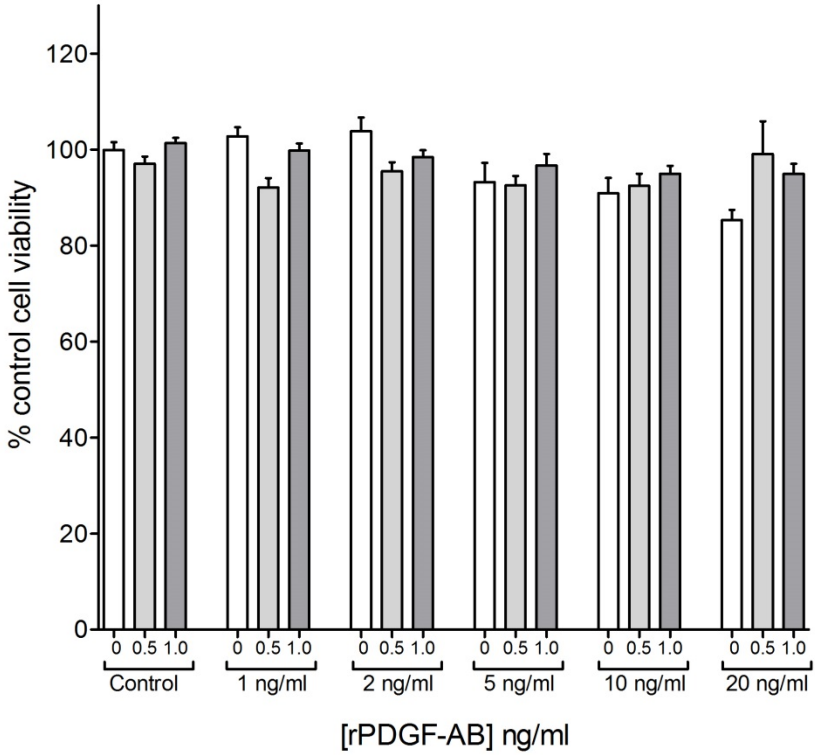

Figure 3.5.1 Effect of rPDGF-AB on chicken embryo fibroblasts in serum free medium (0), medium supplemented with 0.5% HI FCS (0.5) or medium supplemented with 1.0% HI FCS (1.0).[4]

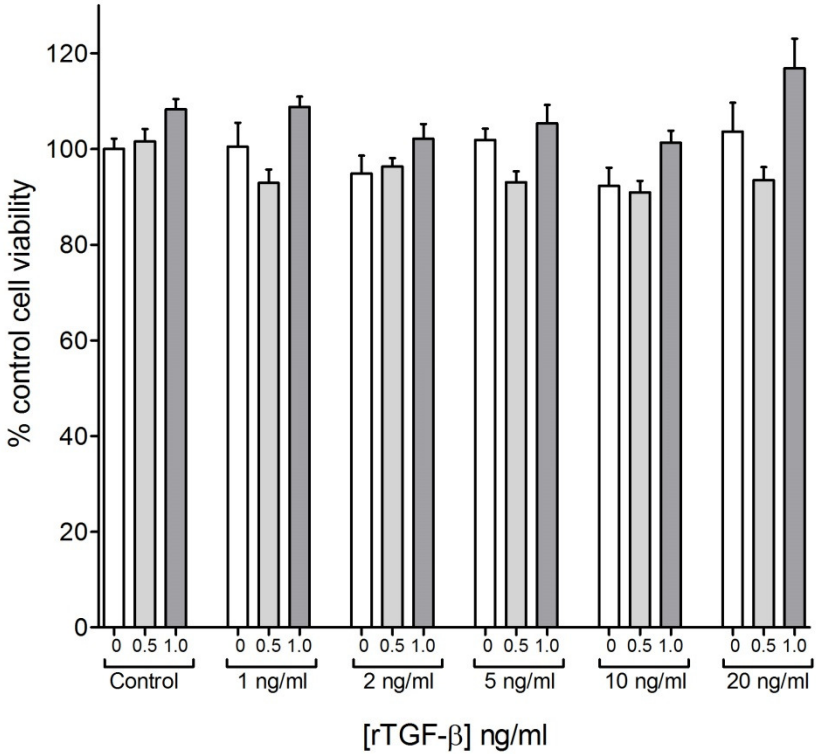

Figure 3.5.2 Effect of rTGF-β_1 on chicken embryo fibroblasts in serum free medium (0), medium supplemented with 0.5% HI FCS (0.5) or medium supplemented with 1.0% HI FCS (1.0).[4]

4. Each endpoint represents the mean of at least three different experiments ± SEM.

3.3.1 *IN VIVO* CASE STUDY RESULTS

To confirm the clinical effect of PRP, a case study registry to investigate the efficacy of activated PRP for various cosmetic and sports related indications was opened. The percentage symptom improvement as well as study product satisfaction for each patient, was recorded. In total 10 patients were treated with the study product, 5 of which were treated for various cosmetic indications and 5 for injury or osteoarthritis related indications. A summary of the diagnosis, VAS scores before and after treatment, the satisfaction score as well as the percentage improvement is given in Table 3.4.

3.3.1.1 Cosmetic indications

Patient 1 presented with severe acne and acne scarring of the face as well as erythema associated with oral isotretinoin treatment. Both the attending physician and patient recorded an initial symptom severity of 3/10 on the VAS. The attending physician administered the PRP obtained from the patient via standard mesotherapy injection technique. At the follow-up visit, 8 weeks post-injection, the attending physician and patient recorded a 50% symptom improvement on post-acne scarring and redness. Both physician and patient gave the study product a maximal satisfaction score of 4. The before and after photos of Patient 1 is shown in Figure 3.6.

Patient 2 presented with healed and scarred burn wound to both legs. Both the attending physician and patient recorded an initial symptom severity of 9/10 on the VAS. The scarred burn wounds were infiltrated with PRP obtained from the patients by means of injection. At the follow-up visit, 3 weeks post-injection, the attending physician recorded a 33% and the patient a 22% improvement of the colour and appearance of the scars. The physician stated that the “indentations had filled up” and that there was an “overall very good result”. The patient received an additional PRP treatment 3 weeks after the follow-up visit following the same administration procedure as described above. On the second follow-up visit, 5 weeks post-injection (second injection) both the physician and patient recorded a 56% symptom improvement on scarring. Both physician and patient gave the study product a satisfaction score of 3.

Patient 3 presented with hypotrophic skin lesions and hypopigmentation of the facial skin. Both the attending physician and patient recorded an initial symptom severity of 9/10 on the VAS. The attending physician administered the PRP obtained from the patient subcutaneously and intradermally with a 30G needle into the skin lesions. At the follow-up visit, 8 weeks post-injection, the attending physician recorded a 13% and the patient 0% improvement. The physician stated that after the initial treatment “plumping out” of the hypothrophic skin lesions was observed. The patient received an additional PRP treatment the same day following the same administration procedure as described above. On the second follow-up visit, 9 weeks post-injection (second injection) the physician recorded a 44% and the patient 56% symptom improvement on scarring. Both physician and patient gave the study product a satisfaction score of 3.

Patient 4 presented with pigmentation and scarring of the facial skin. The attending physician recorded an initial symptom severity of 6/10 and the patient 7/10. The attending physician administered the PRP obtained from the patient via standard mesotherapy injection technique. At the follow-up visit, 6 weeks post-injection, the attending physician and patient recorded a 67% symptom improvement on skin pigmentation and scarring. Both physician and patient gave the study product a maximal satisfaction score of 4.

Patient 5 presented with fine lines and wrinkles around the peri-orbital area and neck as well as pigmentation of the neck. The attending physician recorded an initial symptom severity of 2/10 and the patient 4/10. The attending physician administered the PRP obtained from the patient with nappage, intradermal and point-point technique with a 12 mm, 32G needle and 4 mm, 32G needle. At the follow-up visit, 7 weeks post-injection, the attending physician and patient recorded a 50% symptom improvement. The physician noted that the skin “looked smoother or tighter” and that the “wrinkles are shallower or gone”. Both physician and patient gave the study product a maximal satisfaction score of 4.

3.3.1.2 Injury and osteoarthritis related treatments

Patient 6 presented with poor healing of soft tissue contusion of a right lower leg injury obtained around a year before treatment. The attending physician recorded an initial symptom severity of 6/10

and the patient 5.5/10. The attending physician administered the PRP obtained from the patient over and around the injured area with 4 mm and 12 mm needles. At the follow-up visit 9 weeks post-injection, the attending physician recorded a 67% and the patient 45% improvement of chronic pain and swelling, respectively. Both physician and patient gave the study product a maximal satisfaction score of 4.

Patient 7 presented with chronic right knee pain due to meniscal degeneration. Both the attending physician and patient recorded an initial symptom severity of 7/10. The attending physician injected the PRP obtained from the patient around the right knee area. At the follow-up visit, 4 weeks post-injection, the attending physician recorded a 29% and the patient 14% improvement of chronic pain and flexibility, respectively. Both physician and patient gave the study product a satisfaction score of 3.

Patient 8 presented with osteoarthritis in both knees. The attending physician recorded an initial symptom severity of 7.5/10 and the patient 6/10. The attending physician did not describe the injection technique. At the follow-up visit, 9 weeks post-injection, the attending physician reported a 14% symptom improvement; however, the patient experienced no improvement. The patient experienced post-injection pain 3-4 days after the administration of the PRP. The attending physician stated that osteoarthritis may not be the primary cause of symptoms. The physician gave the study product a partial satisfaction score of 2 and the patient 1.

Patient 9 presented with severe osteoarthritis in the right knee. The attending physician recorded an initial symptom severity of 7/10 and the patient 6/10. The attending physician infiltrated the PRP obtained from the patient via intra-articular injection in the right knee. At the follow-up visit, 10 weeks post-injection, the attending physician reported a 43% symptom improvement and the patient 50%, respectively. The physician gave the study product a satisfaction score of 3 and the patient 2.

Patient 10 presented with a bone fracture in the right arm with typical non-union characteristics and the absence of cortical bone. Neither the attending physician nor the patient recorded an initial symptom severity, or time lapse between the initial break and the PRP injection. The attending physician injected PRP obtained from the patient around the fractured area. At the follow-up visit, 16

weeks post-injection, the physician noted that new cortical bone deposition occurred and bone density was increased dramatically. The before and after photos of patient 10 is shown in Figure 3.7.

Table 3.4 Summary of the diagnosis, VAS scores before and after treatment, the satisfaction score as well as the percentage improvement of patients treated with PRP

Patient number	Diagnosis	Initial symptom severity score	Post-injection symptom severity score	Percentage sympom improvement	Satisfaction score out of 4
1	Severe acne, acne scarring and erythema of the face	a) 3; b) 3	a) 2 b) 2	a) 50% b) 50%	a) 4 b) 4
2	Healed and scarred burn wound to both legs	a) 9 b) 9	a) 4 b) 4	a) 56% b) 56%	a) 3 b) 3
3	Hypotrophic skin lesions and hypopigmentation of the facial skin	a) 9 b) 8.5	a) 5 b) 4	a) 44% b) 56%	a) 3 b) 3
4	Pigmentation and scarring of the facial skin	a) 6 b) 7	a) 2 b) 2	a) 67% b) 83%	a) 4 b) 4
5	Fine lines and wrinkles around the peri-orbital area and neck as well as pigmentation of the neck	a) 2 b) 4	a) 1 b) 2	a) 50% b) 50%	a) 4 b) 4
6	Poor soft tissue healing and skin contusion	a) 6 b) 5.5	a) 2 b) 3	a) 67% b) 45%	a) 4 b) 4
7	Chronic right knee pain due to meniscal degeneration	a) 7 b) 7	a) 5 b) 6	a) 29% b) 14%	a) 3 b) 3
8	Osteoarthritis in both knees	a) 7.5 b) 6	a) 6.5 b) 6	a) 14% b) 0%	a) 2 b) 1
9	Severe osteoarthritis in the right knee	a) 7 b) 6	a) 4 b) 4	a) 43% b) 50%	a) 3 b) 2
10	Bone fracture of right arm with non-union	N/A	N/A	N/A	N/A

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Table 3.4 presents a summary of the results obtained after treatment with one or two PRP injections for a variety of indications. The initial and post-symptom severity score was marked by the physician (a) and patient (b) on a VAS in the case study report form. The VAS was anchored on the left hand side with 0and on the right hand side 10, 0 being healthy or normal or 10 extremely severe. Both physicians and patients were asked to indicate their satisfaction with the product from 0 to 4, 0 being not satisfied and 4 highly satisfied

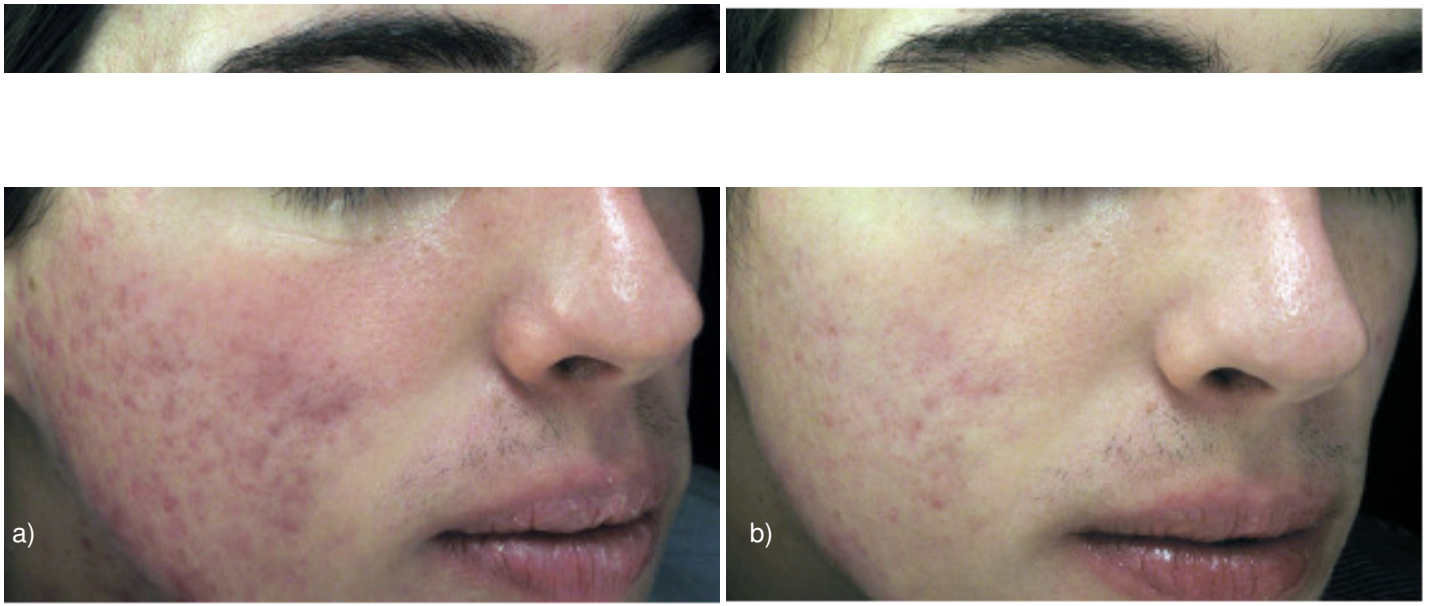

Figure 3.6 Improvement in acne scars and redness associated with oral isotretinoin before (a) and 8 weeks after (b) patient 1 received a PRP injection.

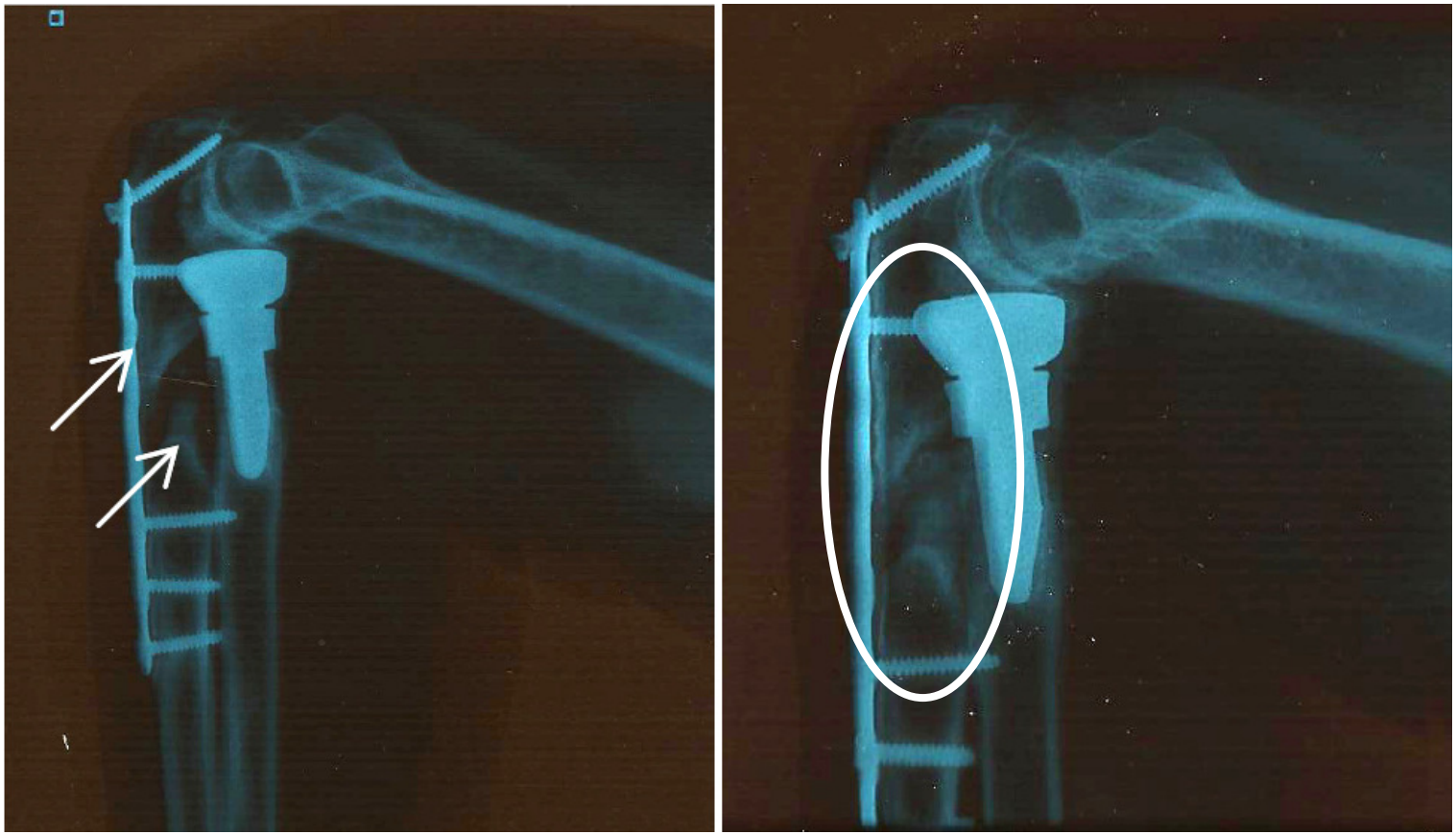

Figure 3.7 X-ray of patient 10 a) before and b) 16 weeks post-injection with PRP around the non-union of the right arm. The arrows in a) indicate the non-union and absence of cortical bone before treatment with PRP. The oval in b) indicates the area 16-weeks post-treatment with an increase in bone density and cortical bone formation.

CHAPTER 4

4.1 DISCUSSION

Although PRP is used in a variety of clinical fields such as the management of chronic wounds or wounds with limited blood perfusion, skin rejuvenation and bone regeneration, the efficacy thereof is debated (Bae *et al*., 2010, Plachokovan *et al*., 2008, Sheth *et al*., 2012). A possible explanation for the variation in results is the fact that no clear-cut set of parameters are available that device manufacturers should follow. Variation in the device specifications and blood collection protocols leads to a range in the volume of whole blood collected from the patient, the centrifugal force with which the PRP is prepared, the number of centrifugation steps and ultimately the volume of PRP and the final platelet content. Variation in platelet content of the PRP preparation unavoidably leads to different concentrations of the platelet-derived growth factors (Yamaguchi *et al*., 2012).

The platelet content of the PRP preparation is crucial and merely concentrating the platelets highly does not indicate a quality PRP product. Work pioneered by Marx *et al*., (1998), reported the regenerative potential of platelets in reconstructing mandibular defects, *if* the PRP is enriched with a platelet concentration up to 10 fold the baseline platelet concentration in whole blood. Marx later reported that PRP preparations should be enriched to a platelet concentration between two-and-a-half and 6-fold the baseline platelet counts and that over concentrating the platelets was not shown to further enhance wound healing (Marx, 2001). In studies where cell proliferation and collagen production were increased, the PRP preparations' platelet enrichment was limited to 3-7 fold the baseline platelet count (Cho *et al*., 2011, Everts *et al*., 2006b, Kim *et al*., 2011, Krašna *et al*., 2007). A PRP preparation enriched with platelets above 4 fold the baseline platelet count may be at risk of premature platelet activation and aggregation due to the close proximity of the platelets to each other (Sister, 2013).

The original protocol employed by Marx *et al*., (1998) and some protocols described by device manufactures, as well as other in house protocols outlined in Table 1.3, made use of two centrifugation steps to prepare PRP. The first or "soft spin" was employed to separate the plasma

from the red blood cells and the second centrifugation or “hard spin” to separate the PRP from the PPP. A “hard spin” has the ability to decrease the integrity of the platelet membrane and centrifugation of platelet preparations above 3000 *g*, has been shown to prematurely activate more than 40% of the platelets in the PRP preparation (Sister, 2013).

To simplify the extraction procedure, avoid additional separation steps and prevent the possibility of premature platelet activation, the number of platelets enriched during a “soft spin” (200 *g*) followed by a “hard spin” (3200 *g*) was evaluated when 300 ml of whole blood was collected. In this study, the “soft spin” or first centrifugation step enriched the platelet concentration to the desired two to four fold the baseline platelet count in whole blood. A second centrifugation step over-enriched the platelet concentration approximately 10 - 27 fold compared to the average platelet count in whole blood. It is therefore unnecessary to employ a second centrifugation step when a large volume of whole blood is collected from a patient, as the platelets are sufficiently concentrated after the first centrifugation step. PRP preparation in the present study used a single “soft spin” centrifugation step for subsequent PRP preparation.

Collecting 300 ml of whole blood to prepare PRP for point-of-care use in a consulting room setting is impractical. The commercially available kits, devices and protocols can collect between 8.5 - 500 ml whole blood per patient as summarised in Table 1.3. In this study, a volume of less than 20 ml was able to produce sufficient volume of PRP with a platelet enrichment of 2 to 4 fold after a single centrifugation step.

The BD Vacutainer® CPT™ Cell Preparation tubes, with sodium citrate, were selected as possible kit components as they contain sodium citrate and blood separation media designed to separate the mononuclear cells form the whole blood after a single centrifugation step (Becton Dickinson and Company, 2009). Sixteen millilitres of whole blood, collected from patients using two 8 ml BD Vacutainer® CPT™ Cell Preparation tubes, processed according to the manufacturer’s instructions, sufficiently separated the buffy coat from other blood components. The buffy coat, which consisted of mononuclear cells and platelets suspended in plasma, was collected as

the PRP fraction. The PRP fraction collected using this methodology was enriched between two and four fold the baseline platelet count.

During the method optimization, the PRP fractions obtained from both the first sample preparation (sonicated) and the second sample preparation (lyophilised) had significantly higher platelet enrichment in comparison to the PPP fractions, indicating that most of the platelets had accumulated in the PRP fraction after a single centrifugation step. No significant differences were observed between absolute platelet recoveries of the PRP fractions prepared using the sonicated and lyophilised PRP preparations (Table 3.1). It was concluded that the BD Vacutainer® CPT™ Cell Preparation tubes were able to provide consistent platelet counts in all the collected PRP fractions.

The platelet yield is a measure of the collection efficiency of a device. Everts *et al.*, (2006), compared three commercially available devices based on their ability to effectively concentrate the platelets in the PRP fraction. The collection efficiency is dependent on the original volume of whole blood collected and the final volume of PRP in which the collected platelets are suspended. The platelet collection efficiency ranged from 32 - 47% for the tested devices. Yamaguchi *et al.*, (2012), manually prepared the PRP with an in house protocol that required two centrifugation steps. Those authors were able to concentrate the PRP between two and 5 fold, but only collected between 12 - 28% of the platelets, as the authors started with 10 ml of whole blood and only obtained 0.5 ml of PRP. Therefore, a high platelet concentration, but low PRP volume would be indicative of a low collection efficiency. With the aid of the BD Vacutainer® CPT™ Cell Preparation tubes, a collection efficiency of 83% and 57% was obtained during the sonicated and lyophilised sample preparation techniques respectively. The results indicate a variation in the final volume of PRP obtained which ranged from 5.4 ± 1.0 (sonicated) to 3.8 ± 0.7 (lyophilised). Using a smaller blood volume did not impair the concentration of platelets as the tubes were able to concentrate the platelets better than reported in the studies described above. The ability of the physician to judge what constitutes the “buffy coat” or PRP layer, strongly influenced the final volume of PRP collected. It would be beneficial to standardise the volume of PRP collected from each tube by providing a visual aid indicating on the tube the level to which PRP should be collected. Further evaluation is required to access the influence of the volume of PRP on the final prepared PRP volume and its biological effect.

The volume of PRP collected ranged from approximately 3 to 6 ml, as described above. A volume of 2 to 6 ml PRP has been suggested to treat an injury or for skin rejuvenation in an office setting (Kon *et al.*, 2009, Zenker, 2010). The BD Vacutainer® CPT™ Cell Preparation tubes therefore produced sufficient volume of PRP for these indications.

Some authors have hypothesized that the presence of mononuclear cells in certain PRP's may be responsible for the reported reduction in pain, while providing an additional source of growth factors (Dohan Ehrenfest *et al.*, 2009, Mei-Dan *et al.*, 2010). In this study, the mononuclear cells were not removed from the PRP fraction, avoiding additional processing steps that carries the risk of contamination. According to the collection tube manufacturer a range of $7.02 \times 10^6 \pm 21.44 \times 10^6$ mononuclear cells are recovered per 8 ml of whole blood (Becton Dickinson and Company, 2009). The concentration of mononuclear cells in the preparations was not measured and should be determined in future studies, as well as their impact on tissue regeneration.

Activated platelets express and release growth factors and cytokines that mediate the different phases of the wound healing cascade (Kuo *et al.*, 2010). Growth factors such as TGF-β are stored in granules in a latent form in the platelet and requires platelet activation resulting in degranulation to release it in its active form (Everts *et al.*, 2006b). During wound healing, platelet receptors bind to exposed collagen, vWF, ADP or TXA_2 which results in release of stored Ca^{2+}, activating several Ca^{2+} dependent proteases which aid in platelet activation (Davì and Patrono, 2007, Fox *et al.*, 1983, Rendu and Brohard-Bohn, 2001, Rožman and Bolta, 2007). Platelet activation leads to a conformational change in the platelet which aids in aggregation by forming bridges with plasma fibrin (Eppley *et al.*, 2006, Everts *et al.*, 2006b, Katzung *et al.*, 2009, Marguerie *et al.*, 1984). During platelet activation, thromboplastin is synthesised from the megakaryocyte derived mRNA (Davì and Patrono, 2007). Thromboplastin aids in the activation of the extrinsic coagulation cascade which ultimately leads to the cleavage of prothrombin into thrombin in a Ca^{2+} dependent manner (Anitua *et al.*, 2004, Gailani and Renné, 2007). Thrombin not only further amplifies activation of surrounding platelets but also cleaves fibrinogen to form fibrin, providing a stable insoluble fibre network forming the basis of a clot.

Citrate anticoagulant is used to collect the whole blood for PRP preparation as it prevents blood coagulation by chelating Ca^{2+}, forming calcium citrate complexes that prevents activation of the Ca^{2+} dependent proteases (Loutit and Mollison, 1943). Although some devices and protocols simply inject the PRP in its anticoagulated form (Biomet, [n.d.], Creaney *et al.*, 2011, RegenLab, 2011, Theodor, [n.d.]), others aim to mimic the wound healing cascade during PRP preparation to ensure a product that would contain active growth factors (Anitua *et al.*, 2004, Knighton *et al.*, 1982, Knighton *et al.*, 1986, Kon *et al.*, 2009, Kon *et al.*, 2010). To reverse the effect of the citrate anticoagulant, Ca^{2+} in the form of 10% $CaCl_2$ or $C_{12}H_{22}CaO_{14}$ is added to the PRP to neutralise the anticoagulant effect (Yamaguchi *et al.*, 2012). Some of these devices and protocols go a step further and ensure that the coagulation cascade further amplifies the platelet activation and guarantee the formation of a fibrin clot or gel by combining the PRP- Ca^{2+} preparation with a exogenous thrombin, autologous thrombin, ethanol and or a negatively charged surface such as glass (Anitua *et al.*, 2004, Knighton *et al.*, 1982, Knighton *et al.*, 1986, Krašna *et al.*, 2007, Mazzucco *et al.*, 2004, Yamaguchi *et al.*, 2012).

Using thrombin from an exogenous source leads to a PRP preparation that cannot be classified as autologous. Furthermore, the PRP would contain a foreign protein with the possibility of inducing immunological reactions in the patient (Anitua *et al.*, 2004, De Somer *et al.*, 2006). To prepare autologous thrombin, additional whole blood is collected from a patient, the PRP or PPP fraction obtained combined with 10% $CaCl_2$ or $C_{12}H_{22}CaO_{14}$ in a glass container and left to form a fibrin gel (Everts *et al.*, 2006a, Knighton *et al.*, 1982, Mazzucco *et al.*, 2004). Glass is used as it initiates the intrinsic blood coagulation pathway by activating Factor XII (Hageman factor) (Schousboe, 1985). The newly formed clot is compressed and the supernatant collected which is combined with the previously obtained PRP fraction in a 1:4 ratio, volume per volume. Although this process ensures the use of an autologous PRP preparation, the additional steps leads to a longer preparation time and the possibility to compromise sterility.

Although not standardised a concentration of 13 - 18.8% ethanol is added to the preparation of autologous thrombin to inactivate antithrombin, prolonging the lifespan of the autologous thrombin (Everts *et al.*, 2006b, Kuo *et al.*, 2010). The final concentration in a preparation of autologous thrombin can range between 3 - 8%, which is further diluted in a 1:4 ratio (Knighton *et al.*, 1982, Kuo

et al., 2010, Mazzucco *et al*., 2004). Furthermore, ethanol, a chaotropic agent, is able to convert latent growth factors, such as TGF-β, into their active form as it disrupts the non-covalent bonds between the associated inhibiting proteins (Sporn *et al*., 1987, Werz *et al*., 1996).

In this study, no significant difference between the concentrations of PDGF-AB obtained from platelets treated with ethanol or with a 10% $CaCl_2$, PPP combination was seen, although the concentration of PDGF-AB obtained with treating the PRP with the 10% $CaCl_2$, PPP combination was approximately double. This finding correlated with that of Everts *et al*., 2006, who demonstrated that the addition of autologous thrombin leads to platelet activation, the formation of a fibrin gel and the release of increased amounts of PDGF and TGF-β from the PRP. A combination of 10% $CaCl_2$, PPP and ethanol was used in this study to activate the PRP and ensure the release of active growth factors during development of the PRP preparation method.

To simplify the preparation of autologous thrombin without the collection of additional blood and preparation steps, the PRP fraction was added to 0.5 ml 5% ethanol and the PPP fraction to 1.5 ml 10% $CaCl_2$. The concept was to use the additional PPP to prepare the autologous thrombin while activating latent growth factors in the PRP fraction. PPP contains adhesion proteins such as fibrin, fibronectin, and vitronectin (Yamaguchi *et al*., 2012). The addition of the $CaCl_2$, PPP preparation not only ensured that the effect of the citrate anticoagulant had been reversed but also added fibrin, fibrinogen, vitronectin and thrombin. The PRP preparation would therefore potentially have the benefit of a fibrin sealant combined with the regeneration potential of platelet-derived factors.

The total concentration of protein in plasma ranges from 65 - 90 mg/ml (Meyer *et al*., 2002). The majority of the protein contained within the plasma is from albumin (> 50%), followed by globulins (approximately 12%) and fibrinogen (approximately 4%) (Cameron *et al*., 1936, Gornall *et al*., 1949, Ingram, 1952, Martin and Morris, 1949, Meyer *et al*., 2002, Tirumalai *et al*., 2003, Travis *et al*., 1976). The measured protein concentrations recovered from the different optimised PRP preparation methods was 22.6 mg/ml and 25.2 mg/ml for the first (sonicated) and second (lyophilised) sample preparations, respectively, approximately 3 - 4 fold lower than the than the normal range. No literature was available to compare the total protein concentration obtained by the sample preparations. The

total protein concentration of the PPP was not assessed. The reason for the lower protein concentration was not elucidated although, it was later postulated that the analytical method may have been out of the linear range. The aim of the study was not to elucidate the full spectrum of proteins and polypeptides obtained in plasma or platelets, but rather to determine the concentration of PDGF-AB and TGF-β_1 obtained. The protein concentrations of the samples used for electrophoresis were diluted and standardised to approximately 1 ug/ul to ensure equal loading for SDS-PAGE analysis.

As shown by the SDS-PAGE, the individual bands were identified according to their migration relative to proteins standards of known molecular mass. As expected, albumin, with a molecular mass of 67 kDa, closely corresponded to the 66.2 kDa band of the marker set, similar to results obtained by previous studies (Anderson and Anderson, 1977, Tirumalai *et al*., 2003). Low levels of high molecular weight proteins were identified after Coomassie staining of the gel. The plasma proteins which are expected to correspond to the protein standards, include the largest fibronectin fragment (116 kDa), heavy chains of immunoglobulins (45 kDa) and light chains of the immunoglobulins (25 kDa), as these are the more abundant plasma proteins, after albumin (Tirumalai *et al*., 2003). Integrin complexes found on the membrane of platelets such as GPIIa/IIIb have been identified in platelet lysates within a molecular mass range of 66.2 - 116 kDa (Moebius *et al*., 2005) and this matched the multiple bands seen in this molecular mass range and may reflect the many intergin complexes.

The bands at position A, B and C on Figure 3.1 are faint and therefore unlikely to indicate the presence of the PDGF A chain, PDGF B chain, and the TGF-β_1 subunits respectively. Fong *et al*., (2011) identified 1048 proteins released from activated platelets, 69 of which were membrane proteins. As none of the proteins visualised by Coomassie staining were further identified, these bands may have reflected a number of low molecular mass proteins such as beta-thromboglobulin (βTG) subunits and cytochrome C. In addition, the low molecular mass bands may be fragments of larger proteins and not solely intact low molecular mass proteins (Tirumalai *et al*., 2003). To quantify the concentration of PDGF-AB and TGF-β_1, enzyme-linked immunological assays were preformed.

During the PRP preparation with the developed method described above, the activated PRP was left to incubate at room temperature until a fibrin clot had formed. The fibrin clot traps the platelets as they release their pre-synthesised growth factors (Enoch and Price, 2004, Macri *et al*., 2007, Roy *et al*., 1993). These growth factors bind to the fibrin and other constituents in the clot. The clot is thereby able to act as a growth factor reservoir, slowly releasing them into the wound area (Eppley *et al*., 2006). Among the many growth factors released by platelets, TGF-β and PDGF-AB are release in the highest concentration (He *et al*., 2009).

The supernatant released from the sonicated and centrifuged clots contained extremely low growth factor concentrations in comparison to the available literature (Table 3.3). It was assumed that the sonication and centrifugation was not able to disrupt the binding of the growth factors to the clot or the growth factors had been denatured in the process. The experiment was repeated and instead the clot was lyophilised and resuspended in PBS. The lyophilised clots contained TGF-β_1 in range of the available literature when the platelet concentration is enriched two to four fold the baseline platelet count. The developed PRP preparation procedure's ability to release growth factors from the platelet stores was comparable to the current available devices and protocols.

Various studies have shown the ability of PRP to induce cell proliferation, although the ideal growth factor concentration and PRP components are not known (Tinsley *et al*., 2012). Graziani *et al*., (2006), demonstrated that the effect of PRP on cells is dose dependent. The authors noticed a significantly higher cell proliferation (85%) in cultures treated with activated PRP with an enriched platelet content two-and-a-half fold the baseline platelet count at 72 h. The results obtained in this study using the MTT-assay are comparable to the results obtained by Graziani *et al*., (2006). Cell proliferation of the experimental wells was compared to a positive control treated with 10% FCS (100% cell viability). A concentration of 10% PRP preparation in the wells was able to induce a cell proliferation from 87 - 105% in comparison to the positive control. The high percentage of proliferation may be due to the large burst of growth factors with which the cells are incubated with as only the supernatants of the fibrin clots were incubated with the cells and not the entire clot. After 72 h of incubation, the percent proliferation of normal dermal fibroblasts slightly decreased. As dermal fibroblasts have a doubling time of 18 - 24 hours (ZenBio, 2011), the decrease in the percent

proliferation may be due to overgrowth of the primary culture, a depletion of available growth factors or nutrients in the media all of which could lead to a slower proliferation rate, a decrease in mitochondrial activity or cell death (American Type Culture Collection, 2012). Normal dermal fibroblasts treated with a 5 or 10% PRP concentration significantly stimulated proliferation when measured by the crystal violet assay in comparison to the positive control. The proliferation followed a dose-response relationship, with cell treated with a 5% PRP concentration showing the highest percentage proliferation. Similar to the MTT-assay, after 72 h incubation, the percentage proliferation of the normal dermal fibroblasts decreased slightly. The decrease in the percent proliferation may be due to overgrowth of the primary culture, a depletion of available growth factors or nutrients in the media all of which could lead to a slower proliferation rate or cell death. The overgrowth hypothesis is further supported by the lower proliferation rate in the 10% PRP concentration treated cells in comparison to the 5% PRP concentration treated cells. It is postulated that the cells in the 10% PRP concentration treated group reach confluence earlier than the other groups and therefore, further growth has inhibited.

A study by He *et al.*, (2009) demonstrated that thrombin activated PRP release more than 80% of the platelet growth factors on day one. This sudden release of growth factors is followed by a decline in growth factor concentration at 3, 7 and 14 days after activation. The authors compared these results to a platelet rich fibrin clot and found that the fibrin clot demonstrate the highest release of growth factors 14 days after preparation. The fibrin clot had a prolonged release of growth factors, similar to normal wound healing, with a delayed peak release of growth factors. The authors further mentioned that the massive fibrin content within the clot protected the growth factors from proteolytic degradation. The concentration of TGF-β obtained after day one was 100 ng/ml and 50 ng/ml for the exudates of activated PRP and platelet rich fibrin clot obtained from cell culture medium, respectively. On day 14, the concentration of TGF-β obtained from the exudates of activated PRP was 25 ng/ml compared to 150 ng/ml for the fibrin clot obtained from the cell culture medium. As a concentration of approximately 50 ng/ml TGF-β was obtained with the optimised PRP preparation, it is plausible that the remaining growth factors was still bound to the fibrin clot. As only the supernatant of the fibrin clots were used during the cell proliferation assays, future studies on cell proliferation should entail

the use of freshly prepared and activated PRP. To determine whether this PRP preparation would mimic the slow release of growth factors from the fibrin clot the freshly prepared and activated PRP should be incubated with the normal dermal fibroblasts as soon as possible and the cell proliferation and growth factor concentration measured at 3, 7, and 14 days after incubation.

The present study proved that the developed extraction and activation method induces growth factor release that can stimulate primary cell proliferation.

When the percentage of viable cells measured with the crystal violet cell proliferation assay was compared to the MTT-assay, the number of viable cells in the crystal violet assay was considerably higher in the 5% and 10% PRP concentration treated groups.

The MTT-assay is a quantitative colorimetric assay that measure viability and proliferation of cells based on the capacity of the cellular mitochondrial dehydrogenase enzyme to reduce the yellow substrate into a dark purple formazan product in living cells (Mosmann, 1983). The amount of formazan produced is directly proportional to the number of viable cells in the assay. The crystal violet assay a colorimetric method based on the use of the basic crystal violet dye to bind to negatively charged nuclear structures of adherent cells (Bonnekoh *et al*., 1989, Vandersickel *et al*., 2011, Vega-Avila and Pugsley, 2011). The O.D. measurements of extracted dye provides a measure of the relative number of viable cells. It is therefore questioned why the percentage of proliferation in the crystal violet assay is considerably higher than in the MTT-assay. Vandersickel *et al*., (2010), measured cell survival of MCF10A cells after radiation exposure with the crystal violet cell assay compared to the standard colony formation assay. The authors noted a higher estimation of cell survival with the crystal violet assay and attributed this finding to the measurement of both dividing and non-dividing cells. As cells reach confluence, their proliferation rate slows down, paired with a decrease in mitochondrial activity, which may reflect the decreased in percentage proliferation seen in the MTT-assay (which requires an active mitochondria) compared to the crystal violet assay.

A study by Bonnekoh, *et al*., (1989), demonstrated the ability of crystal violet to bind to proteins. The water-soluble protein fraction of cultured epidermal cells was separated by SDS-PAGE and stained with crystal violet. The authors noted a less intense but similar staining pattern to that of Coomassie

blue. Furthermore, crystal violet is also used as a protein stain to enhance the appearance of bloody fingerprints (Saviers, 2000). It is therefore possible that the crystal violet assay not only measured the percentage of viable cells but also stained proteins produced by these cells as well as sub-cellular proteins. It is unlikely that the crystal violet stained any of the residual proteins of the added PRP as this would have been reflected in the 10% PRP concentration treated group and the positive control. During wound healing the platelet-derived growth factors not only promote cell proliferation and migration but the deposition of new extracellular matrix proteins needed to support the cells that fill the wound bed (Fitzpatrick and Rostan, 2003, Haukipuro *et al*., 1991, Rutkowski *et al*., 2008, Stojadinovic *et al*., 2008, Wroblewski *et al*., 2010). The initial material produced by fibroblasts within a wound are granulation tissue with predominantly Type IV collagen. As the proliferation phase of wound healing advances, the granulation tissue is gradually replaced by Type I collagen. It is therefore possible that the crystal violet assay used in this study indicated the presence of "new tissue" formation induced by the growth factors released by the activated PRP. Crystal violet has been shown to bind to DNA (Heng *et al*., 2006), it is speculated that crystal violet might also be able to stain other negatively charged compounds and lysosomes.

In this study, a 5 - 10% PRP preparation contained 2.3 - 4.6 ng/ml TGF-β_1 in combination with a variety of other growth factors, cytokines and adhesion proteins. Many studies have investigated the ability of a single growth factor to induce cell proliferation. Seppä *et al*., 1982, supplemented serum-free culture medium with 3.1 ng/ml EGF, FGF or PDGF introduced at the beginning of cell culture experiments. The authors noted that at 24 h, cell proliferation of normal dermal fibroblasts were increased in the presence of serum-supplemented culture medium. The authors stated that "the replication of mesenchymal cells can be activated by low concentrations of growth factors such 1 ng/ml PDGF", but mentioned that "a significant increase in cell growth was observed when growth factors were incorporated within fibrin". In contrast to the literature, the present study was unable to induce cell proliferation by the addition of a single growth factor to serum-free medium, or medium supplemented with a low percentage of serum.

Members of the medical fraternity showed interest in the use of a locally developed and produced PRP extraction kit, which would easily obtain activated PRP. As the developed method yielded

positive *in vitro* results, a case study registry was opened whereby physicians interested in the use of PRP could treat a variety of cosmetic indications and injuries. The study product and instruction leaflet was prepared in a kit, which contained all the necessary equipment, except a centrifuge, to easily prepare autologous PRP from a patient. From data reported by physicians and patients in a series of case studies it is evident that PRP prepared using the method developed during this study augmented soft tissue regeneration and bone formation. Patients treated with the activated PRP for skin rejuvenation reported an improvement in skin texture and smoothness while decreasing scars, fine lines and wrinkles. The simple PRP preparation method was able to produce similar skin rejuvenation results as reported in peer reviewed literature (Cho *et al.*, 2011, Na *et al.*, 2011, Sclafani, 2010, Zenker, 2010).

Furthermore, treatment with the activated PRP resulted in symptom reduction and accelerated healing of various injuries and symptom management in most of the patients diagnosed with osteoarthritis. A single injection with activated PRP was able to induce increased bone density and cortical bone formation in a non-attaching bone fracture.

The patients that were treated with the prepared PRP in the present study did not report any product related side effects. However, two patients (one with osteoarthritis of the knee and one with plantar fasciitis) reported severe pain at the injection site 3 - 4 days post treatment. With the current developed method, physicians were pleased with the easy of PRP extraction method and the study product which able to easily produce PRP in a clinical setting without prior training and limited equipment.

The hypotheses can therefore be accepted as the developed thrombin free extraction method of platelet-rich plasma does yielded therapeutically usable concentrations of growth factors.

4.2 CONCLUSION

The method developed during this study to isolate PRP, was a simplified method, which will enable physicians to easily obtain autologous PRP in a consulting room setting for point-of-care use with sufficiently concentrated growth factors such as TGF-β and PDGF. The prepared PRP has been shown to initiate soft tissue augmentation by cell proliferation and extracellular matrix production. These results correlate with clinical findings of skin rejuvenation and accelerated wound and internal injury healing rates. The PRP prepared by the current method is a safe and effective treatment for improvement of scars, fine lines and wrinkles, pain and symptom management of chronic injuries and osteoarthritis, and overall skin revitalisation.

Future research may be directed toward elucidating the complete spectrum of growth factors, adhesion proteins and mononuclear cells contained within the PRP preparation as well as the role these components play in soft tissue augmentation and bone regeneration, as their inclusion in the PRP preparation is only speculated. Another area where this PRP preparation can be tested is with respect to the ability to induce enhanced wound healing in chronic wounds where a protocol needs to be developed for the optimal number of treatments needed to augment soft tissue regeneration and bone formation.

According to the SAMED, all medical devices will need to be registered in the future and the Medicines Control Council of South Africa (MCC) is drafting the new regulations thereof. The developed PRP preparation procedure and components used will need to be registered to conform to the proposed standards set out by the MCC. The use of commercially available blood collection tubes and a well-defined protocol for the PRP preparation would allow relatively easy registration of the kit that has been developed during this study.

The simplified, developed PRP preparation method combined with the study product would aid physicians to easily obtain autologous PRP in a clinical setting.

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APPENDIX A

Non-intervention research 2

PARTICIPANT'S INFORMATION LEAFLET & INFORMED CONSENT FORM

Dear Mr. / Mrs. / Ms ..... date ...../...../....

THE NATURE AND PURPOSE OF THIS STUDY

I understand that I am being asked to take part in an experimental *in vitro* research study. Thus the research is conducted entirely outside of the body in a laboratory. The aim of this study is to draw blood from the participant. By collecting blood, we wish to extract growth factors and add it to cell lines (skin cells grown in the laboratory) to study the possible treatment for skin that has been exposed to Ultra Violet light damage (sun damage). Sun damage produces abnormalities in the dermal fibroblasts, these cell do not function the same as they produce less collagen than normal cells, resulting in wrinkles. Collagen is a protein found normally in the skin and is produced by cells in the skin. Collagen keeps the skin supple and firm. Skin lacking normal collagen appears wrinkled. Growth factors (proteins found in the blood) have the ability to increase collagen production in cells thus have the potential to treat wrinkles.

EXPLANATION OF PROCEDURES TO BE FOLLOWED

This study involves collecting blood in the form of a venipuncture. A competent medical person will clean the area on your arm with an antiseptic wipe and gently insert a needle into your vein, and blood collected.

RISK AND DISCOMFORT INVOLVED

The only possible risk and discomfort involved is the collection of blood in the form of a venipuncture which involves inserting a needle into your vein.

POSSIBLE BENEFITS OF THIS STUDY

This study has no direct benefit to you as the volunteer but it will aid in the research of possible new treatments for sun-exposed skin.

I may at any time withdraw from this study

You may choose to withdraw from participating in this study at any time without stating a reason.

HAS THE TRIAL RECEIVED ETHICAL APPROVAL?

This clinical trial Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2000), which deals with the recommendations guiding doctors and investigators in biomedical research involving human subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

INFORMATION

If I have any questions concerning this study, I should contact: Ilze Laurens cell: 082 448 9153

CONFIDENTIALITY

All records obtained whilst in this study will be regarded as confidential. Results will be published or presented in such a fashion that patients remain unidentifiable.

CONSENT TO PARTICIPATE IN THIS STUDY

I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given opportunity to ask questions and am satisfied that they have been answered satisfactorily. I hereby volunteer to take part in this study.

I have received a signed copy of this informed consent agreement.

…… ……

Volunteer's signature Date

…… ……

Person obtaining informed consent Date

…… ……

Witness Date

APPENDIX B

PATIENT INFORMATION LEAFLET AND INFORMED CONSENT DOCUMENT

CASE STUDY TITLE

Case study / Patient Registry to investigate the efficacy of the PRP kit for various cosmetic and sports related indications

DAYTIME AND AFTER HOURS TELEPHONE NUMBER

Doctor:_____

PATIENT NAME

PATIENT IDENTIFICATION NO.

To the Participant: *This consent form may contain words or concepts that you do not understand. Please ask the doctor or the staff to explain any words or information that you do not clearly understand. You may take home* ***an unsigned copy of this consent*** *form to think about or discuss with family or friends* ***before making your decision.***

INTRODUCTION:

You are invited to consider participating in a case study. Your participation in this study is entirely voluntary.

Before agreeing to participate, it is important that you read and understand the following explanation of the purpose of the study, the study procedures, benefits, risks, discomforts, and precautions as well as the alternative procedures that are available to you, and your right to withdraw from the study at any time.

This information leaflet is to help you to decide if you would like to participate. You should fully understand what is involved before you agree to take part in this study.

If you have any questions, do not hesitate to ask.

- Should not agree to take part unless you are satisfied about all the procedures involved.
- If you decide to take part in this study, you will be required to sign this document to confirm that you understand the study. You will be given a copy to keep.

CASE STUDY AIM:

The aim of this case study is to determine the efficacy of the platelet rich plasma (PRP) kit for various cosmetic and sports related indications. This kit is designed to concentrate growth factors and other substances in the platelet rich part of your blood. The PRP is then injected into a desired area in order to stimulate specific cell growth, which will help to repair/improve the desired area. PRP can be used to assist in the healing of sports injuries, improve appearance of wrinkles and fine lines, healing of scars, wound healing etc. PRP is collected from the patient's own blood and re-injected using a sterile technique. This therapy is not new; however the way in which the PRP is prepared is new. Your doctor has decided that PRP would be a good choice for the treatment of your condition and you are a candidate to consider the use of your clinical data and pictures to demonstrate the effect of treatment using the new technique to yield PRP.

LENGTH OF THE CASE STUDY AND NUMBER OF PARTICIPANTS:

The study will be performed at your doctor's office.

The number of participants has not been determined because this is a case study, which means that patients can be evaluated only if the doctor prescribes treatment with the PRP kit

The participants must be over the age of 18 or must obtain consent from a legal guardian or parent to participate

Treatment with the PRP kit will be administered in your doctor's office and the procedure will take ± 30 - 60 minutes.

PROCEDURES:

If you volunteer as a candidate to take part in this study, you will have to agree to take part by signing this document.

Your doctor will then start with the procedure as described below:

1. Your doctor will take a "before" picture of the area that is going to be treated. This photo will be taken in such a way that you will remain anonymous (no one will be able to recognize you from the photo)

2. Your doctor will draw 2 tubes (18ml) of your blood

3. He/she will then process the blood before injecting the PRP in the treatment area

4. You will need to go back to your doctor after ± 4 - 6 weeks so that you and he/she can assess the results and take an "after" picture of the treated area.

WILL ANY OF THESE STUDY PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE?

Venipunctures (i.e. drawing blood) and injection of the PRP into the treatment area are normally done as part of routine medical care by your doctor and present a slight risk of discomfort. Drawing blood

may result in faintness, inflammation of the vein, pain, bruising or bleeding at the puncture site. There is also a slight possibility of infection. Your protection is that experienced personnel perform the procedures under sterile conditions. A total of 18 ml of blood will be collected from you.

RISKS OF THE TREATMENT:

No side effects are expected. The PRP kit is similar to other PRP kits on the market that has been used successfully and without side effects for many years. Because your doctor is going to use your own blood to obtain the PRP it cannot elicit an allergic reaction. The content of the PRP kit is sterile (before opening) and your doctor is instructed to maintain sterility. Therefore the risk of infection is minimal. Although the safety of this type of kit is well established, this is the one of the first times that the PRP is tested and there might be side effects, which are unforeseen or unknown. You should immediately contact your doctor if any side effects occur after the application of the PRP.

BENEFITS:

Your participation in this study will contribute to medical knowledge and there will be no other benefits other than the treatment selected by your doctor.

RIGHTS AS A PARTICIPANT IN THIS CASE STUDY:

Your participation in this study is entirely voluntary and you can decline to participate, or stop at any time, without stating any reason. Your withdrawal will not affect your access to the medical care you are receiving.

CONFIDENTIALITY:

All information obtained during the course of this study, including medical records, personal data and photographs will remain confidential. Data that may be reported in scientific journals will not include any information or visual material that can identify you as a participant in this study.

ETHICAL CONSIDERATIONS:

This is a case study; not a clinical trial; therefore approval from an ethics committee is not deemed necessary if the data is not given out by your doctor, however in order to register the benefit on a registry your data will be entered anonymously on this registry and reported as such in medical literature, you will not be identified and none of the photographs will be used unless you sign specific consent for that. Consent from the participant should be obtained before inclusion in the study to release data on to a registry. This is a standard way of obtaining platelets for reinjection. The only difference to existing kits is the exposure of platelets to Calcium and ethanol to activate the platelets and to reverse the effect of citrate. The process does not include drug therapy or exposure to drugs as the in vitro treated platelets are then re-injected in to the patient. This is a devise for obtaining platelets and falls outside the MCC mandate (note only the FDA has a process for screening). The device however needs to comply with certain specifications; i.e. quality and sterility therefore the sterile repacking of the containers. The latter process needs certification.

INFORMED CONSENT:

I hereby confirm that I have been informed by my doctor about the nature, conduct, benefits and risks of the clinical study:

I have also received, read and understood the above written information (Participant Information Leaflet and Informed Consent Document) regarding the case study.

I am aware that the results of the study, including photographs, personal details regarding my gender, age, date of birth, name, initials or diagnosis will be anonymously processed into a study report.

I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the case study.

I understand that this is medical research and not treatment and that I might not benefit from taking part.

PARTICIPANT:

Printed Name Signature Date and Time

LEGAL GUARDIAN IF PARTICIPANT IS YOUNGER THAN 18 YEARS:

Printed Name Signature Date and Time

I, (person obtaining consent), herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study.

STUDY DOCTOR/EVALUATOR/MEDICAL APPOINTEE:

Printed Name Signature Date and Time

WITNESS:

Printed Name Signature Date and Time

APPENDIX C

CASE STUDY REPORT FORM FOR THE USE OF THE PRP KIT

Contact details for queries and / or reporting of results
Ilze Laurens Cellphone: 0820583818 E-mail address: ilze@dermav.co.za

Patient name and surname:

……

Page 2: Date of start of treatment:

……

Page 4: Date of follow-up consultation:

……

Page 6: Date of follow-up / 2nd treatment (if any):

……

Page 8: Date of 2nd follow-up consultation (if any)

……

The following page should be completed ON THE DAY OF TREATMENT by the respected persons as indicated below

A) Completed by physician:

Name:……**Signature:**……

Date: ……

Detailed patient diagnosis (for which indication is the patient treated for):

……
……
……
……
……

Describe step-by-step the technique used for applying the PRP:

……
……
……
……

Did you take a photograph / MRI of the treatment area before commencement of treatment?

Tick: [YES / NO]

Please insert the photograph/ MRI in the file and give an interpretation of the photo/ MRI

……
……
……
……

What is the severity of the patient's condition/injury according to your diagnosis? Please mark the severity according to you on a number with an X on the scale below:

Healthy/normal Mild severity Moderate severity Extremely severe

B) Completed by patient:

Patient identification number: ……**Date:** ……

What is the severity of your condition/injury according to you? Please mark the severity according to you on a number with an **X** on the scale below:

Healthy/normal | Mild severity | Moderate severity | Extremely severe

The following page should be completed ON THE DAY OF THE FOLLOW-UP VISIT by the respective persons as indicated below:

A) Completed by patient:

Name:……**Signature:**……

Date: ……

Did you take a photograph / MRI of the treatment area before commencement of treatment?

Tick: [YES / NO]

Please insert the photograph/ MRI in the file and give an interpretation of the photo/ MRI

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Please describe your impression of the outcome of the PRP treatment below:

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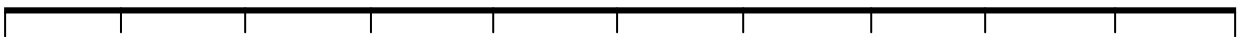
What is the severity of your condition/injury according to you? Please mark the severity according to you on a number with an **X** on the scale below:



Healthy/normal Mild severity Moderate severity Extremely severe

How would you rate your satisfaction with the product? Please indicate with an X on the scale below:

Not at all satisfied Highly satisfied

B) Completed by patient:

Patient identification number: ……**Date:** ……

What is the severity of your condition/injury according to you? Please mark the severity according to you on a number with an **X** on the scale below:

Healthy/normal Mild severity Moderate severity Extremely severe

How would you rate your satisfaction with the product? Please indicate with an X on the scale below:

Not at all satisfied Highly satisfied

The following page should be completed ON THE DAY OF THE COMMENCEMENT OF 2nd TREATMENT (if any) by the respective persons as indicated below:

A) Completed by physician:

Name:……**Signature:**……

Date: ……

Detailed patient diagnosis (for which indication is the patient treated for):

……
……
……
……
……

Describe step-by-step the technique used for applying the PRP:

……
……
……
……

Did you take a photograph / MRI of the treatment area before commencement of treatment?

Tick: [YES / NO]

Please insert the photograph/ MRI in the file and give an interpretation of the photo/ MRI

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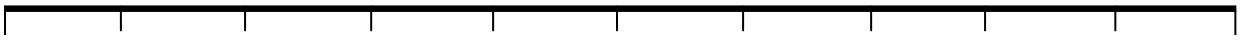
What is the severity of the patient's condition/injury according to your diagnosis? Please mark the severity according to you on a number with an X on the scale below:

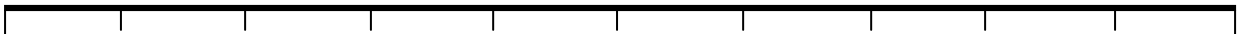


Healthy/normal Mild severity Moderate severity Extremely severe

B) Completed by patient:

Patient identification number: ……**Date:** ……

What is the severity of your condition/injury according to you? Please mark the severity according to you on a number with an **X** on the scale below:



Healthy/normal | Mild severity | Moderate severity | Extremely severe

---

The following page should be completed ON THE DAY OF THE 2nd FOLLOW-UP VISIT by the respective persons as indicated below:

A) Completed by patient:

Name:……**Signature:**……

Date: ……

Did you take a photograph / MRI of the treatment area before commencement of treatment?

Tick: [YES / NO]

Please insert the photograph/ MRI in the file and give an interpretation of the photo/ MRI

……
……
……
……

Please describe your impression of the outcome of the PRP treatment below:

……
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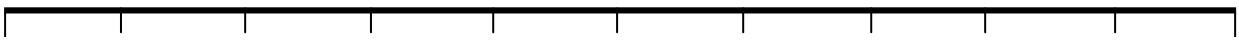
……

What is the severity of your condition/injury according to you? Please mark the severity according to you on a number with an **X** on the scale below:



Healthy/normal Mild severity Moderate severity Extremely severe

How would you rate your satisfaction with the product? Please indicate with an X on the scale below:

Not at all satisfied Highly satisfied

B) Completed by patient:

Patient identification number: ……**Date:** ……

What is the severity of your condition/injury according to you? Please mark the severity according to you on a number with an **X** on the scale below:

Healthy/normal Mild severity Moderate severity Extremely severe

How would you rate your satisfaction with the product? Please indicate with an X on the scale below:

Not at all satisfied Highly satisfied

APPENDIX D

FACULTY OF HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria comply with ICH-GCP guidelines and has US Federalwide Assurance. FWA 00002567, Approved dd 22 May 2002 and Expires 24 Jan 2009.
IRB 0000 2235 IORG0001762 Approved dd Jan 2006 and Expires 21 Nov 2008.

Universiteit van Pretoria
University of Pretoria

Faculty of Health Sciences Research Ethics Committee
University of Pretoria
HW Snyman Building, (South)
Level 2-34
Pretoria

Private Bag X169
Pretoria
0001

Date: 21/08/2007

PROTOCOL NO.		**78/2007**
PROTOCOL TITLE		An investigation to determine whether transforming growth factor beta and platelet-derived growth factor can be carried across the stratus corneum with electoporation.
INVESTIGATOR		Ilze Laurens
	Phone:	012-3192243
	Fax:	
	E-Mail:	ilzelaurens@yahoo.com
	Cell:	082 448 9153
DEPARTMENT		Pharmacology; University of Pretoria
STUDY DEGREE		M.Sc (Pharmacology)
SUPERVISOR		Prof J R Snyman
SPONSOR		Applied for NAVKOM funding
MEETING DATE		27/06/2007

This Protocol and Informed Consent and all the attatchments have been considered by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria on 25/07/2007 and found to be acceptable.

*Advocate AG Nienaber	(female)BA(Hons) (Wits); LLB; LLM (UP); Dipl.Datametrics (UNISA)
*Prof V.O.L. Karusseit	MBChB; MFGP (SA); M.Med (Chir); FCS (SA): Surgeon
*Prof M Kruger	(female) MB.ChB.(Pret); Mmed.Paed.(Pret); PhDd. (Leuven)
Dr N K Likibi	MB.BCh.; Med.Adviser (Gauteng Dept.of Health)
Snr Sr J. Phatoli	(female) BCur (Et.Al) Senior Nursing-Sister
Dr L Schoeman	(female) Bpharm, BA Hons (Psy), PhD
*Dr R Sommers	(female) MBChB; M.Med (Int); MPhar.Med;
*Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path) Senior Specialist; Oral Pathology
Dr A P van Der Walt	BChD, DGA (Pret) Director: Clinical Services of the Pretoria Academic Hospital
*Prof C W van Staden	MBChB; Mmed (Psych); MD; FTCL; UPLM; Dept of Psychiatry

DR R SOMMERS; MBChB; M.Med (Int); MPhar.Med.
SECRETARIAT of the Faculty of Health Sciences Research Ethics Committee - University of Pretoria

* = Members attended the meeting on 25/07/2007.

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 27/08/2009

Prof V Steenkamp
Department of Pharmacology
University of Pretoria

Best Prof Vanessa Steenkamp

RE.: Application for Blood Collection utilizing lymphocytes, macrophage, neutrophils and plasma.

Herewith acknowledgement that the above Application for blood collection has been received and tabled on 26/08/2009, and found to be acceptable by the Faculty of Health Sciences Research Ethics Committee.

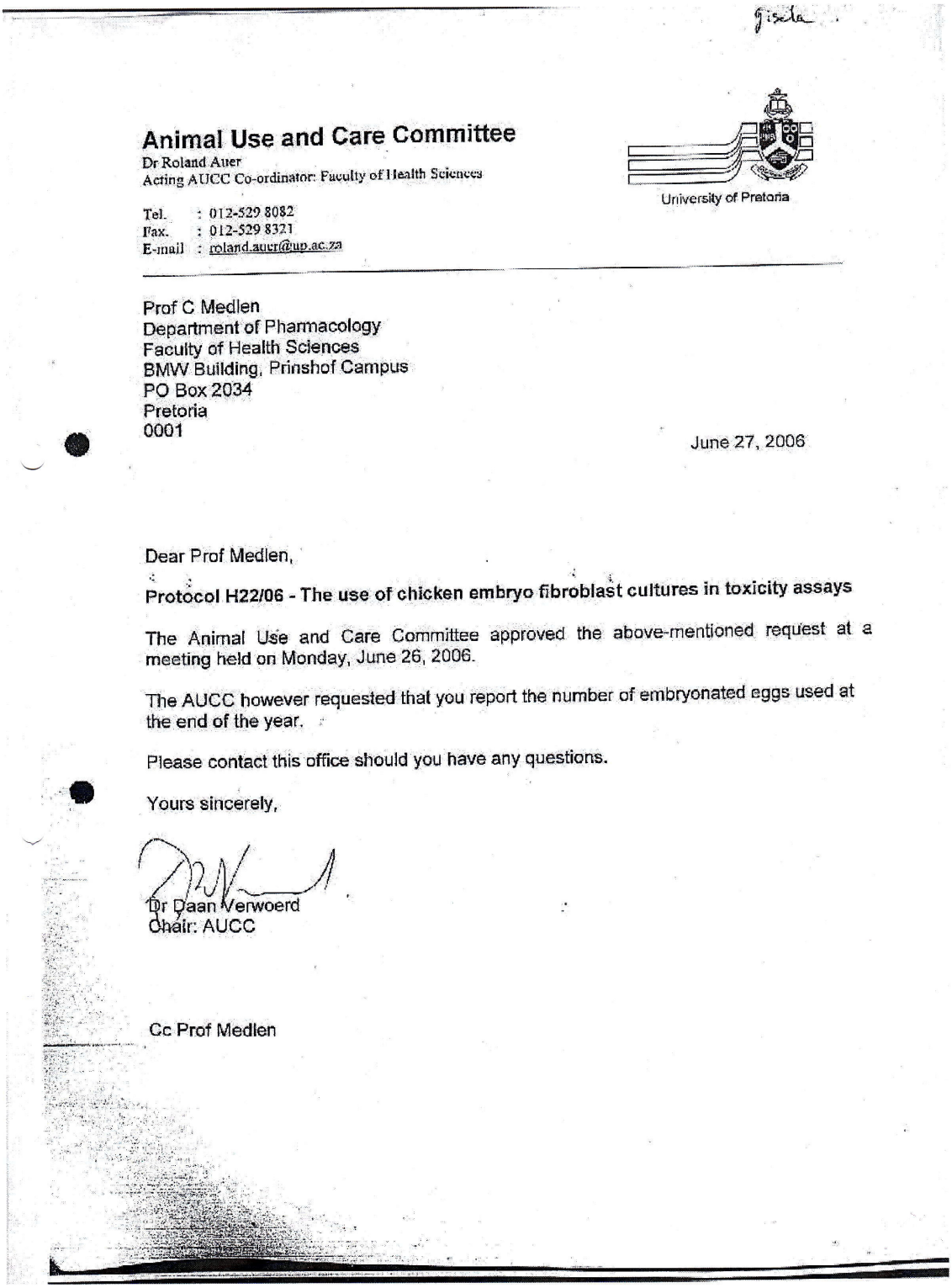
With regards

DR R SOMMERS; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

31 Bophelo Road ♦ H W Snyman Building (South) Level 2-34 ♦ P.O.BOX 667, Pretoria, South Africa, 0001 ♦ Tel:(012)3541330 ♦
♦ Fax: (012)3541367 / 0866515924 ♦ E-Mail: manda@med.up.ac.za ♦ Web: //www.healthethics-up.co.za ♦

MS: dd 2009/09/28: C:\DOCUME~1\User\LOCALS~1\Temp\XPgrpwise\Blood Collection~Approval.doc

ANIMAL USE AND CARE COMMITTEE



Gisela

Animal Use and Care Committee
Dr Roland Auer
Acting AUCC Co-ordinator: Faculty of Health Sciences

Tel. : 012-529 8082
Fax. : 012-529 8321
E-mail : roland.auer@up.ac.za

University of Pretoria

Prof C Medlen
Department of Pharmacology
Faculty of Health Sciences
BMW Building, Prinshof Campus
PO Box 2034
Pretoria
0001

June 27, 2006

Dear Prof Medlen,

Protocol H22/06 - The use of chicken embryo fibroblast cultures in toxicity assays

The Animal Use and Care Committee approved the above-mentioned request at a meeting held on Monday, June 26, 2006.

The AUCC however requested that you report the number of embryonated eggs used at the end of the year.

Please contact this office should you have any questions.

Yours sincerely,

Dr Daan Verwoerd
Chair: AUCC

Cc Prof Medlen

THE SOUTH AFRICAN MEDICAL ASSOCIATION

THE SOUTH AFRICAN MEDICAL ASSOCIATION
Tel: +27 (0)12 481 2000 | Fax: +27 (0)12 481 2100 | www.samedical.org
Block F, Caslte Walk Office Park, Nossob Street, Erasmuskloof, Ext 3, Pretoria
PO Box 74789, Lynnwood Ridge, 0040

27 October 2010

DermaV Pharmaceuticals (Pty) Ltd
P O Box 167
Cornwall Hill
Irene
Ext 10

Attention : Wilna Bouwer
wilna@dermav.co.za

Dear Wilna

APPROVAL : PRP KIT – REGISTRY

Registry Title	A case study / patient registry to investigate the efficacy of the celluVance™ PRP kit for various cosmetic and sports related indications

Resolution

After noting and considering the input of the evaluators on the above-mentioned study, the committee **RESOLVED** that the application be **approved**.

1. **PATIENT INFORMATION AND INFORMED CONSENT DOCUMENT**

   The Patient Information and Informed Consent Document were approved.

2. **CASE STUDY REPORT FORM**

   The Case Study Report Form was noted and approved.

3. **CelluVance™ PRP Kit Brochure and Instructions for Use**

   The CelluVance™ PRP Kit brochure and Instructions for Use was noted and accepted.

4. **NHREC APPLICATION dated 24 October 2010**

   The NHREC application was noted. Kindly submit the Department of Health number to SAMAREC, when available.

Yours sincerely

Maureen Otto
SAMAREC Co-ordinator
SAMA Research Ethics Committee
Tel: (012) 481 2046
Fax: (086) 631 0566
E-mail: maureeno@samedical.org