Elucidating the Biological Role of Autologous Derived Platelet-Rich Plasma Gel in the Treatment of Chronic Diabetic Foot Ulcers.

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Elucidating the Biological Role of Autologous Derived Platelet-Rich Plasma Gel in the Treatment of Chronic Diabetic Foot Ulcers.

A Thesis Submitted in accordance with Regulations for the Degree of MD (*Res*)

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ABSTRACT

The molecular basis for the use of synthetic growth factors (GFs) in tissue reparation has been poorly investigated. More recently, autologous derived platelet rich growth factor has gained popularity in the field of regenerative/ reparative medicine, mostly because it fits the description of an ideal naturally existing constellation of GFs. However, its efficacy remains controversial. Hence, this study is designed to further elucidate the physiological role of PRP in treating chronic diabetic foot ulcers.

Platelet -rich plasma (PRP) and Platelet -poor plasma (PPP) were prepared from blood samples taken from healthy donors and diabetic patients through the use of platelet collecting and concentrating system. The GFs released were measured through immunoassay technique. The effects of the varying concentrations of PRP/PPP in culture media was assessed through tissue culture assay (proliferation, cell migration and angiogenesis assay) on human epithelia keratinocyte, dermal fibroblast and umbilical vein endothelia cell. Furthermore, immuno-histochemistry technique was used to evaluate the differentiation, proliferation, migration and extracellular matrix alterations occurring along wound margins of patients with chronic diabetic ulcers following PRP treatment.

A significant difference was observed when the expression of platelet derived growth factor-AA, epidermal growth factor, vascular endothelia growth factor, transforming growth factor and thrombospodin-I released from PRP/PPP were compared between the two groups. There was a significant proliferative, migratory and angiogenic effect of PRP over PPP in the tissue culture assay; however this effect was most prominent with 5% PRP. Overall, hyperproliferative keratin, CD44 and β1-integrin were upregulated in diabetic ulcer keratinocytes as compared with normal foot skin. The clinical study showed that 3 of the 7 diabetic foot ulcer patients treated with PRP achieved complete wound re-epithelisation. We have been able to demonstrate through *in vitro* studies that PRP has a positive biological effect which mimics normal physiological tissue reparation process.

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State of Copyright

Declaration

The work contained in this thesis is the independent work of the author with advice and guidance from his supervisors; Mr. Constantinos Kyriakides and Prof Harshad Navsaria.

ABSTRACTS AND PUBLICATION

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ABBREVATIONS

List of Abbreviations

α	Alpha
AB	Alamar Blue
aa	Amnio Acid
AEC	3-amino-9 ethylcarbazol
APC	Autologous derived platelet concentrate
ABC	Avidin: Biotinylated enzyme Complex
β	Beta
BMZ	Basal membrane zone
BAS	Bovine Albumin serum
C	Centrigrade
Cm^2	Centimetre squared
CO_2	Carbon dioxide
CRUK	Cancer Research U.K
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl-sulphoxide
DEJ	Dermo-epidermal junction
DAB	3, 3'- diaminobendizine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl-sulphoxide
dH_2O	Distilled water
EBM	Endothelial Cell Basal Medium-2
ELISA	Enzyme linked immuno-absorbent assay
EGF	Epidermal growth factor
ECM	Extra-cellular matrix
EHS	Engelbreth-Holm Swarm
EHS	Engelbreth-Holm Swarm
EDTA	Ethylediaminetetraacetic acid
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FAD	Flavin adenine dinucleotide

FITC	Fluroescein isothiocyanate
FADH2	Reduced Flavin adenine dinucleotide
FMNH2	Reduced Flavin mononucleotide
FMN	Flavin mononucleotide
g	Gram
GF	Growth Factor
GM-CSF	Granulocyte /macrophage colony-stimulating factor
HEK	Human epithelia keratinocyte
HDF	Human dermal fibroblast
HUVEC	Human umbilical vein endothelial cell
IF	Intermediate filament
IGF-1	Insulin-like growth-1
IMS	Industrial methylated spirit
IL-1	Interleukin
KSC	Keratinocyte stem cell
KGF	Keratinocyte growth factor
kDa	Kilo Dalton
L	Litre
MMP-9	Matrix metalloproteinase
MTT	(3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
µL	Microlitre
MFI	Multiplex fluorescent immunoassay technique.
ng	Nanogram
NADH	Nicotinamaide adenine dinucleotide
NADHP	Nicotinamadie adenine dinucleotide phosphate
NO	Nitrous oxide
HUGO	Nomenclature of the Human Genome Organization
ORS	Other root sheath
PDGF	Platelet derived growth factor
PF-4	Platelet Factor-4
PRP	Platelet rich plasma gel
PPP	Platelet poor plasma gel
PSS	Platelet separation system
PBS	Phosphate buffer solution
r-hFGF-B	Recombinant human fibroblast growth factor-B

rhEGF	Recombinant human epidermal growth factor
rhVEGF	Recombinant human vascular endothelial growth factor,
R3-IGF-1	Recombinant long releasing insulin-like growth factor
RADIL	Research animal diagnostic laboratory
NaOH2	Sodium Hydroxide
SDF-1	Stroma cell-derived factor -1
K-SF	Serum free keratinocyte
TSP-1	Thrombospodin-1
TA	Transit amplifying cells
TGF	Transforming growth factor
TNF	Tumour Necrosis Factor
VEGF	Vascular endothelia growth factor
V/V	Volume for volume
VWF	Von Williebrand factor

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CHAPTER 1

INTRODUCTION

1.1 Structure of the Human Skin

The integument comprises the skin together with its appendages (**Figure 1.1**). These include the hair, sebaceous and sweat glands, nails and hair follicles. It covers a surface area of more than 1.8m^2, making up in total 16% of the normal body weight. It has a range of functions that support survival which include acting as a physical barrier to biological, chemical and ultra violet radiation. Other functions of the skin include homeostasis regulation for fluid, tactile function, thermoregulation, vitamin D synthesis, immune surveillance, excretory function, sociosexual communication and reproduction via production of pheromones. Embryologically, the skin is derived from the ectoderm and mesoderm respectively. The skin can be divided into two parts namely, the hairy skin and non-hairy skin. It comprises of 2 layers, the outer most layer is called the epidermis and the innermost part is called the dermis.

1.1.1 The Epidermis

The epidermis is said to be the stratified squamous epithelium made up of several cell types. These are melanocytes, langerhans cells, merkel cells and keratinocyte, of which keratinocytes make up to 80% of the cellular population. The epidermis has four distinct layers, each showing a pattern of keratinocyte proliferation, differentiation and maturation. The layers are strata basale (germinativum), the spinosum, the granulosum and corneum (Watt, 1989). A fifth layer exists in very thick skin called the lucidum, which is present between the granulosum and corneum layers. The cell layers represent the sequential differentiation process of keratinocytes as they migrate from the basal to the terminal differentiation. The process of terminal differentiation involves a series of biochemical and morphological changes, which results in the production of the cornified outer most layer. The average epidermal turnover takes about 14-30days (Latkowski and Freedberg, 1999). The stratum basale consists of a single layer of cuboidal basal cells attached to the basement membrane by hemisdesmosomes that contains intergrins. Each adjacent cell is attached by desmosomes containing cadhein. The cells in this layer are mitotically active and are involved in continuing replacement of keratinocytes by upward displacement. These mitotically active cells are believed to have originated from the tip of the dermal papillae and the deep rete ridges of the hairy skin (Jensen et al., 1999) and the bulge region of the outer root sheath (ORS) of the adult human hair follicles in hair skin (Cotsarelis et al., 1990; Lyle et al. 1998). Basal cell contains cytokeratins organised in bundles around the nucleus and insert into desmosomes peripherally.

The stratum spinosum is the cellular layer above the basal layer. It consists of several layers of irregular polyhedral shaped cells that display spiny projection. As the basal layer cells move upwards

and away from their blood supply, their cell content and shape changes, and the cells become irregular in shape and form the spinous layer. However, some cells in this layer are still mitotically active. Cells become progressively flattened as they move up towards succeeding layers, these cells contains lamella granules and more demosomes for cell to cell adhesions. Above this layer, cells move into the granular layer which is a 3-5 cellular layer. The cells are distant from the blood supply in the dermis, hence these cells begin to flatten and die. The cell cytoplasm contains lamellar granules, which consists of lipid and keratohyalin. Keratohyalin granules contain pro-filaggrin, which is a precursor of filaggrin that binds the keratin filaments together.

The stratum lucidum consists of several flattened anuclei cells and keratin rich cytoplasm. The stratum corneum ('horny layer') is the top layer of the epidermis. Cells here are flat and scale-like ('squamous') in shape. These cells are dead and contain lots of keratin and are arranged in overlapping layers that impact a tough and waterproof character to the skin's surface. Dead skin cells are continually shed from the skin's surface. This is balanced by the dividing cells in the basal cell layer to produce a state of constant renewal. Other proteins found as components of the cornified cell envelope include keratolinin, loricin, filaggrin-linked segment peptide and envoplakin.

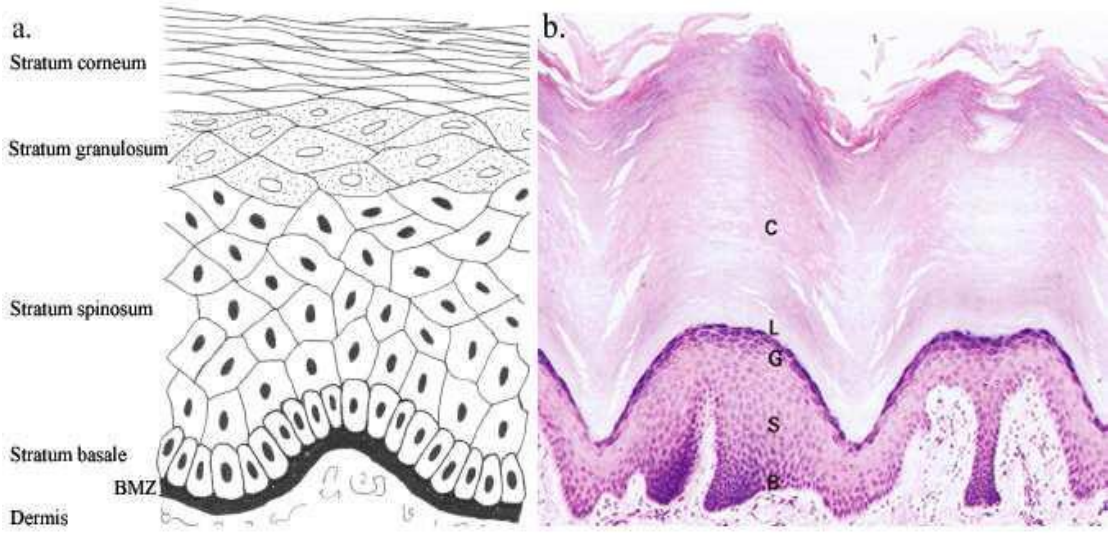


Figure 1.1 a) Schematic representation of epithelia structure and b) H&E (x20) of human epidermis *(*Young B, Wheater PR. Wheater's functional histology: a text and colour atlas. 5th ed. [Oxford]: Churchill Livingstone Elsevier 2006, *Wickett* RR. Structure and function of the epidermal barrier. American Journal of Infection Control.2006 December 2006; 34(10):S98-S110 *C-corneum*, only found in thick Skin, L-lucidum*, G-granulosum, S-spinosum, B-basale).*

1.1.1.1 Keratinocyte Stem Cell

Skin homeostasis and regeneration are maintained by the presence of keratinocyte stem cell (KSC) which gives rise to the differentiating cells of the interfollicular epidermis, hair follicles and sebaceous glands. The proliferative reserve of these cells is enormous; they can maintain 1-2m^2 of skin for decades (Alonso, 2003). The pioneering *in vitro* study by Barrandon and Green demonstrated that keratinocytes were capable of generating three distinct colony types based on their differing capacities for multiplication; these include holoclones, meroclones and paraclones (Barrandon, and Green, 1987).

Holoclones are generated by KSC which show the greatest proliferative capacity and can undergo more than 140 doublings before senescence (Pellegrini et.al 1999). Meroclones form intermediate colonies of limited growth potential and are committed to a maximum of 15 divisions, which represent the largest group of dividing cells. Transit amplifying (TA) cell are the progeny of the stem cells, with a limited proliferative capacity identified as a pool of rapidly proliferating cells that are lost from the basal layer to terminal differentiation within 4–5 days.

Finally, paraclones form the smallest colonies and are typical of mature, differentiated basal cells. The transition from holoclone to meroclone to paraclone is an irreversible, unidirectional process which occurs slowly during ageing as well as during repeated keratinocyte sub-cultivation (Pellegrini et.al 1999, Lajtha 1979). This transition process is often accelerated by inappropriate culture conditions causing a rapid decline in KSC number (Pellegrini et.al 1999).

KSCs and TA cells can be isolated from terminally differentiated basal cells on the basis of protein expression. Stem and transit amplifying cells have been shown to express high levels of Keratins 5 and 14 and the integrins α6, β1 (Jones, 1993; Li, 1998). Integrins are a family of heterodimeric transmembrane receptor proteins, some of which help attach basal cells to the underlying basement membrane. Integrin (α6) is found exclusively in hemi-desmosomes and partners with β4 integrin to attach to the basement membrane component laminin 5 (Alonso, 2003). Fractionation of cultured keratinocytes via fluorescent-activated cell sorting (FACS) has shown that cells expressing high levels of β1 integrin (Jones, 1993)and α6 integrin (Li, 1998) have the greatest proliferative capacity *in-vitro* and produce holoclones. In addition, β1 integrin, like α6 is also involved in determining the strength of attachment of basal cells to the basal membrane zone (BMZ). Stem cells express high levels of β1 integrin and therefore exert stronger binding to the basement membrane.

1.1.1.2 Epidermal differentiation

Epidermal differentiation begins in the stratum basale. Basal cells commence on a journey towards the skin surface by breaking away from the underlying basement membrane. During transit, the cell undergoes a series of morphological and biochemical changes which culminate in the production of dead, flattened, enucleated squames called cornified cells (Fuchs, 1990). The stratum basale represents the only mitotically active area of the epidermis and consists of a single layer of cuboidal or low-columnar keratinocytes separated from the dermis by the BMZ (Fuchs, 1990). These are then shed from the skin surface and replenished by the next generation (**Figure 1.2**). Regulation of the process is largely controlled by growth factors such as epidermal growth factor (EGF) (Rheinwald, 1975), transforming growth factor a (TGF-a) (Coffey, et.al 1987), keratinocyte growth factor (KGF) (Finch et.al 1989) and the cytokines IL-6 (Grossman, et.al 1989), IL-1a (Ansel, et .al 1988). The stratum basale represents the only mitotically active area of the epidermis and consists of a single layer of cuboidal or low-columnar keratinocytes separated from the dermis by the BMZ (Fuchs, 1990).

Basal cells are secured to the BMZ and each other through specialised connections termed hemi-desmosomes and desmosomes, respectively. Both exhibit similar structural characteristics containing

plaque structures which are specialised for the anchorage of keratin 15 intermediate filaments (Green, 1996). Desmosomes are calcium activated membrane junctions that fix cells into a three dimensional lattice (Franke, 1987). Conversely, hemi-desmosomes contains just one plaque connected to the basement membrane component laminin 5 by integrin α6β4 (Sonnenberg, et al.,1991). In order for cells to be liberated from the basal layer and commit to terminal differentiation, down-regulation of α6β4 must occur.

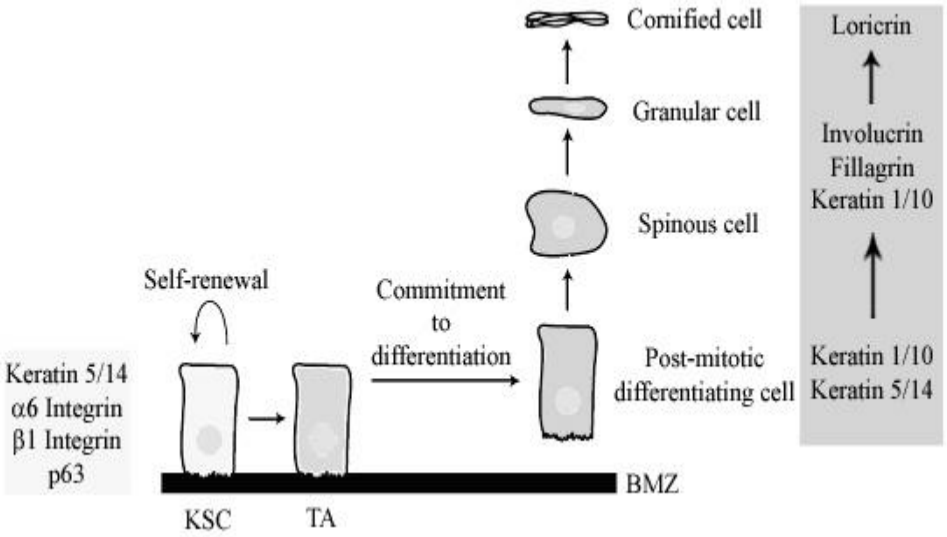


Figure **1.2 Schematic illustration of epidermal differential programme (**Adolphe C, Wainwright B. Pathways to improving skin regeneration. Expert Rev Mol Med. 2005 Sep 23; 7(20):

1.1.1.3 Structure of Keratin

The cytoskeleton of all epithelial cells including keratinocytes is formed from the three groups of filaments; actin (microfilaments), tubulin (microtubules) and intermediate filaments. The keratin is a typical intermediate filament protein of epithelia origin. The keratin gene family consists of the highest members of numbered human keratin with 54 distinct functional genes (Moll, 2008). Keratin belongs to a multi-gene family of proteins that form filaments of 10nm in diameter, with keratin forming the two largest groups. They are heteropolymeric filaments formed by pairing of type I (acidic) and type II (basic) molecule. Keratins can only constitute their filamentous stage by heteropolymeric pair formation of type I and type II (1:1). They contain central rod domain of approximately 310 amino acids with helical confirmation flanked by non –helical rod domain of variable length. The molecular weight of human keratins ranges from approximately 22 to 66 kDa. Keratins are among the subfamilies of intermediate filament (IF) proteins with high molecular

diversity. IF are usually very stable, long with un-branched filaments of approximately 10nm in diameter.

Keratin represents the typical IF category of epithelial cells. Inside the cell, keratin braids the nucleus, spans through the cytoplasm and is attached to the cytoplasmic plaques of the typical epithelial cell-cell junctions (the desmosomes). This feature already suggests that they are involved in structural integrity and mechanical stability of both the single epithelial cells and that of the epithelial tissue (Waschke, 2008). The keratins are clustered at 2 different chromosomal sites: chromosome 12q13.13 (type II) and chromosomes 17q21.2 (type I keratin except K18). The keratin genes are designated as KRT2, KRT3 etc. (Rogers et al., 2006). In the cell they braid the nucleus, span through the cytoplasmic plaques of the typical epithelial cell-cell junctions, the desmosomes. This feature already suggests that keratins play a major functional role in the integrity and mechanical stability of both the single epithelial cells and via cell-cell contacts, of the epithelial tissues. There are 28 types I of keratin genes (17 epithelial keratins and 21 hair keratins) and 26 type of II keratin genes (20 epithelial keratins and 6 hair keratins), **Table 1** shows the location and the types of Keratin.

1.1.1.4 Function of Keratin

Keratins are part of the epithelial cytoskeleton, they are important for the mechanical stability and integrity of the epithelial cell and tissues. Their expression is highly specific in relationship to the epithelial type and cellular differential stage. Thus keratin expression has been used as specific epithelial cell markers to assess epithelial proliferation and differentiation with the aid of immuno-histochemistry.

Other keratins are involved with intracellular signalling pathways, wounding, and apoptosis. Keratins also contribute to basement membrane attachment and the connective tissue compartment of a given epithelium. New functional roles of keratin are being defined and still emerging. These include the protection from apoptosis (Caudin et al., 2003), the regulation of protein synthesis and cell size during wound healing involving intracellular signalling pathways (Kim et. al., 2006). Beyond their biological functions, keratin expression patterns not only characterise cells as epithelial layer but also they are also characterises for distinct event– including the terminal stages during cellular epithelial differentiation from embryonal to adult cell or of internal maturation program during development. Epithelial tumours – including metastases most widely retain the keratin patterns of their original epithelia, thus the determination of the keratin patterns of tumours are being widely exploited for cell and tumour typing. Keratins have also been used in immuno-histochemistry for diagnosis of carcinomas. Therefore keratins have evolved to be one of the most potent epithelial differentiations

and tumour markers in cell biology, embryology and surgical pathology (Oshima 2007, Chu and weiss 2002b).

Type I (acidic)	Type II	Epithelia location
K10	K1	Suprabasal epidermal keratinocytes
K9	K1	Palmoplantar suprabasal keratinocytes
K10	K2e	Granular layer of the epidermis
K12	K3	Cornea
K13	K4	Non- keratinizing stratified squamous epithelial
K14	K5	Basal layer keratinocytes
K15	K5	Basal layer of non –keratinizing epithelial
K16	K6a	Outer root sheath (hair), hyper-proliferative keratinocyte, oral epithelium
K17	K6b	Nail bed, myoepithelium, inflammatory conditions
	K7	Various partners in transformed cells
K18	K8	Simple epithelial
K19		Bulge cells (hair follicle), simple epithelial
K21		Intestinal epithelium

Table 1.1 Cytokeratin protein with their primary location of expression. Freeberg I. Keratinocytes. Education 2009; Available on www. Aad.org/education/students/ keratinocytes

1.1.2 The Basement Membrane

The cutaneous basement membrane is also called the dermo-epidermal junction, (DEJ) which separates the epidermis from the dermis. DEJ is a complex structure, composed of a membrane of different macromolecules, which form an important network to provide structural stability between the epidermis and underlying dermis. The organization of basement membrane to form a morphologically identifiable structure results from a cooperative effort of both keratinocytes and

fibroblasts (Fleischmajer et al., 1993; Marinkovich et al., 1993; 1998; Moulin et al., 2000). Marinkovich et al. studied the cellular origin of various basement membrane molecules by probing skin equivalents that contains bovine keratinocytes and human dermal fibroblasts with species specific antibodies (Marinkovich et al., 1993).

The basement membrane consists of lamina densa which is associated with hemidesomes, lamina Lucida and anchoring filaments and anchoring fibrils (Uitto & Christiano, 1992). The hemidesmosomal proteins associated with keratin filaments in the intracellular space of the basal keratinocyte extend through to the extracellular lamina Lucida (Pulkkinen and Uttio 1999). There are two inner and an outer hemidesmosomal proteins that are the transmembrane proteins which include the bullous pemphigoid antigen and the intergrin which is the receptors for laminin 5. These proteins function to anchor the basal keratinocytes to the underlying basement membrane. The lamina Lucida is traversed by anchoring filaments which are composed of laminin 5, which is made from three polypeptide chains, α3, β3 and γ2 chains. Furthermore, the lower portion of the network involves anchoring fibrils which are composed of collagen type VII. These anchoring fibrils extend from the lamina densa to the papillary dermis. Thus, securing the basement membrane to the underlying dermis.

1.1.2.1 Integrins

Integrins are a family of heterodimetric (transmembrane receptors) cell surface receptors composed of α and β subunits, which mediate cell-cell and extracellular matrix function. Eighteen different α and 8 different β subunits are known in mammals, which can combine to 24 different integrin receptors (Hynes, 2002). The largest subgroup is formed by the β1 subunit containing integrins which consist of 12 members with different ligand binding properties. All β1 integrins bind to extracellularmatrix molecules and some β1 integrin containing receptors also interact with cellular receptors.

Integrins plays a significant role in cell adhesion to the surrounding extracellular matrix and to other cells for maintenance of tissue integrity. In addition to this structural function, cell adhesion induces intracellular signalling mechanisms that regulate proliferation, apoptosis, cell polarity, differentiation and other processes. Analysis of β1 integrin function *in vivo* demonstrated that β1 integrin are important for attachment to and integrity of the basement membrane by mediating binding to basement membrane components. Various intracellular signalling pathways are elicited following the banding of ligands to integrin at the extracellular side. Finally, β1 integrin could be important for the migration of skin stem cells from the bulge region of the hair follicles to the interfollicular

epidermis and to the hair matrix region (Brakebusch et.al, 2000). Severe defects in adhesion and migration of β1-deficient keratinocytes were observed *in vitro* as well as during wound healing *in vivo* (Grose et.al, 2002).

1.1.2.2. Anchoring fibrils

Anchoring fibrils are specialised structures in the skin, measuring 20-60nm in width, and 200 - 800nm in tlength. They are flexible, banded, fibrillary structures arising in the lamina densa and extending into the dermis. They are made up of aggregates of collagen type VII anti-parallel dimers as early studies demonstrates the correlation between the length and banding pattern of anchoring fibrils. The amino terminals domains attach to the lamina densa as well as condensing with the ends of the other anchoring fibrils and basement components to form anchoring plaques in the dermis (Burgeson, 1993).

1.1.2.3 Collagen type VII

Collagen type VII is the main component of anchoring fibrils present in the basement membrane of stratifying squamous epithelia of the skin, mucous membrane and cornea (Burgeson 1993). It is synthesized by keratinocytes and fibroblasts, although epidermal keratinocytes are thought to be the major source of collagen type VII *in vivo* (Rynanen et. al, 1992). Each molecule of Collagen VII consist of a triple helix of three identical subunit of alpha chain and each alpha chain is produced as a precursor polypeptide, procollagen type VII (α1) (Uitto and Christiano, 1992). Collagen VII gene is known as COL7A1 and resides on Chromosomes 3p21 (Uitto and Richard), with a molecular weight of 300kDa. Each molecule consists of a central collagenous domain flanked by non–collagenous domains on either side (Lunstrum et .al 1986).

Collagen VII is the major structural component of the anchoring fibrils that stabilize the basement membrane on the dermis in chronic human wounds and scars. The instability of the basement membrane zone observed in epidermolysis bullosa dystrophica, a blistering skin disorder that is often associated with mutations in the collagen VII gene (Uitto, 1992), could be a pointer to the structural role of collagen VII. The deposition of collagen VII in healing human surgical wounds has been demonstrated from day 7 following wounding (McGrath, 1992) and over a similar time course in healing human acute oral wounds (Larjava, 1993). Chronic wounds such as lower-limb venous ulcers are usually characterized by basement membrane destruction and it is likely that collagen VII synthesis is required for the restoration of a functional basement membrane zone. The presence of fibronectin type III, cartilage matrix protein and von Willebrand factor domains in the collagen VII

molecule indicates that collagen VII may have a role in modulating cell behaviour and matrix–matrix interactions at the wound site (Hopkinson, 1997).

It has been observed that cutaneous wounds in which the basement membrane remains intact, hast an uninterrupted wound healing process. In addition, the intact basement membrane provides part of the cellular signals required for direct re-epithelialization. On the other hand, ulcers from which the basement membrane is histologically absent are usually slow to heal and restoration of the basement membrane is prerequisite to epithelialization. Synthesis and incorporation of Collagen VII into the anchoring fibrils is probably a prerequisite for the synthesis of a stable basement membrane in these ulcers and is required in order that healing can proceed (Hopkinson, 1997).

1.1.3 The Dermis

The dermis is a dense, irregular fibroelastic connective tissue composed of extracellular matrix (ECM) and thicker than the epidermal layer. The dermis can be subdivided into the papillary and reticular dermis. The papillary dermis and reticular dermis differ in both the composition and organization of their respective extracellular matrices. The papillary dermis is characterized by thin, poorly organized collagen fibre bundles, consisting primarily of type I and type III collagens, which contrast with the thick, well-organized fibre bundles in the reticular dermis (Cormack, 1987). The papillary dermis interdigital with the epidermal ridges along the DEJ and it contains more loosely arranged collagen and more cells. The reticular dermis contains more tightly packed coarse collagen fibres. There is a sub-papillary plexus of blood supply at the arbitrary interphase. Collagen fibre bundles in the papillary dermis contain more type III collagen than do those in the reticular dermis (Meigel et al., 1977).

Other matrix molecules are also differentially apportioned between the papillary and reticular dermis. These regions differ in cell density as well as in the composition and organisation of ECM (Sorell and Caplan, 2004). Other ECM that makes the dermis includes the dermatan sulphate, chondroitin-6-sulphate, chrondoitin -4-sulphate, heparin sulphate, amorphous ground substance that is made of up of glycosaminoglycans (GAG). Other skin appendages such as nerve, complex vascular and lymphatic networks and hair follicles pass through the dermis and often extend into the underlying subcutaneous layer. The cells that are resident in the dermis are the fibroblast, mast cells and the macrophages (Haake AR and Holbrook K,1999).

1.1.3.1 The Extracellular Matrix

The ECM is a form of connective tissue, composed of a mixture of proteins and polysaccharides in which cells are embedded. The main components of the ECM are the fibrous connective tissues, elastin and the collagen. The fibroblast found the ECM secretes the matrix macromolecules. The collagen component are mainly type I,III and V. The type I is the most abundant, it constitute about 80-90% of the total body collagen although the ration of type I: III collagens is higher in the reticular dermis compared with the papillary dermis. The papillary dermis also has smaller bundles of collagen fibres compared with the reticular dermis (Sorrell JM and Caplan AI, 2004). The elastin fibres lie between the collagen bundles and are also present in blood vessels. They are important for skin pliability and account for about 5% of the dermal matrix. The elastins fibres are structurally made up of a microfibrillary component embedded in an elastic matrix. The ECM's main function is to provide structural support and tensile strength for the skin. Its also provides substrates for cell adhesion and cell migration. Furthermore, it regulates cell behaviour, migration, proliferation and maintains their shape. There are 2 main types of extracellular macromolecules.

- **The structural proteins and the adhesive proteins**: Collagens are part of the structural proteins that maintains the tensile strength; whereas elastins which are also structural proteins are know to give resilience to the matrix. The adhesive proteins (laminin and fibronectin) link components of the ECM which serves as the linkage to the cell surface Other cell surface receptor proteins are the glycoproteins and integrins

- **The proteoglycans**: They are the main polymorphic macromolecules consisting predominantly of molecules with core proteins and glycosaminoglycans (GAG) side chain extensions consisting of repeated disaccharide units. They are divided into the intracellular, membrane–bound and extra-cellular group. They function to regulate several cellular processes, such as morphogenesis, tissue repair, cellular proliferation and differentiation.

1.1.3.2 Collagen I

The extracellular spaces in tissues are filled with organized extracellular matrix (ECM), which is composed of proteoglycans like decorin and fibromodulin; Fibrous proteins like collagen, elastin, and fibrillin; adhesion molecules like fibronectin and laminin; and different types of matrix metalloproteinase's, and play important roles in cell signaling and cellular activities (Raghow,1994). Collagens are the major fibrous proteins in ECM. Different combinations of different α-chains make triple-stranded helical structure of different collagens. To date 19 to 20 types of collagens have been identified. The molecular nature and functions of several collagens have been characterized (Myllyharju, 2001). Type I collagen, the major component of ECM in skin, bone, ligaments, etc., is

composed of glycine- and proline rich two α -1 (I) and one α -2 (I) chains, α1 (I) and α 2 (I) are products of two genes.

The pro-COL1A1 and COL1A2 polypeptide chains are synthesized by fibroblasts, osteoblasts, or odontoblasts and enter into endoplasmic reticulum where specific proline and lysine residues are hydroxylated to form hydroxyproline and hydroxylysine, respectively, which help the pro-α chain to combine with other chains by hydrogen bonds and form the triple helix pro-collagen structure. Pro-collagens are secreted by the fibroblasts through the Golgi apparatus in the extracellular space where the N-terminal and C-terminal pro-peptides are cleaved by specific proteases. The mature processed collagen molecules aggregate to form larger collagen fibrils and help to form the ECM with other components. Therefore, normal structural and functional Type I collagen production and deposition in ECM to make normal physiological connective tissue needs regulation at several steps.

1.1.3.3. Collagen III

Collagen Type III is initially produced as pro-collagen, a protein which consist of three pro-alpha 1(III) chains that form the triple-stranded, rope –like molecule. The main function of Collagen III is to add structure and strength to connective tissues. Collagen III is found in many places in the body, especially in the skin, lungs, intestinal walls and blood vessel walls. COL3A1 is the gene responsible for producing the pro-alpha1 (III) chain of type III procollagen. After being synthesized, the procollagen molecule is modified by the cell. Enzymes modify the amino acids lysins and proline in the protein strand by adding chemical groups that are necessary for the strands to form a stable molecule and then later to crosslink other molecules outside the cell. Other enzymes add sugars to the protein. The type III pro-collagen molecules are released from the cell and are processed by enzymes that clip small segments off either end of the molecules to form mature collagen. The mature collagen molecules assemble into fibrils. Cross-linking between molecules produces a very stable fibril, contributing to collagenous tissue strength function.

1.1.4. Fibroblast

The fibroblast is the predominant cell type in the dermis and they are heterogenous cell population of mesenchymal origin. Fibroblast exists as a highly diverse population such as the papillary and reticular dermis fibroblast. Dermal fibroblasts represent a heterogeneous population of cells defined according to their location within the dermis. Two subpopulations of fibroblasts reside in distinct dermal layers: the papillary and reticular dermis (Cormack, 1987).

Fibroblasts cultured from each of these layers have different characteristics (Harper and Grove, 1979; Azzerone and Macieira-Coelho, 1982; Sorrell et al., 2004). A third group is associated with

hair follicles. These cells synthesise and degrade connective tissue matrix proteins, and a number of proteins involved with structural support and basement membrane framework. They also synthesise growth factors that promote interactions between the epidermis and the dermis (Smola et al 1993: Maas-szabowski and Fusenig 1996), which are important in the regulation of normal skin physiology as well as in wound healing. Some of the growth factors involved are Keratinocyte growth factor (KGF 1, 2), transforming growth factor-beta-1 (TGF-B1), vascular endothelia growth factor (VEGF) and granulocyte / macrophage colony-stimulating factor b (GM-CSF). They are spindle-shaped cells with a prominent rough endoplasmic reticulum and have an elliptical nucleus.

1.1.4.1 Fibroblast heterogeneity and Subtypes

Primitive mesenchyme cells are multipotential stem cells that differentiate and give rise to fibroblasts as well as other connective tissue cells such as mast cells, adipocytes, chrondrocytes and osteoblasts. Fibroblast from different anatomical sites has all been shown to have similar morphology and express the mesenchymal intermediate filament marker, vimentin (Chang et al., 2002). However studies using microarray technology have demonstrated that fibroblast from different anatomical sites have their unique gene expression profile and characteristic phenotype, synthesizing extracellular matrix proteins and cytokines in a site specific manner (Chang et al., 2002). Human skin fibroblast cultured in modified conditions differentiate along seven terminal cell lineage pathway; the mitotic activity (I- III) to the post mitotic stage (IV- VIII) (Bayreuther et al., 1988b). Furthermore, as the cumulative population time increases, the proportion of mitotic and post mitotic fibroblast cell type changes and it has been suggested that theses proportions also change similarly in vivo with increasing age (Bayreuther et.al, 1988b).

Cutaneous fibroblasts can be subdivided according to their location; papillary or reticular dermis or associated with hair follicles. There are no distinction in the *in vivo* characteristics but have different expression in the *in vitro* environment. Through mechanical separation of skin (dermatome) into defined papillary and reticular layers allows for establishment of explant cultures of cells from each layer. Papillary fibroblasts divide at faster rates, have a longer replicative lifespan, are smaller and exhibit less of contact inhibition than do site-matched reticular fibroblasts (Harper and Grove, 1979; Azzerone and Macieira-Coelho, 1982; Schafer et al., 1985; Sorrell et al., 2004).

Reticular dermal fibroblasts seeded into type I collagen lattices faster than do papillary dermal fibroblasts (Schafer et al., 1985; Sorrell et al., 2004). When grown to confluence in monolayer culture, the papillary cells attain a higher cell density partly because they are not fully contact

inhibited (Schafer et al., 1985; Sorrell et al., 2004). Co-culturing papillary dermal fibroblast with keratinocytes produce a more differentiated and organised epidermis with complete formation of dermoepidermal junction. More of GM-CSF is produced by papillary fibroblast and relatively less of KGF as compared with reticular fibroblast. Production of type I and III collagen are similar for both fibroblasts *in vitro* whereas type III is said to be more in papillary *in vivo* (Tajima and Pinnell 1981).

Myofibroblast are a specialised form of fibroblast found in granulation tissue that plays a critical role in wound contraction. They are distinct from other fibroblast ultrastructurally, biochemically and functionally (Moulin et al. 2000). They are characterised by the presence of intracellular bundles of alpha smooth muscle actin, which is the actin isoform expressed by smooth muscle cells (Grinnell, 1994)

1.1.4.2 Fibroblast in Wound Healing

The process of wound healing involves inflammation, granulation tissue lay down and tissue remodelling. Fibroblast contributes significantly to these stages. Activation of fibroblast occurred following cutaneous injury, whereas it remains in the quiescent state in the unwounded skin (Clark 1996). Fibroblasts play a crucial role in cutaneous wound repair (Martin, 1997). These cells are attracted to wound sites by the localized release of growth factors/cytokines such as platelet derived growth factor and TGF- β (Pierce et al., 1991; Konig and Bruckner- Tuderman, 1992). The first wave of fibroblasts enters the wound site along with sprouting vasculature. These cells differentiate into a specialized, but non-lasting, cell type called the myofibroblast (Sappino et al., 1990; Grinnell, 1994). In addition, they lay down extracellular matrix and contributes to basement membrane formation, partly by producing collagens types IV and VII , laminin 5 and nidogen, and through the secretion of cytokines that have a stimulatory effect on keratinocyte that in turn produce basement membrane components. (Marinkovich et. al., 1993)

Myofibroblasts in response to monocyte/macrophage derived factors, produce a provisional wound matrix that is enriched in fetal-like fibronectin and hyaluronan (Clark, 1990; Gailit and Clark, 1994; Juhlin, 1997; Singer and Clark, 1999). These cells also provide the motive force to contract the wound (Sappino et al., 1990). The wound is predominately populated with myofibroblast and display a different morphology compared with normal fibroblasts. Myofibroblast has the alpha smooth muscle actin filaments, deep folders in the nuclear membrane and numerous cellular connections all of which enhance wound contraction. Myofibroblast disappear from the wound site, apparently by apoptosis, and are replaced by a second wave of fibroblasts that initiate the formation of a collagenous matrix (Grinnell et al., 1999). Fibroblasts/myofibroblasts are also intricately involved in

transforming granulation tissue into a mature scar, through continual collagen remodelling. However, their ability to seamlessly organise this process is often impaired, hence scar tissue formation occur (Gailit and Clark, 1994; Singer and Clark, 1999). On the other hand, fetal skin is repaired without scar formation (Liechty et al., 2000). This is mainly due to differences in fetal and adult fibroblast phenotypes (Olsen and Uitto, 1989; Gosiewska et al., 2001). The low level of growth factors/cytokine production by fetal cells, especially TGF-b1, appears to be a major factor in the absence of scar formation (Shah et al., 1995; Eckes et al., 2000).

1.1.4.3 Fibroblast Ligands

Cell-cell, cell-matrix and matrix-matrix linkages are all important for regulating fibroblast activity and bringing about appropriate responses to changes in the environment. Fibroblast expresses receptors for binding of growth factors and cytokines, which leads to intracellular signal transduction, gene expression and a variety of cell responses. These responses include activation, proliferation, migration, differentiation, as well as ECM production. Integrin receptors facilitate the binding of fibroblast to the surrounding ECM. The Alpha-1 beta 1($\alpha 1\beta 1$) and alpha-2 beta 1($\alpha 21\beta$) integrin receptors are the main receptors in the processing of facilitating the binding of collagens I, IV, V and VII (Chen et al, 1999, Ruggiero et al 1996). The binding of collagen I to $\alpha 1\beta 1$ receptors on fibroblast results in a negative feedback that down regulates collagen synthesis. Fibroblasts are capable of forming cell-cell connections with other fibroblasts and collectively, these cellular mini-networks are thought to be important in transmitting the contractile force across wounds, which ultimately enhance wound closure (Welch et al.1990.)

1.1.4.4. Fibroblast Proliferation and Migration during Healing

Fibroblast proliferation is influenced by growth factors, hypoxia, cytokines and physical factors such as radiation and laser therapy. After tissue injury, the hypoxic environment and the presence of cytokines such as platelet derived growth factor (PDGF), insulin like growth-1 (IGF-1) and transforming growth factor –beta (TGF- β) directly stimulates fibroblast proliferation (Clark,. 1996). Movement of fibroblast occurs in 2 days, either by haptotaxis or by contact guidance (Clark,. 1996). Haptotaxis refers to the movement of cells along an adhesion gradient whilst contact guidance represents the movement of cells guided by substances, such as fibronectin. Following skin injury, the provisional matrix is rich in fibrin, fibronectin and hyaluronic acid and consists of relatively little collagen. For cell migration to occur degradation of ECM is needed and the release of ECM degrading proteases is produced by a number of cell types including keratinocytes, fibroblast and macrophages. The activities of these proteases and their expressions (matrix metalloproteinase

MMP, serine and cystein proteinases) are controlled by growth factors and cytokines (pro-inflammatory IL-1 and Tumor necrosis factor- alpha), following cell contact with ECM (Clark, 1996).

In the presence of PDGF and a provisional matrix environment, α5β1 integrin receptor is up-regulated on their surface which enables them to adhere to fibronectin and migrate into the wound (Xu and Clark, 1996). Once the wound site is filled with fibroblast, the provisional matrix is replaced with granulation tissue, this environment leads to increased expression of collagen receptors α5β1 and reduced α1β1 (Xu and Clark, 1996). Binding of fibroblast to collagen is enhanced under this condition. Hypoxic wound environment has also been shown to stimulate fibroblast migration on collagen gel (Li W et. al., 2005)

Subcutaneous Fat

The innermost layer of the skin is the layer of subcutaneous fat, and its thickness varies in different regions of the body. The fat stored in this layer represents an energy source for the body and helps to insulate the body against changes in the outside temperature.

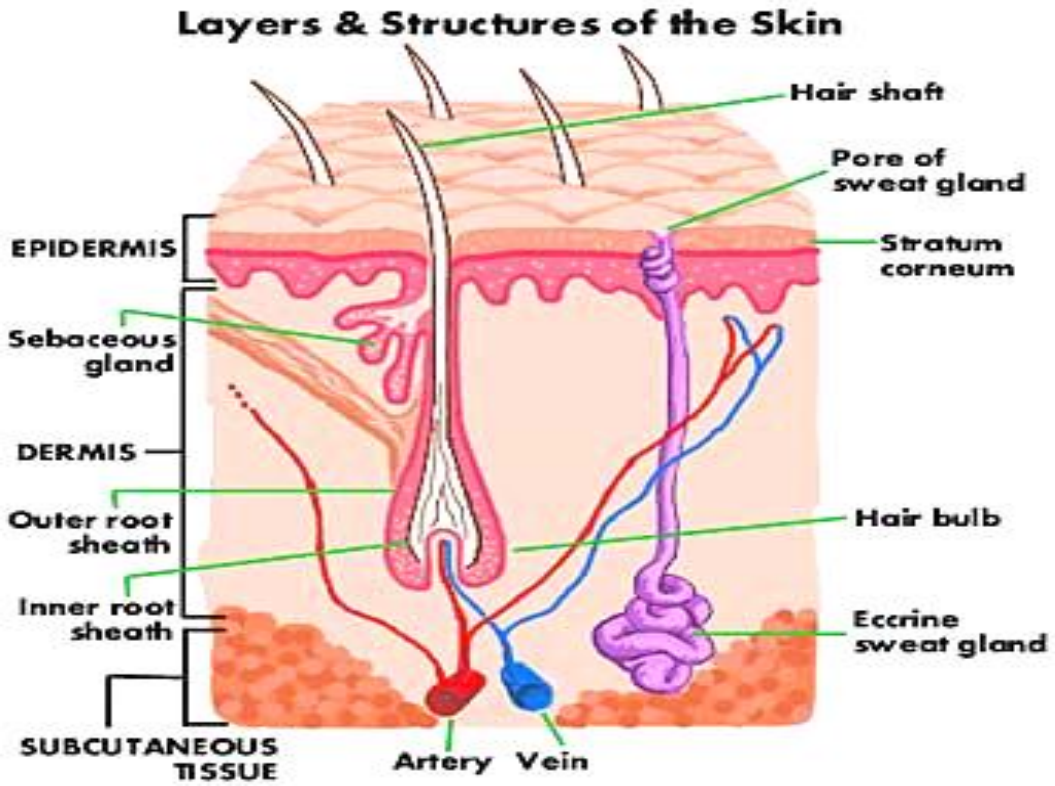


Figure 1.3. Schematic diagram representing cross section of the Human skin

1.1.6 The Principle of Wound Healing

1.1.6.1 Cellular and Molecular events Occurring during Acute Wound Healing

The process of post inflammatory wound healing and tissue regeneration is characterized by a complex, multicomponent cascade of degradative and biosynthetic reactions that orchestrate underlying cell-cell and cell-ECM interactions (Howell, 1992). Theoretically, tissue remodelling can be divided into three somewhat overlapping phases, each stage exhibiting its own unique cellular and molecular interactions (**Figure1.4**). Tissue injury and wounding is promptly followed by the inflammatory phase, which eventually merges into the second phase characterized by granulation tissue formation; the third and final phase of wound healing is dominated by massive biosynthesis and restructuring of the ECM (tissue remodelling and repair).

1.1.6.2 Inflammatory Phase

Tissue injury from vascular damage triggers the inflammatory phase initiated by haemorrhaging; various cellular components of blood come in direct contact with other cells and constituents of ECM, with the net outcome of activation of the clotting cascade leading to hemostasis (**Howell, 1992**). Clotting releases thrombin which in turn activates platelet, followed by the release of a host of potent chemotactic and growth-promoting cytokines through degranulation (e.g., PDGF, TGF I3, Tumour necrosis factor(TNF), and Interleukin-1(IL-1) at the site of inflammation. The stimuli that initiate clotting diminish with time; this process is further facilitated by rapid proteolytic degradation of the coagulation factors V and VII by protein C. Concomitant release of plasminogen activator along with plasminogen helps dissolve the thrombus and prostaglandins, which inhibit platelet activation. Fibrinogen and collagen activate platelets and also interact with thrombospondin, which binds to platelet-specific tyrosine kinase-containing receptor. This concerted action on platelet surface results in strong activation and adhesion of platelets, culminating in degranulation and release of their contents (Terkeltaub, 1988).

Liberation and activation of potent cytokines are crucial to the overall process of tissue regeneration and remodelling. In addition, intact TGF/3 and its proteolytic sub-fragments are potent chemoattractants which attracts massive influx of inflammatory phagocytes (e.g., polymorphonuclear cells and macrophages) toward the site of inflammation (Martin, 1992, Helmer, 1990). Combined action of different cytokines within the dynamic milieu of ECM thus determines the directional migration (Helmer, 1990) and function of the phagocytes (Berton, 1991). Furthermore, the adhesive interactions among various cells and the surrounding ECM profoundly modify the phenotypic features of mesenchymal and nonmesenchymal cells attracted to the site of tissue injury (Howell,

1992; Mast 1992). Differential proliferation and differentiation of various cells (e.g., fibroblasts vs. keratinocytes) by autocrine and pracrine processes are hallmark features of the inflammatory phase of wound.

1.1.6.3 Formation of the Granulation Tissue

The components of the provisional ECM, laid down during the clotting cascade, are modified and rearranged to form the granulation tissue (Howell, 1992). Macrophages, in addition to keeping wound aseptic by active phagocytosis and debridement, release several potent cytokines and hydrolytic enzymes. Granulation tissue is made up of many mesenchymal and nonmesenchymal cells with distinct phenotypes, and an extensive neovasculature embedded within the loosely assembled matrix composed of collagens, fibronectin, and proteoglycans.

Thus, reorganization of the granulation tissue is carried out by the components of the provisional ECM and the actions of the newly released cytokines. In contrast, the process of disassembly is accompanished through the action of collagenases, proteoglycanases, and other proteases, whereas the restructuring and renewal of ECM by the activated myofibroblasts. Phenotypic modification of cells present in the granulation tissue as exemplified by the formation of blood vessels and enhanced ability of fibroblasts to contract collagenous matrix, contribute to the quality of the healing process and the strength of the healed wounds. All of these processes are dependent on cell-ECM interactions in the peculiar milieu of the granulation tissue.

1.1.6.4 Biosynthesis and Remodelling of Extracellular Matrix

The last and easily identifiable phase of wound healing involves a systematic dissolution of the granulation tissue. This phase of tissue regeneration is typically accompanied by a gradual loss of cells and vasculature and exhaustive restructuring of the ECM (Howell, 1992). During this phase of the repair process, macrophages continue to phagocytose and carry out extensive debridement, a necessity for the inward migration of the vasculature and mesenchymal cells deeper into the wound. The granulation tissue is gradually replaced with more organized and elastic ECM. Many observers suggest that appropriate healing and tissue remodelling, as well as fibrosis, are two sides of the same equation, and a delicate balance in the biosynthetic and degradative pathways of ECM restructuring may swing the pendulum toward one or the other.

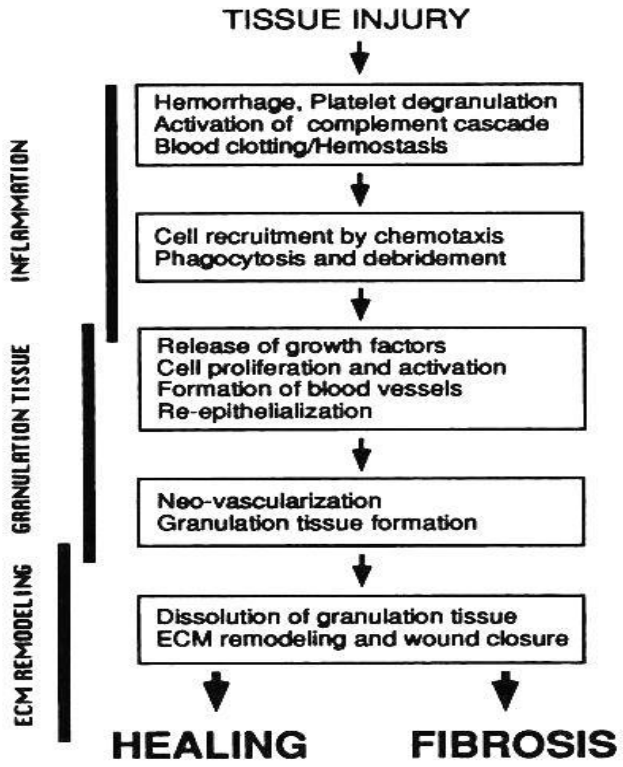


Figure 1.4 Shows succession of the major cellular and biochemical reactions encountered during post-inflammatory tissue regeneration. Cell-ECM interaction, in conjunction with a variety of cytokines/growth factors actively modulate these reactions at all stages of tissue regeneration and determines outcome of wound repair. The complexity and feedback regulation involved in the entire process is not depicted in this schematic diagram. Diagram from Raghow 1994, FASEB J; 8(11):823-31.

1.1.6.5 Extracellular Matrix in Post-inflammatory Wound Healing

The process of wound healing after an acute injury regardless of whether the cause of injury is physical, cytotoxic or immunological sum up the major event that occur during embryogenesis(Tranquillo, 1992). The various stages of wound healing are characterized by massive cell migration, proliferation, phenotypic differentiation and exaggerated biosynthetic activity at the site of repair (remodeling). Extracellular matrix (ECM) has been shown to play a central role in orchestrating the key steps in the programmed wound healing and repair (McGown, 1992). ECM has the ability to generate extraordinary large arrays of structure through dynamic interactions with protein and non protein component. ECM modulates cellular behaviour and phenotypes, able to bind specific cytokines and growth factors, thus sequestering potent biological response modifiers in the wound microenvironment (**Figure 1.5**). In addition, ECM organizes the geometric framework that facilitates cell migration and modulates cell-cell interactions. Furthermore, the releases of cytokines during this process are responsible for continuous dynamic cell-ECM interaction. This process is subject to a feedback regulation which is mediated by transcriptional, post transcriptional, translational and post translational mechanism elaborated during wound repair (Raghow, 1994).

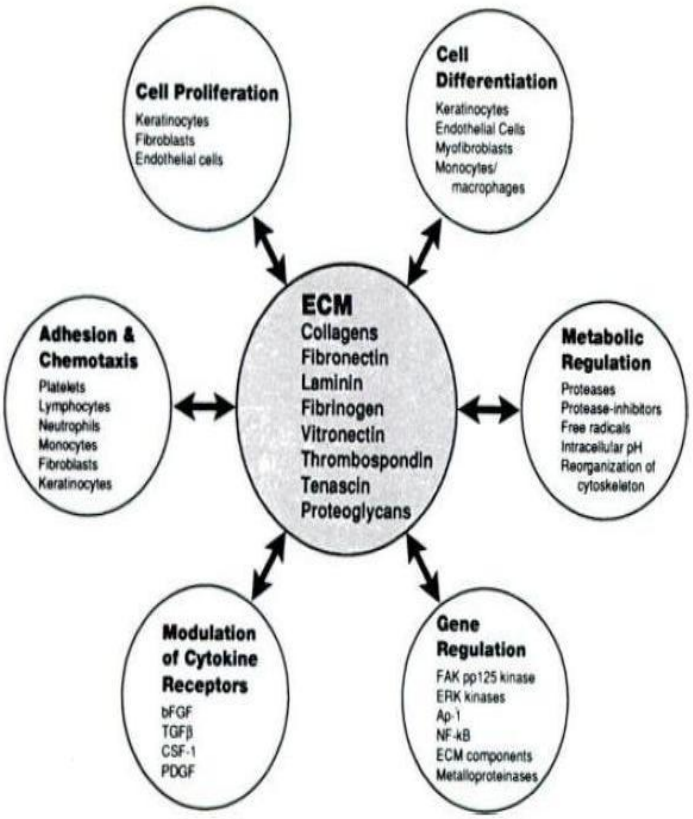


Figure1.5. Cell-ECM interactions modulates many key mechanism involved in post-inflammatory wound healing and fibrosis. The two way arrows connecting ECM to various cellular responses reflect the dynamic reciprocity between cells and ECM. Diagram from Raghow 1994, FASEB J; 8(11):823-31.

1.1.6.6. Cell Cycling in Wound Healing

Most cell-cycling studies in wound repair to date have focused on keratinocytes in models of reepithelization. Other studies have described a defect in fibroblast cell-cycling activity in chronic pressure ulcers (Vande Berg, 1998, Vande Berg, 2001). It was suggested that an abnormality in the proportion of wound repair cells restricted to cell cycle quiescence, senescence, or even apoptotic pathways might explain delays in wound healing **(Figure 1.6)**. Fibroblasts confined to G1 arrest were measured by the expression of the cell cyclin inhibitor p21, and the proportion of fibroblasts capable of DNA synthesis was measured by the level of expression of the proliferating cell nuclear antigen (PCNA). The recruitment of fascia fibroblasts out of the quiescence into a functional cell cycle remains unknown. It is possible that defects or delays in the recruitment of fascia fibroblasts into the cell cycle might contribute to delays in fascia repair and ultimately fascia dehiscence and incisional hernia formation. The mechanism that controls the balance between uninjured fascia fibroblast quiescence, cell cycle arrest, or functional cell cycle progression is unknown.

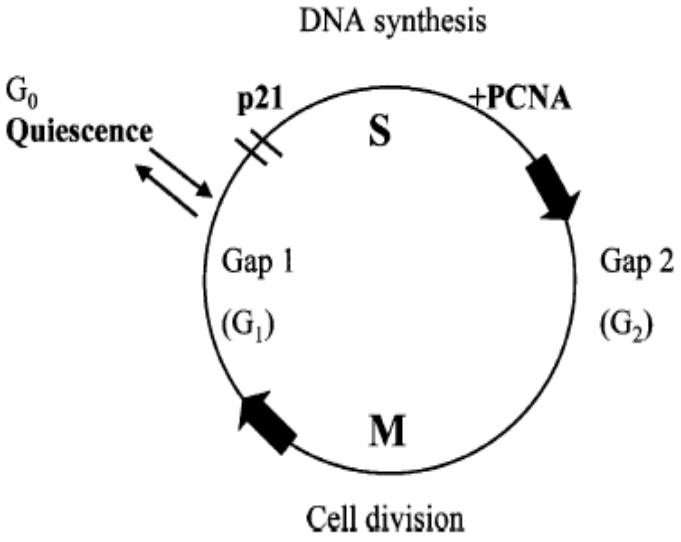


Figure 1.6. Abnormalities in the proportion of wound repair cells restricted to cell cycle quiescence, senescence, or even apoptotic pathways might explain delays in wound healing. Increased numbers of repair fibroblast confirmed to G1 arrest have been measured in non-healing ulcers and in a model of incisional hernia (thin arrows). Proliferating cell nuclear antigen (PCNA) is a classic maker of cells capable of DNA synthesis and cell cycle progression, whereas p21 is cell cycle inhibitor. Increased numbers of arrested fibroblast expressing p21 and reduced number expressing PCNA have been observed in non healing ulcers and in model of fascia wound failure leading to incisional hernia formation. M, mitosis, S, synthesis, Thick arrow showed normal progress of cell cycle. (Dubay DA, Franz GM, acute wound healing failure. Surg clinic Am (2003):463-481.

1.1.7 Aetio-Pathogenesis of Diabetic Foot Ulcers

The physiological alteration in diabetic foot ulcer could partly explain the chronicity and/or the recalcitrant nature of the ulcer. The triad of neuropathy, vasculopathy, and immunopathy forms the cardinal point on which the chronicity of diabetic wounds rest (Boulton et.al, 2005, Reiber, 1999). The neuropathic effect on the foot produces a biomechanical abnormality, which could predispose areas of concentrated pressure to significant risk of ulceration, repetitive trauma, vasodilatation, and decreased sweating (Shaw et.al, 1998). Atherosclerotic plaque deposit contributes to the development of diabetic peripheral vasculopathy resulting in either occlusion or stenosis of the vessels. The vasculopathy causes decreased blood flow and ultimately ischemia will ensue. Infection has more deleterious effect in this group of patients, whose impaired immunity increase their risk for local and systemic infections. The wound infections may either be superficial or deep and are commonly poly-microbial in aetiology. Superficial wound infection may be treated with antibiotic therapy only whilst the deep-seated wound infection might require surgical debridement which may be followed by antibiotic and or other wound care adjunct.

1.1.8 Physiological Changes in Diabetic Foot Ulcers

Aggressive sharp wound debridement is believed to convert chronic wounds to acute ones and allows growth factors to function more effectively. This allows the wound to progress through the normal phases of wound healing. Regular and frequent wound debridement removes collagenase, matrix metalloproteinase, and elastase which are inhibitors of wound healing (Steed, 1996, and Nwomeh, 1999). The cellular and molecular alteration has a direct link with the major physiological changes mentioned earlier and there is evidence to support hyperglycaemia—related deleterious molecular and cellular alteration in the cellular environment. Hyperglycaemic non-enzymatic glycosylation has an inhibitory effect on structural and enzymatic proteins. In addition, glycosylated collagen is resistant to enzymatic degradation and less soluble than the normal protein products, which makes connective tissue inelastic (He, 2004). High deposition of sorbitol has been implicated in diabetic vasculopathy. This is associated with increase in dermal vascular permeability and results in peri-capillary albumin deposition (Bucalo, 1993), which impairs oxygenation and nutrient distribution (Bennet, 1993). Bennett et.al. demonstrated increased destruction of growth factors by elevated levels of pro-inflammatory cytokines and metallo-matrix proteins following repeated trauma and infection (Bennet 1993, Yager, 1997).

Most chronic wounds are characterized by increased protease levels, particularly matrix metalloproteinase's (MMPs) and neutrophil elastases. Furthermore, tumour necrosis factor–alpha (TNF-alpha) has been shown to increase the production of MMPS, whilst inhibiting the production of tissue inhibitory metalloproteinase (Palolahti; 1993, Chen et.al 1997). The response mounted from the host immune system following critical wound colonization by bacteria is followed by polymorph nuclear leukocyte reaction that releases protease and oxidant species. This is followed by degradation of cytokines and extra-cellular matrix which distort the internal cellular milieu, thereby contributing to the non-healing of wounds. The cellular response to injury is poorly coordinated in diabetic wounds, most of which are complex and inter-related biochemical activities.

1.1.9 Molecular Mechanisms Involved in Chronic Wounds

The understanding of the molecular mechanisms involved in chronic wounds holds the key to solving the huge problem posed by this disease entity. Several efforts made at understanding this process have been undermined by the absence of an easily reproducible animal model that mimics the human chronic wound. Keratinocyte biology has attempted to explain the mechanism involved in chronic wound healing through cellular injury. Keratinocytes are the first cells to respond to injury; they are programmed to maintain skin integrity based on their specific attachment to extracellular matrix and vice versa. The absence of normal migration and proliferation of cells caused by failure of

keratinocyte activation during wound healing results in non-healing (Morasso, 2005). The activation of C-myc affects epidermal biology directly and its relevance to wound healing. C-myc is required for transition from the G1 to S1 phase of the cell cycle and it also promotes proliferation of transit amplifying cells.

Deregulation of c-myc results in depletion of epidermal stem cells thereby making the tissue non-responsive to injury (Waikel et. al, 2001, Arnold, 2001 and Stojadinovic 2005). Stojadinovic and colleagues discovered that the stabilization of β-catenin (a subtype of keratinocyte adhesion molecule) inhibits keratinocyte healing of human skin in organ culture (Stojadinovic et.al, 2005). This is done through different mechanisms: (a) activation of c-myc, (b) blocking of epidermal growth factor (EGF) effects and by synergizing with glucocorticoids. Beta-catenin also participates in glucocorticoid signaling and repression of keratin genes, which are involved in cytoskeletal network and keratinocyte migration. The main features of chronic wound are; persistent inflammation, decreased activity of growth factors, and angiogenesis may be affected by the activities of β-catenin and c-myc. Other authors suggested that C-myc was found to inhibit the expression of platelet derived growth factor (PDGF)-BB and its receptors (Oster et.al, 2000), whereas fibroblast growth factor (FGF)-basic and EGF were shown to induce C-myc expression (Wang et.al 1999, Izadnegahdar, 1999). In contrast to their stimulatory effects, these growth factors may also stimulate negative feedback in chronic wounds by sustaining C-myc expression.

1.1.10 Platelet Derived Growth Factors in Wound Healing

Wound healing is a well-orchestrated and complex series of events involving cell-cell and cell-matrix interactions, with growth factors acting as messengers to regulate the various processes. The role of growth factors in wound healing has been well described in acute wound healing; growth factors are stored in the form of α-granules in platelets and when these platelets are activated, they in turn release a multiplicity of growth factors. After the formation of platelet coagulum, the activated platelets are interspersed among the fibrin strands forming a matrix within the clot, which helps to keep the growth factors within the mesh. They eventually diffuse out into the surrounding tissue.

Growth factors act locally to recruit undifferentiated cells to the injury site by chemoattraction and also stimulate mitosis in the undifferentiated cells. Stem cells are attracted to areas of high concentration of growth factors and cellular movement occurs by forming attachments to the matrix/ scaffold. Growth factors attach to receptors on the stem cell membrane, thereby activating genes controlling cell division. They also attach to cell receptors and control the genetic expression of stem cells via the modulation of signal transduction pathways of secondary proteins, resulting in cellular division and differentiation. Mitosis occurs via a signal transduction pathway through the tryosinase

kinase located on the cell membrane. Receptor activation leads to the activation of secondary messenger proteins, which enter the cell nucleus and influence the expression of the genes responsible for triggering mitosis, angiogenesis, and macrophage activation (Alder, 2002). The growth factor that was first identified was the platelet derived growth factor, which has other isomers (αα, ββ, αλ).

PDGF has been synthesized through recombinant DNA technology and has been used topically for treating chronic diabetic foot ulcers. The first efficacy study documented showed complete healing of non-ischemic foot ulcers in 25% of the placebo-treated group and 48% in the PDGF-treated group (p <.01) (Steed, 1995). The second efficacy study found complete healing in 35% of the placebo-treated group, and 50% of the PDGF-treated group, which represented a 43% improved healing (p <.007). The PDGF-treated group also showed a reduced healing time by 6 weeks (p <.013) (wieman, 1998). Other identifiable growth factors include transforming growth factor (TGF-β), which has β1 and β2 as its isomers; platelet derived angiogenesis factor; platelet derived epidermal growth factor; fibroblast growth factor; keratinocyte growth factor; insulin like growth factor; interleukin-1; vascular endothelial growth factor; epidermal growth factor; thrombospodin and platelelt factor-4, osteocalcin; osteonectin; fibrinogen; and fibronectin.

The summary of the roles of the super-family growth factors in wound healing cascade is shown in **Table 3.4 of Chapter 3**. Growth factors have special roles they play at different phases of the wound healing, and they also appear to have synergistic effects on one another. The other groups of growth factors not discussed in the table are collectively called the adhesive proteins, these includes fibrinogen and fibronectin. They are known to participate in thrombus formation and some mitogenic action (Anitua, 2004). More recently, Kubota et al. 2004 described a new platelet growth factor known as connective tissue growth factor (CTGF). They showed in their experiment that CTGF is released after the activation of platelet rich plasma. They are said to be expressed along with the platelet coagulation process. The expression of CTGF following autologous derived platelet rich plasma gel (APG) activation is said to be more than a 20-fold increase when compared with any other platelet growth factors.

1.1.11 Role of Exogenous Growth Factors in Acute or Chronic Wounds (Evidence to support our hypothesis)

It has been shown that the messenger RNA for proteins may be expressed in some wounds, but the protein itself may be deficient, trapped or destroyed quickly. It has been postulated that repeated

trauma and infection increase the presence of pro-inflammatory cytokines and the levels of matrix metalloproteinase (TIMP), and decrease the level of growth factors (Bennett 1993). It has also been shown in some chronic wounds that the levels of PDGF, BFGF, EGF or TGF-b are markedly decreased when compared with acute wound. Furthermore, a marked difference has also been observed in the quantity and quality of cytokines, proteases and their inhibitors when acute and chronic ulcers were analysed (Trengrove, 2000; Tarnuzzer, 1996; Trengrove, 1997). Proliferation abnormalities of fibroblast cell have been demonstrated in diabetic wounds and the use of exogenous growth factors on diabetic wounds has been found to stimulate fibroblast proliferation and facilitate wound healing. These exogenous growth factors may act to induce endogenous growths factors and their gene expression or as stimulator for tissue repair which in turn accelerate acute or chronic wounds (Grazul-Bilska et. al, 2002)

1.1.12 Proposed Gene Therapy

A few studies have shown growth factors to be efficacious in treating chronic wounds, but a less dramatic effect than those predicted based on animal studies. Some authors therefore hypothesized that adenovirus containing EGF, PDGF-BB, TGF-B and other growth factors genes may produce large amplification of these growth factors if transfected into wound bed and may enhance wound healing (Andree et .al, 1994, Magrogolis et.al ,2000). Phase 1 clinical trials using gene therapy transfer have been conducted; as of date no wound healing gene transfer technique has been approved for use in extensive human trials. Gene therapy might eventually find it application in promoting wound healing in chronic ulcers but may be difficult to popularize in clinic before it is proven to be safe, simple and effective. For this to be effective, multi-level gene therapy at specific wound healing stage will be required because different stages are modulated by different cytokines and growth factors during wound healing process.

1.1.13 Dose related Issue with Growth Factors.

It is worth mentioning that the final concentrations of GF used in topical applications are many times higher than those found normally in tissues and blood. There have been no reports of significant systemic absorption from topical application of GF significant enough to cause renal or liver impairment (Robson, 1992).

1.1.14 Scientific basis for Wound Measurement

Most wounds are caused by trauma and a large percentage of these wounds heal without any complications. Wound repair process is frequently impaired by the presence of significant co-morbidity such as circulatory dysfunction and diabetic's mellitus. The rate of wound healing has been traditionally measured by conducting a physiological and or biochemical laboratory

measurement. Successful healing is evident by complete wound closure on the surface, a satisfactory endpoint so desired is to have the structure and function of the ulcerated area restored to original state before wounding. In most cutaneous lesions, wounds are measured by tracing the contour/ edges of the wound, which reliably estimate healing despite the errors introduced by flattening a curved surface.

Wound photographs may be quantified simply by placing a linear scale within the field of view of the lens. Stereo-photograph can be used to measure wound volumes as well as area (Bulsstrode, 1986). More recently a novel method of video camera attached to a computer was developed (Jone, 1995). Measuring the square root of wound diameter have been proposed as possible tool in prognosticating to a degree of accuracy the wound process of a wound (Lawrence, 1995). Predicting outcomes of wound treatment is very useful clinically as well as from a resource management perspective.

1.1.15 Aim of Project

The molecular basis for the use of synthetic growth factors in tissue reparation has been rationalised. However, its application as not been fully explored in all fields of surgical practice. It has also been proposed that use of combination growth factors might be more beneficial than the use of single growth factor in wound healing. More recently, autologous derived platelet rich growth factor has gained popularity in the field of regenerative/ reparative medicine, mostly because it fits the description of an idea naturally existing constellation of growth factors. However, there is conflicting evidence from published series on it clinical efficacy. Furthermore, there are insufficient studies that have investigated its *in vitro* and *in vivo* biological effect. Hence, this study is designed to test our hypothesis on the biological effect of autologous derived platelet rich plasma gel on *in vitro* cultured cell and in treating chronic diabetic foot ulcer

- To characterise and quantify the expression of growth factors present in the autologous derived platelet rich plasma gel and as well to compare the pattern of expression of these growth factors between the healthy volunteers and diabetic patients.
- To determine the proliferative and migratory effect of autologous derived platelet rich plasma on Human derived keratinocyte, human derived dermal fibroblast and human umbilical vein endothelial cell. Furthermore, to determine the angiogenic effect of autologous derived platelet rich plasma on Human umbilical vein endothelial cell.
- To investigate the immunohistochemistry alteration occurring at the epithelial and extracellular matrix margin of treated chronic diabetic ulcers.
- To determine the average time to achieve complete re-epithelization in chronic diabetic ulcers patients treated with autologous derived platelet rich palsma

CHAPTER 2

MATERIALS AND METHODS

2.1 Histological Procedures

The steps described for both the immunohistochemistry and Haematoxylin and Eosin are in accordance to the operational standards of the Cellular and experimental pathology department of the institute of Cell and Molecular Science, Barts and The London School of Medicine and Denistry (ICMS) where experiments were performed.

2.1.1 Preparation of Formalin Fix Tissue and Frozen Sections

After taking a 6mm punch biopsy from a diabetic wound ulcer, the biopsy material was divided into two equal halves. One halve of the biopsy sample fixed in 10% (w/v) formal saline, which was later embedded in paraffin and then cut at 3μm using a rotary microtome. Sections were then picked up on 2% 3-aminopropyltriethoxysilane (APES) in acetone (VWR, UK) coated slides. The sections on the slides were stained with Gill's H&E or stored at 40°C in an oven for immuno-histochemistry use. The other halve of the specimen was immediately snap frozen in liquid nitrogen and air dried for an hour before being stored at -20°C. The frozen sections were cut on a cryostat with Reichert-Jung 2035 microtome at 5μm, after which the sections are air dry and picked up on Super Frost slides (VWR, UK) until required for H&E or immuno-histochemical staining.

2.1.2 Haematoxylin and Eosin (H&E)

For frozen sections, slides were air-dried for 30 min at room temperature and then rehydrated by transferring slides through absolute ethanol, 2x3min; 90% (v/v) ethanol, 3min 70% (v/v) ethanol, 3min and tap water, 3min. on the other hand paraffin embedded sections were de-paraffinised through 2x5min xylene and then rehydrated as a frozen sections. All slides were then stained as follows:

- 15 min Gill's Haematoxylin
- 1min tap water
- 2 dips in acidified alcohol to differentiate satin
- 5min in tap water
- 2min Eosin
- 10-20 dips In water

Slides were then dehydrated in ascending grades of ethanol 70% (v/v), ethanol 2min 90% (v/v) ethanol , 2min; absolute ethanol, 2x2 min and xylene 2x 5min slides were mounted on DePeX mountant (BDH Laboratories Supplies, U.K) under 22x64 mm coverslips (Chance Propper, Warley, U.K) .

2.2 Principle of Immuno-histochemistry

2.2.1 Introduction

Immuno-histochemistry allows the identification and localisation of antigens/proteins in tissues. This method is based on the utilisation of antibodies raised against the protein/antigen of interest for their subsequent detection. Immuno-hsitochemistry can be performed as a single step, two or three-layer method. During my research, the method employed was the two layer method. With the two-layer method, tissue sections were incubated with normal serum from the same species the secondary antibody was raised in. The function of the normal serum is to block any cross-reactivity with endogenous immunoglobulin that would otherwise non-specifically bind to the primary or secondary antibodies. Following serum blocking, the section was incubated with an unconjugated IgG primary antibody raised against and with high binding affinity to antigen of interest. Washing steps with a buffer was used to remove any unbound primary antibody.

A secondary antibody was directed against the IgG in order to bind to primary antibody, which is bound to the antigen. The secondary antibody can be covalently linked to an enzyme, for example, horseradish perioxidase such as 3, 3'- diaminobendizine (DAB) or 3-amino-9 ethylcarbazol (AEC) are applied which are converted by the enzyme to yield an insoluble (former) or soluble (latter) coloured product that indirectly stains the bound antigen and can be visualised using standard light microscopy. Alternatively, a molecule that fluoresces such as fluroscein isothiocyanate (FITC) can be attached to secondary antibody, which can be detectable by ultraviolet (UV) light microscopy. For the three-layer method, the secondary antibody is usually biotinylated (covalently linked with biotin). Following a washing step with buffer, a tertiary antibody covantley linked with an avidin or stretavidin (that has high affinity for biotin) linked to enzyme /FITC molecule is then applied. Visualisation of protein localisation can be achieved by standard light or UV microscopy.

2.2.2 Immunoperoxidase

Immunoperoxidase techniques were originally introduced by **Hsu, et al**. which was aim for localizing a variety of histologically significant antigens and other markers. This procedure employs biotinylated antibody and a preformed Avidin: Biotinylated enzyme Complex and that has been termed the "ABC" technique. Because avidin has such an extraordinarily high affinity for biotin (over one million times higher than antibody for most antigens), the binding of avidin to biotin is essentially irreversible. In addition, avidin has four binding sites for biotin, and most proteinsincluding enzymes can be conjugated with several molecules of biotin. These properties allow macromolecular complexes (ABC's) to be formed between avidin and biotinylated enzymes.

2.2.3 VECTASTAIN® ABC Kits

Vectastain contains Avidin DH and biotinylated enzyme, which are matched reagents and have been specially prepared to form ideal complexes for immuno-histochemical staining. Although the structure of the Avidin DH: biotinylated enzyme complex is still not completely understood, evidence suggests that it consists of many biotinylated enzyme molecules cross-linked by avidin into a three-dimensional array. The complex apparently has few exposed biotin residues but has at least one remaining biotin binding site. Formation of the complex is achieved by merely mixing Avidin DH and biotinylated enzyme in dilute solution and in defined amounts prior to use. After the initial incubation, there appears to be little change in the complex. The complex remains stable for many hours after formation and in general, can be used for several days after preparation. VECTASTAIN® ABC has several advantages over the other commercially available kit. The kit is very simple to use and is highly sensitive. Figure 2.1 illustrates the main guiding principles involved in immunoperoxidas

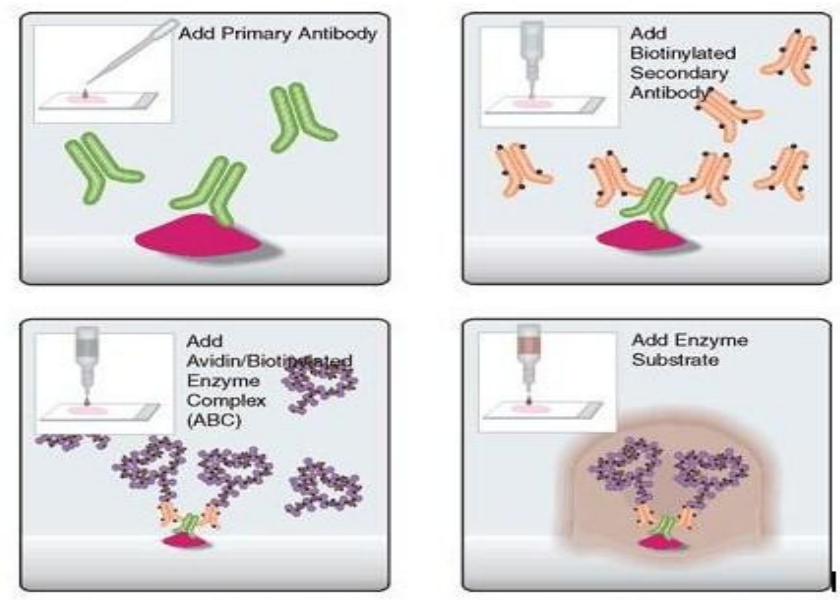


Figure 2.1 Step involved in Immunoperioxidase .The first step is to incubate the section with primary antiserum raised against the antigen of interest. Secondly, a biotin-labelled secondary antibody is added (biotinylated anti-rabbit IgG). This introduces many biotins into the section at the location of the primary antibody. The avidin: biotinylated enzyme complex (ABC) is then added and binds to the biotinylated secondary antibody. In the final step, the tissue antigen is localized by incubation with a substrate for the enzyme (Vectastain universal elite ABC kit).

2.2.3.1VECTASTAIN® UNIVERSAL Elite ABC Kit

- 3ml blocking serum (Normal Horse serum)
- 2ml containing 2.1mg Biotinlylated Universal Antibody that recognizes mouse and rabbit IgG
- VECTASTAIN Elite Regent; 2ml Reagent A (Avidin DH solution) and 2ml of Reagent B (Biotinylated enzyme). {Vector Laboratories, Inc, 30 Ingold roads, Burlingame CA, 94010 USA.

- Enzyme substrates; the chromogen used to localize peroxidase in tissue section is Di-aminobenzidine (DAB) which produces a brown color.

2.2.4 Antibody Antigen Optimization Procedure

All sections were stained using indirect immuno-histochemistry with the R.T.U Vectastain Universal Elite® Procedure ABC kit® (Vector Laboratories, UK). Wax sections were dewaxed through xylene and descending grades of ethanol to distilled water. Antibodies were first optimized by testing a range of dilutions and antigen retrieval methods. The table below illustrates the different optimizing antigen retrieval technique used for the different antibodies in these experiments. This optimization technique has the advantage that it uses a single antigen retrieval technique with varying dilutions and it tests twelve possible combinations of the treatment in one go, giving an immediate indication of the most suitable method. Fourteen normal paraffin skin slides were used, except in some peculiar circumstances when antigen retrieving needs changing. Each of the slides is labeled from numbers 1 to 12 and 2 negative control slides. The numbered slide corresponds to the individual optimizing condition (Table 2.1).

Table 2.1 Antibody optimization layout

Antibody Details : Date :
Dilution : based on manufacturer recommendation
Control Tissue : Human Skin

pH	Microwave 10 mins	Microwave 40 mins	Water-bath 10 mins	Water-bath 40min
Citrate buffer (6.0)	Slide 1	Slide 2	Slide 3	Slide 4
Citrate buffer (8.1)	Slide 5	Slide 6	Slide 7	Slide 8
Citrate buffer (9.0)	Slide 9	Slide 10	Slide 11	Slide 12
		Negative control		Negative control

Table 2.1 The standard antibody optimization layout used for all the different antibodies during the immuno-histochemtry experiment. This optimization protocol was adapted from the cellular and experimental pathology department of the ICMS.

There are few except to the optimization protocol. Anti Ki67 and antihuman endothelial cell (CD31) were initially optimized with appendix tissue section before been tested in normal human skin. Six paraffin normal skin sections were used with microwave setting at 10 minutes and 40 minutes, with varying concentration of the citrate buffer i.e. pH 6, 8, and 9 respectively. Another set of 6 sections were optimized in water bath at 97℃ for 10 minutes and 40 minutes. A single negative control was used separately for the microwave and water bath respectively. Positive and negative skin controls were used to confirm antigen specific binding and identify non-specific staining.

After the antigen retrieving step, the slides were left under running tap water bath before the application of the endogenous peroxidase, which is met to prevent non specific binding of the antibody. This step was followed by blocking with normal horse serum, which helps to eliminate background staining. The biotinlylated anti rabbit and mouse secondary was added after the application of the specific primary antibody. Diamino-bendizidine DAB, which was mixed with 1 ml substrate buffer and 1 drop of DAB chromogen were applied at the final stage to allow for localization of peroxidase in tissue sections. The stepwise approach for the immuno-histochemistry can be found in Appendix 4, which is a modification of the VECTASTAIN universal instruction that have been adapted for use in the Cellular and experimental pathology laboratory of the Barts and the London School of Medicine and Dentistry.

Monoclonal & Polyclonal Antibodies used for Immuno-histochemistry.					
Antibody	**Tissue**	**Dilution**	**Endogenous block**	**Antigen retrieval Method**	**Source**
keratin 1&10 **(LH1)**	Wax	1/150	Water/H2O2/ 15min	PH 8.1 citrate Buffer 40 min microwave	CRUK, London, UK
keratin 10 **(LH2)**	Wax	1i/50	Water/ H2O2/ 15min	PH 8.1 citrate Buffer 40 min microwave	CRUK, London, UK
Keratin 14 **(LL001)**	Wax	1in 100	Water/H202/ 15min	PH 6.0 Citrate Buffer 10 min microwave	CRUK, London, UK
keratin 16 **(LL025)**	Frozen	Neat	Water/H2O2/ 15min	Antigen Retrieval	CRUK, London, UK
keratin Ki67 **(NCL-Ki67-MMI)**	Wax	1 / 300	Water/H2O2/ 15min	PH 6.0 Citrate Buffer 30 min microwave	Novocastra
Integrin β1 **(CD29)**	Wax	1/100	Water/H2O2/ 15min	PH 9.0 citrate Buffer 10 min water bath	CRUK, London, UK

Cell surface Maker Monoclonal**(CD44)**	Wax	1 in 75	Water/ H2O2/ 15min	PH 6.0 Citrate Buffer 30min microwave	Dako, Glostrup, Denmark
Antibody	**Tissue**	**Dilution**	**Endogenous block**	**Antigen retrieval Method**	**Sources**
Endothelia Cell **Monoclonal (CD31)**	Wax	1 in	Water/ H2O2/ 15min	PH 6.0 Citrate Buffer 25min microwave	Dako, Glostrup, Denmark
Collagen I polyclonal**(ab292)**	Wax	1 in 50	Water/ H2O2/ 15min	PH 6.0 Citrate buffer 10 min microwave	Abcam, Cambridge, UK
Collagen III **polyclonal(ab7778)**	Wax	1/ 200	Water/ H2O2/ 15min	PH 6.0 Citrate buffer 20 min microwave	Abcam, Cambridge, UK
Collagen VII **(NCL-COLL-VII)**	Frozen	1 in 50	Methanol/H202/ 30min	Antigen retrieval not used	Newcastle, UK

Table2.2 Shows varying optimization conditions for the different antibodies employed in the immunoperoxidase, their sources, the antigen retrieving methods and the dilution ratios.

For visualization of DAB slides, (Bio Genex, Inc, San Ramon, CA) sections were counterstained in Gill's Haematoxylin, washed in water, differentiated in 1% acid alcohol then washed again in water. Sections were dehydrated in industrial methylated spirit (IMS) cleared in xylene and then mounted on xylene-based mountant. (This automated machine was available for use in the department and the details of the steps involved can be found in Appendix 4). Frozen sections were air dried for an hour before use and then fixed with acetone (-20°C). These were then cleaned in wash buffer and an endogenous block applied (as for wax sections with the exception of diluting hydrogen peroxide in methanol instead of water), the antigen retrieving steps were skipped. The remaining steps followed the standard R.T.U Vectastain Universal Elite protocol (Appendix 4).

After the complete immuno-histochemistry staining, the slides are covered with a cover slip and allowed to air dry. Each slide were then examined under the microscope and graded based on the specified and expected characteristic staining pattern recommended by the manufacturer for the antibody. The slides were graded as; poorly stained, faint stained, average, good, very good and too strong stained, these grading has a directly relation to the optimizing condition for the antibody tested. The optimization condition used for the slide that comes out as the very good stain was then adopted as the gold standard for that particular antibody. The same was repeated for all antibody used for the immunoperoxidase staining.

2.2.4.1 Enzymatic Antigen Retrieval

Enzymatic antigen retrieval is one of the earliest ways of unmasking formaldehyde-fixed antigens, before exposing the slide to primary antibody, in a solution of a proteolytic enzyme. The rationale for using a proteolytic enzyme is on the basis that breaking some peptide bonds will make holes in the matrix of cross-linked proteins, allowing for the entry of antibody molecules. Enzymes other than trypsin have been used in similar ways, these includes pronase, proteinase, protease pepsin. Most of the antibodies employed in these experiments worked with the earlier described optimization method (See Table 2.2) with the exception of antibody keratins K16 and K5/K14 that needed enzymatic antigen retrieval step. The proteinase and protease from streptomyces griseus type XIV (3.5 units/mg) solid power, Sigma Aldrich, UK, were used for enzymatic antigen retrieving when the earlier described optimization method was unsuccessful.

2.2.4.2 Optimization Condition for K16

The antigen retrieval protease (from streptomyces griseus type XIV, ≥3.5 units /mg solid power) and proteinase were weighed (0.1g) and added to 100ml of PBS X1, which was mixed until all the powers have completely dissolved in PBS at room temperature. Three separate methods were employed in order to achieve the best possible immunoperoxidase staining protocol. Two slides were tested per optimizing condition, one as the positive and the other negative control.

1. Each slide was dipped into a trough of proteinase and was quickly transferred into citrate buffer solution at PH of 6 and micro-waved for 10 and 40 minutes respectively. The same process was repeated with protease (WGK, Germany).

2. The tissue section was left in a trough of proteinase mixture for 15 min at room temperature and each slide was then transferred into citrate buffer solution at PH of 6 and micro-waved for 10 and 40 minutes respectively. The same process was repeated with protease

3. The tissue section was dipped quickly into a trough containing proteinase mixture and the slide was then transferred into citrate buffer solution at pH 6 and left in water bath for 10 and 40 minutes respectively. The same process was repeated with protease.

4. After the antigen retrieval step, the other steps described in the Vecstain ABC protocol were followed. The antigen retrieval optimization process that produced the best and desired staining result was the slide left in proteinase mixture for 15 minutes and heated in water bath for 40minutes. This was adopted for the rest of the immunoperoxidase staining

2.2.4.3 Optimization Condition for K5/K14

The optimization process described for K16 was adapted for K5/K14 by using buffer solution at PH of 6 and 10, heating in microwave and water bath at 10 and 40 minutes respectively. The combination of slide left in protease for 15 minute and heated in water bath for 40minute produced the desired immunoperoxiadse staining pattern for K5/K14 cytokeratin antibody. K5/K14 antibody were out of stock (not available commercially) at the time of the study, hence the antibody was not tested on all the wound biopsy samples.

2.3 General Principle of Cell Culture

All cell types described were handled using an aseptic technique standard to all tissue culture laboratories. All procedures were carried out in sterile laminar hoods. Sterile disposable tissue culture dishes were purchased from Corning Inc USA. All tissue culture instruments, including tips and forceps were autoclaved. Cells were incubated in a humidified atmosphere at 37°C 90% air / 10% CO_2. Centrifuging was carried out at room temperature in an IEC Centra-3C Centrifuge (International Equipment Company, UK) at 1200 RPM. A Nikon Eclipse TE2000-S (Nikon, Japan) was used for light microscopy (LM).

2.3.1 Mouse 3T3 Fibroblast Cell line

Mouse 3T3 cell line was obtained from Cancer Research U.K (C.RU.K). This cell line was first established by G. Todaro and H. Green in 1962 from disaggregated embryos of Swiss mouse (Todaro and Green, 1963). The Swiss 3T3 strain was used for the purpose of this research. The 3T3 cells were cultured in T75cm^2 polystyrene tissue culture flasks with Dulbecco's modified Eagle's medium (DMEM) {Invitrogen Life technologies, Paisley, Scotland, U.K}, supplemented with 10% Fetal Calf Serum (FCS) {PAA Laboratories GmbH, Austria, 5ml of 1% L- glutamine (concentration) and a mixture of 5ml of Penicillin Streptomycin. The seeding density for 3T3 depends on number needed for the planned experiment. On the average 200,000 3T3 cells were seeded in to a 75cm2 flask with DMEM of 15ml. The prepared flasks were incubated at 37^0C in a humidified 10% CO_2. The first media change was after 24hrs and subsequently on alternate days. When the cells reached 80-90% confluence, they were passage before contact inhibition sets in.

For further sub-culturing, old culture media was aspirated from the flasks, the cells are bath /washed in trypsin, the trypsin is then aspirated off. The trypsin acts to chelate the divalent cations (Calcium and magnesium ions), weakening the adhesion of cells to each other and to the flasks. Five millimetre of trypsin (0.0- ethylenediamino-tetraacetic acid (EDTA) (PAA, Laboratories GmbH) was added to the flask of T-75 and left in the incubator for 5 to 10 min. Incubation with trypsin cleaves extracellular cell adhesion proteins required for cell to cell attachment and cell to flask attachment. The flask can be given a gentle tap to enhance further detachment; the cells are checked under the microscope to determine the degree of detachment of the cells. If all the cells have detached, equal volume of E4 medium (5mL) to inhibit the protease activity of the trypsin was added. If the cells are still attached, the flask was put back in the incubator or the old trypsin-EDTA is removed and a fresh warm trypsin-EDTA (37^0C) was added to the cells and allows to incubate. Leaving the cells too long in trypsin-EDTA is harmful to the cells. An additional 10mL E4 medium were added to the solution of the cells to make a total of 20mL in a falcon tube (BD Bioscience, Oxford U.K.). At this point a cell count was performed before passaging or the 3T3 cells are treated as the case may be.

The cells were re-suspended in the falcon tube; cell count was done by pipetting a few drops under the cover slip of a Neubauer haemocytometer (Weber Scientific International, West Sussex, and U.K). Cell concentration was calculated by taking an average of cell count in each of four 4 x4 square grids under the microscope. The average number was then multiplied by 10^4 to give the final cells/mL. The solution of cells and medium in the falcon tube were centrifuged at 1,000rpm for 5min using the IE 215A rotor (International equipment company, U.K). The supernatant were removed and the pellets were left at the bottom of the tube, which was suspended in fresh volume of E4 media. The cells are now split into new fresh T-75 flask and return to the incubator at 37^0C and 10% of CO_2. Media change is done after 48hrs and the cells are maintained in culture accordingly.

2.3.1.1 Treating 3T3 for Feeder

This involved treating known amount of 3T3 (NIH) with mitomycin in preparation for subculturing keratinocyte.

2.3.1.2 Mitomycin Preparation

Mitomycin C (Sigma Saint Louis, Missouri 63103 USA) is produced by a strain of actinomyces named streptomuces caespitous. Mitomycin C inhibits DNA synthesis; it reacts covalently with DNA, *in vivo* and *in vitro*, forming cross-links between the complementary strands of DNA. This interaction prevents the separation of complementary DNA strand, thus inhibiting DNA replication. Mitomycin C is soluble in water; the 2mg vial is in a crystal form. Mitomycin C was dissolved in

4mL of water to make 0.5mg /mL. The recommended concentration for eukaryote cell culture is 10-50 µg/mL. For the purpose of treating 3T3 or for scratch assay experiment we used 20 µg/ ml. Mitomycin C undergoes rapid degradation in acidic solutions with pH less than 6.0. The reconstituted vials are wrapped in aluminium foil, kept in the dark and stored at -20^{0}C, this is because it light sensitive.

2.3.1.3 Preparation of 3T3 Fibroblast for keratinoctye Isolation

Murine 3T3 (NIH) fibroblasts were obtained from cancer research UK. Cells were cultured in T-175 flasks with E4 medium (See Appendix 1) and incubated. Cells were maintained by media change every 3 days and passage once 80% confluences were reached (See Appendix 2). Cells were splitted into new flasks to generate stocks or frozen for long-term storage in liquid nitrogen (See Appendix 2). 3T3 cells were splitted by washing in 5mL of 1x versene/EDTA (Invitrogen, UK) for 30 seconds. EDTA chelates calcium ions thereby disrupting calcium-dependent cell junctions such as the cadherins found in desmosomes. It allows trypsin to digest these junctions, causing the dissociation of cells from each other and the flask. EDTA was then aspirated and replaced with 5mL of 0.25% trypsin-EDTA (Sigma Aldrich, UK). Flasks were then incubated for 5-10 minutes and checked by light microscope (LM) for cell detachment. Once all cells had detached an equal volume of E4 medium was added to the flask. Serum (FCS) within the medium inhibits trypsin activity. Cells were then re-seeded at a lower density. Growth arrested 3T3 "feeder" cells were produced through Mitomycin-C treatment or irradiation (Appendix 2 stepwise process of Mitomycin treatment). This inhibits cell division but maintains cell viability therefore allowing 3T3s to produce paracrine growth factors to encourage keratinocyte growth.

2.4 Preparing RM+

RM+ is a supplementary chemical needed to complement the keratinocyte media- Dulbecco's modified of Eagle's medium (DMEM). The reagents needed for RM+ was prepared individually and then mix together to produce the final product. The insulin was re-suspended in 0.05M HCL and dH_2O to make a final concentration of 5µg/ml in DMEM medium of 500ml. The other reagents were prepared after dissolving them in dH_20 to the following required concentrations as shown in table **2.3**. The volume of the stock needed to make 200ml 100 x RM+ is as shown in the table, was added to the cylinder, which was then toped up until it reached 100ml with distilled water, it was then filtered. Another 100mls of E4/F12 (without serum) was added to the cylinder to give a total volume of 200ml. The mixture was again filtered into another bottle and aliquot into 4ml of bijous

and store in -20^{0}C. One of the bijoux was tested for bacterial infection by putting it in the incubator for 1 week and checking regularly for turbidity or cloudiness of the solution- this is the sterility check of the procedure.

Product Name	Company	Stock Concentration	Volume in stock needed for 200ml 100x Rm+	Final concentration in 100X Rm+
Cholera toxin	Sigma	100µg/ml	2ml	0.84µg/ml
Epidermal growth factor (Mouse)	Serotec	100 µg/ml	2ml	1µg/ml
Hydrocortisone	Sigma	4mg/ml	2ml	40µg/ml
Insulin(dissolved in 0.05M HCl)	Sigma	50mg/ml	2ml	500µg/ml
Transferrin	Sigma	50mg/ml	2ml	500µg/ml
3,3',5 Triiodo-L-thyroxine sodium Salt	Sigma	2.6 µg/ml	100µl	1.3µg/ml

Table 2.3 Detailed description of RM+ and it constitutes. Details of the varying concentrations and the suppliers of the ingridents are shown in the above table.

2.4.1 Isolation of Primary Cells.

Human epidermal kerationcyte and dermal fibroblast were extracted from either redundant skin from breast reconstruction, abdominoplasty, and foreskin from circumcision and facial skin from facelift, using the method initially described by Rheinwald and Green in 1975. Ethical approval and inform consent was obtained from the patient before the procedure.

2.4.2 Human Epithelial Keratinocyte

Keratinocytes were isolated from redundant facelift skin obtained from plastic surgery (Appendix 2**). Ethical committee approval was obtained from East London and The City research committee Authority (No. 08/H0704/17)** and informed **consent obtained. Skin was transferred** to the laboratory in transport medium at 4^{0}C, upon arrival; skin was removed from its container by sterile forceps and placed into a 100 mm^2 cell culture dish. It was covered with 2–3mL of EDTA in preparation for keratinocyte extraction (**Appendix 2**).

Primary keratinocytes were cultured based on the original methods described by Rheinwald and Green, 1975. Keratinocytes were seeded at a density of $2x10^6$ onto a mitomycin-C treated layer of $2x10^6$ 3T3 "feeders". These were grown in T75 flasks, suspended in 20mL of RM+ medium (Appendix 1). Flasks were then incubated and checked daily for healthy cell growth. Keratinocytes were maintained at sub-confluence with medium changes every 2 days, additional 3T3 feeders were added as necessary. Primary cultures were serially passage at 75% confluence, in order to prevent stratification and differentiation. Only passages one to three (p1-3) were used for the purpose of this research. Keratinocytes were passage by trypsinization (Appendix 2).

First, culture medium was aspirated and the cells washed in PBS (PAA, Laboratories UK). This was then replaced with 5mL versene and the flask returned to the incubator for 5-10 minutes. Cell detachment was ascertain under light microscope (LM) every minute. As 3T3 adhesions were affected first, this method allows for the selective removal of all feeders before splitting the keratinocytes. If any 3T3 cells remained, the flask was given a gentle tap and re-incubated until only keratinocyte colonies were present. Once all 3T3's had detached, the versene was then aspirated and 5mL of trypsin-EDTA added for 5-10 minutes at 37°C.

Again cell detachment was confirmed by examination under light microscope, once all keratinocytes had detached, an equal volume of RM+ medium was added to the flask to neutralize the protease activity of trypsin. The flask contents were then transferred to a sterile 50mL falcon tube and centrifuged for 5 minutes. The supernatant was then aspirated and the pellet re-suspended in10mL of RM+. Cells were then counted using trypan blue (Sigma-Aldrich Ltd, UK) and a haemocytometer (Weber Scientific International) (Appendix 2) under LM and then re-seeded in T75 flasks with new 3T3 feeders. Keratinocytes were routinely split at a ratio of 1:5. These cells grew in colonies and were serially passage every 8- 10 days. After the 4th passage the doubling rate of keratinocyte slow day.

2.4.3 Prevention of Fibroblast Contamination in Keratinocyte Cultures

The addition of 3T3 feeder cells discourages the growth of dermal fibroblast in keratinocyte cultures but if the densities of 3T3 feeders are low, fibroblast contamination can occur. For the removal of these fibroblasts, the flasks were washed once in 0.02% EDTA and then incubated with 0.05% trypsin in 0.02% EDTA (1:4 dilutions) at 37^0C for about 30-60 sec or until the feeders and human fibroblast had detached from the flask. Tapping the sides of the flask gently aided in the detachment

of fibroblast. Fresh medium was then pipette into the flasks that still contained attached keratinocyte and fresh feeders were re-added.

2.5 NEB-1 Immortalised Cell line

Neb-1 cells human cutaneous keratinocyte immortalize by human papilloma virus (HPV) - type 16 (Morley, D' Alessandro et. al., 2003). They were obtained fron the Centre for Cutaneous Research ICSM, London UK), and cultured in DMEM/Hams F12 medium in T75flasks. The cell medium were refreshed every two to three days and cells passaged according to already described protocol. Neb-1 cells were used because they have similar biological behaviour as keratinocyte, easy to grow and maintain in culture with RM+ medium, which is changed every 48hrs. Passages from 6-10 were used in this set of experiments

2.6 Isolation of Human Dermal Fibroblast

There are 2 different techniques that could be employed in the isolation of fibroblast; the explants and the D- Collagenase methods.

2.6.1 The Explants Technique

The 6- 8 pieces of skin left over from keratinoctye extraction was placed in a scored treated sterile Petri-dish (Corning, Inc, USA) to create a rough surface and a small amount of 10% FCS was added to the dish and left in the hood to air dry (10-15 min) until the skin piece sticks to the scored dish. Twenty mills of E4 media was then added to the dish, which was left to incubate at 10% CO_2 and 37^0C. The media changed was carried out every 2-3 days, until the formation of new fibroblast which may take up to 7-10 days were formed. The Petri-dish was checked for new fibroblast daily under the light microscope. The skin pieces were discard once sufficient yield of fibroblast was established. Culturing of the fibroblast was continued until 70-80% confluent was reached and the same passage according to protocol for 3T3 mouse feeder cell line. Passages one to seven were used for study.

2.6.2 Collagenase D Technique

Collagenase D (Boerhringer Mannheim GmH, Germany) from clostrium histolyticum, 0.05g of it was weighed under a sterile condition and added to 50ml of DMEM media with streptomycin penicillin without the other entire supplement, to make final concentration of 0.5µg/mL. A micropore filter (0.2µm) was used to filter the dissolved solution. About 6-8 piece of left over skin from keratinocyte extraction was added to 10- 15ml of Collagenase D solution in the Petri dish (untreated dish) and left over night in the incubator at $C0_2$ and 37^0C, following which the suspension are collected into a 15ml falcon tube and are neutralized with equal volume of E4 media. The tube was gently vortex to release as many cells as possible. The suspension was then filtered using a sterile cell strainer (100µm) and the filtrate was centrifuged at 1200 rpm for 5min.

After aspiration of the supernatant, the pellet left at the bottom of the tube was re-suspended in DMEM containing 10% FCS and the cells seeded at a density of $1x10^6$ per T75 cm flask. This was then further sub-cultured by incubating at 37^0C in a humidified 10% CO_2/ 90% atmospheric air with full complement of E4 medium. Confluent cultures of fibroblasts were trypsinied using 0.05% trypsin in 0.02% EDTA (1:4 dilutions) and subculture using the same protocol as for 3T3 mouse feeder cell line. Passage one to five was used for the experiments in this study.

2.7 Passaging Cells

Cells that were not grown on feeder layer were passage when they were approximately 70% confluent by washing once with 5ml sterile PBS. The cells were once again washed with 5ml of versene and incubated at 37^oC for 5min with trypsin-EDTA in the case of 3T3 mouse fibroblast, or trypsin in the case of NEB-1cells. The process of trypsinisation was monitored under the light microscope and the flask was tapped to aid cell detachment. Once all cells had lifted off, the trypsinisation was halted by adding 5ml culture medium containing 10% FBS and the solution decanted into 5ml centrifuge tube. The cells were centrifuged at 200g for 5min at room temperature. The supernatant was aspirated, the cell pellet resuspended in 10ml culture medium and the cells counted using haemocytometer as previously described. Cells were then seeded for experiments, propagated at 10% of their original density or cyropreserved for storage.

2.7.1 Cryopreservation and recovery of Cell types.

Cells (3T3, HDF) for frozen storage were suspended at a density of $2x10^6$/mL in freezing medium of 90% FCS and 10% dimethyl-sulphoxide (DMSO) (BDH Laboratory Supplies, UK), whereas for Neb-1 cell and HEK 10% DMSO, 30% RM+ media and 60% of FCS was used. The DMSO protects against ice crystal formation which can rupture cell membranes (Lovelock, 1959). 1mL of suspension was pipette per cryogenic vial (Corning Inc, USA). These were then wrapped in six layers of paper towels which allowed the cells to freeze down slowly to -80 °C in a freezer. Cells were then transferred to liquid nitrogen for long-term storage. When retrieving cells from storage, vials were defrosted in a 37°C water bath, swabbed with industrial methylated spirit (IMS) and the contents then transferred to a 50mL falcon tube containing 10mL of medium. This helps to dilute the DMSO. The tube was then centrifuged for 5 minutes at 1200 rpm and the pellet re-suspended in a fresh medium and re-seeded.

2.8 Preparation of the culture media for the HDF, HEK and HUVEC

The stored aliquots of PRP and PPP in -20^0C were carefully defrosted in water at 37^0C and reconstituted into varying concentration of 5%, 10%, 25% and 50% were mixed with different culturing media. Studies have shown that storing growth factors in -20^0C to -80^0C does not affect the level of the growth factors released. For proliferation assay with keratinocyte; Phenol red free DMEM/ Ham's F-12 with 1% FCS / 1% Bovine Albumin serum (BAS) and antibiotic,without the RM supplement (choleratoxin, epidermal growth factor, hydrocortisone, insulin, transferrin and thyronine sodium salt were used **(Table 2.3)**. For fibroblast; phenol red free high glucose DMEM with 1% FCS /1 % BAS and antibiotic without L-glutamine was used in diluting the PRP and PPP into the desired concentrations.

For HUVEC, varying elimination and combination of the EMG-2 and its supplement was tried with PRP and PPP before arriving at a final concentration which allowed HUVEC to be sub-cultured successfully for a period of 5 days. The EMG media was reconstituted using the EMG only + heparin+ gentamycin + hydrocortisone +ascorbic acid and or 1% BAS and the remaining supplement were not used in the final concentration of PRP and PPP. The supplement recommended for optimal culturing of HUVEC are Hydrocortisone 0.2ml,fetal bovine serum 10ml(FBS), recombinant human fibroblast growth factor-B 2ml (r-hFGF-B),heparin 0.5ml, recombinant human epidermal growth factor in buffered bovine serum albumin 0.5ml (rhEGF) , recombinant human vascular endothelial growth factor 0.5ml (rhVEGF), Gentamicin sulphate Amphotericin-B 0.5 ml (GA-100),

recombinant long releasing insulin-like growth factor-1 0.5ml (R^3-IGF-1) and ascorbic acid 0.5ml; which is the full complement for EMG media.

2.9 Human Umbilical Endothelial Cell

Clonetics Umbilical Vein Endothelial Cell Systems contains normal human Vein Endothelial cells (HUVEC); this system has the capability to generate HUVEC cultures for experimental application. The cryopreserved HUVEC (Lonza Walkersville, Inc. USA) are shipped as frozen primaries. A cryo-vial on the average contains a total of 500,000 cells/ ml, stored at -80^{0}C.

The systems consists of umbilical vein endothelial cell product (cryopreserved) isolated in (endothelial growth medium) EGM, endothelial cell medium (Bullet-kit- 500ml), clonetics EGM-2 Bullet-kit contains one 500ml bottle of Endothelial Cell Basal Medium-2 (EBM) and the following growth supplements: Hydrocortisone 0.2ml,fetal bovine serum 10ml (FBS), recombinant human fibroblast growth factor-B 2ml (r-hFGF-B),heparin 0.5ml, recombinant human epidermal growth factor in buffered bovine serum albumin 0.5ml (rhEGF), recombinant human vascular endothelial growth factor 0.5ml (rhVEGF), Gentamycin sulphate Amphotericin-B 0.5 ml (GA-100), recombinant long releasing insulin-like growth factor-1 0.5ml (R^3-IGF-1) and ascorbic acid 0.5ml,The HUVEC cells were cultured according to the manufacturers advice. Passages 2 and 3 were used for all the assays types with HUVEC, this is because the older passages have been shown to have less doubling rate compared to the earlier ones.

2.9.1 Using the Clonetics Endothelial Cell System.

1. Cryovials containing HUVEC are removed from dry ice pack and place into the liquid nitrogen until its ready for use. When the cells are to be used immediately, they are thawed quickly in water bath and are transferred to the ready prepared flasks.

2. The cryovial are wiped with ethanol before opening, the vial was opened in a sterile field, the cap of the vial was twisted a quarter turn to relieve pressure, and then retighten. The cryovial was thawed in water bath at 37^{0}C quickly, this was not for more than 2minute and the vial was closely monitored to avoid submerging.

3. A cryovial contains about 500,000 cells; tryphan blue was used to determine the accurate number of viable cells. The cells were divided into equal volume/ number of culture vessel; for example, 250,000 cells was seeded into a T-25 flask (25cm^2).

4. Before the addition of cells to the flask, appropriate amount of medium (1ml/5 cm^2) required were added and allowed to equilibrate in humidified incubator for at least 30 minute in 37^0C, 5% $CO_{2.}$ Once the flask was ready for use (T-25 cm^2 flask), the cells are re-suspended and dispensed into the culture vessels set up earlier. The cells from the cryovial are not centrifuged; this effect is more damaging than removing the residual DMSO in the cultured cell.

5. The growth factors are allowed to thaw after removal from dry ice and added to a decontaminated bottle of basal medium (EGM-2), which can then be stored in 4 ^{0}C

2.9.2 Subculturing HUVEC

Sub-culturing will be needed once the cell have reached about 70-80% confluent and contains many mitotic figures throughout the flask.

- The Reagent Pack ^{TM} subculture reagents are sterile –filtered and stored at -20^0C. This pack consists of Trysin/ EDTA solution, HEPES –BSS (Buffered Saline Solution) and trypsin neutralizing solution (TNS). After thawing, they are immediately aliquot into a 15ml tube and stored in -20^0C, for a longer shelf live.

- To subculture an 80% confluent flask, each flask is done at a time, before removing the culture media; 5ml of HEPES-BSS, 8ml of Trypsin/EDTA and 4ml of TNS are allowed to thaw at room temperature. After removing the flask from the incubator, the media was aspirated off the cells and are rinse with HEPES-BSS, which helps to prevent the complex proteins and calcium from neutralising the action of trypsin. The HEPES- BSS is aspirated off the flask and the cells are cover with 4ml of Trypsin/ EDTA solutions and left in the incubator for 4-6minutes, this allows for trypsinization, until 90% of the cells are rounded up.

- The flask was rapped against the palm to allow majority of the cells to be released from the culture surface. After cells are released, 4ml of TNS was added to neutralise the newly released cells. If some of the cells are not detached the reminding are re-trypsinized with fresh, warm trypsin/ EDTA solution and returned into the incubator until the remaining are well rounded up. The trypsinized cells are transferred into a sterile 15 ml centrifuge tube. The harvested cells were examined under the microscope to briefly assess for cell viability.

- The tube containing the harvested cells was centrifuged for 700prm for 5 minute, after centrifuging, the supernatant was aspirated off and the pellet was left at the bottom of the tube. The pellet was diluted with fresh media to a total volume of 5ml for easy of cell counting. At this point, 20µl of the re-suspended harvested cells were collected into a separate sterile 15ml tube and 20µl of trepan blue was added to the cells and left for 5-10minute before determining cell viability as previously described. A uniform suspension was ensured and the cells were re-seeded into already prepared flask depending on total number of viable cells. When further sub-culturing was desired, the number of flasks needed depends upon cell yield and seeding density needed. For my experiment, the cells are seeded at 250, 000cells/T-25c^{m2} flasks, which will reach full 80-90% confluent in 5- days**.**

- To maintain the cell in culture, the media was changed after 24 hrs of seeding and then every alternate day. Once the cells become more confluent, the volume of the media for maintenance in cultured was increased by 2-5ml/25cm^2 flask to cope with the metabolic demand of the proliferating cells. It is important to avoid repeated warming of the media. To prevent this occurrence only the required volume of media was taken out from the -20^{0}C freezer and warm in separate sterile container. The clonetics cells can be use for up to ten population doublings. Although additional population and doubling are possible but the growth rate, biological responsiveness and function deteriorate with subsequent passages. For this reason, all the experiments were carried out with the HUVEC in 2nd or 3rd passage.

- The HUVEC cells were cryo-preserved after establishing a larger enough cell population beyond the number needed for the planned experiments. The freezing media (which is made of 80% EGM-2, 10% FBS and 10% DMSO) was filtered using a sterile cell filter of 0.2micron size. The cells were harvested and centrifuged as described above. The pellets collected are re-suspended in a cold freezing solution at 500,000 to 2,000,000 cells per ml quickly. The cell suspension was stored at 1ml per cryovial and sealed off. The cryovials were then transferred into a propanol freezing canister and stored in -80^{0}C overnight, the cells were then transferred to the liquid nitrogen (- 180^{0}C) after the initial 12-24 hours storage in propanol freezing canister.

2.10 EA.hy 926 Cell line

EA.hy 926 cells are endothelial cell line obtained by hybridization of human umbilical endothelial vein cells with the A549/8 human lung carcinoma cell line, This cell line has maintained the phenotype of endothelial cell line, has high differentiation functions that are characteristics of human vascular endothelium, stable through passage number and offers reproducibility of results. Bauer et al. 1992, showed that EA. hy929 cells are capable of undergoing tube formation when grown on matrigel. The cell line is very easy and cost effective to maintain in tissue culture, EA.hy 929 was maintained with DMEM medium used for fibroblast, and they were obtained from Translational Medicine department, William Harvey Research Institute, Queen Mary University of London.

2.11 Seeding Density for the different Cells ypes and the Assay

At the initial stage of the research, the seeding densities for all the different cell types and assay was determined through optimisation experiments.

- Optimization experiment with fibroblast cell were seeded at 1000, 3000, 4000, 6,000, 8000, 10,000, 20,000, 40,000, 80, 000, 100,000 and 200, 000 respectively
- Optimization experiment for HUVEC was seeded at 10,000, 40,000, 80,000,100,000, 400,000 and 800,000/ well.
- Seeding density of Fibroblast for experiment in 12 well plate; Proliferation assay is $1x10^4$/well, Scratch assay of $5x10^5$ and Transwell assay $5x10^4$
- Seeding densityfor HUVEC / EA.hy. 926 in 24 well plate; proliferation assay is $1x10^4$ transwell assay is $1x10^4$ and angiogenesis assay $2x10^5$ respectively.
- Seeding density for Neb- 1 cell and primary keratinocyte; proliferation is $2x10^4$ /well, Scratch $6x10^5$ / well, transwell $5x\ 10^4$ /well-plate respectively.

2.12 Principles of Cell Viability Assay with Alamar Blue

Cellular activities can be detected directly or indirectly by measuring plasma membrane integrity, DNA synthesis, DNA content, enzyme activity and presence of ATP. Cellular reducing conditions are known indicators of cell viability and cell death. Alamar blue cell viability reagent functions as a cell health indicator by using the reducing power of living cells to quantitatively measure the proliferation of various human and animal cell lines. When cells are alive they maintain a reducing environment within the cytosol of the cell. Resazurin, the active ingredient of AB reagent, is a non toxic, cell permeable compound that is blue in colour and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, a compound that is red in colour and highly fluorescent.

Viable cells continuously convert resarzurin to resorufin, thereby increasing the overall fluorescence and colour of the media surrounding cells.

Alamar Blue (AB) is designed to quantitatively measure the proliferation of various human and animal cells by detecting the level of oxidation during respiration. Alamar blue (AbD serotec, Kidlington, Oxford, UK) is an indicator dye which incorporates an oxidation-reduction (REDOX) which both fluorescences and changes colour in response to chemical reduction of growth medium, resulting from cell growth. AB is able to detect oxidation by the whole of the electron transport chains. The midpoint potential of AB is greater than that of any of the other component of the electron transport chain **(Table 2.5).** The mid potential for any half reaction is defined as the voltage at equilibrium is obtained between the concentration of oxidized and reduced products. Midpoint potential gives a measure of the strength of an electron donor. AB will be reduced by $FMNH_2$, $FADH_2$, NADH, NADPH and cytochromes, since their midpoint potential are lower than that for alamar Blue. Therefore, AB is an excellent detector of reduction of all elements of electron transport chains.

Alamar Blue (AB) was added to cells in culture media at a 10% of the sample volume (i.e., add 10 µL AB reagent to 10µL sample), followed by 4-8hrs incubation at 37^oC. Longer incubation times may be used for greater sensitivity without compromising cell health. In our study the optima incubation period was between 16-24hrs. The resulting fluorescence or absorbance was read on a plate reader or fluorescence spectrophotometer. Finally, the results are analyzed by plotting fluorescence intensity or absorbance versus the compound concentration.

Half –Reaction			Eo' (mV) PH 7.0 25^0C
NAD $+2H+2e^-$	←-------→	$NADH+ H^+$	-320
$NADP+2H+2e^-$	←-------→	$NADPH+H^+$	-320
$FAD+2H+2e^-$	←-------→	$FADH_2$	-220
$FMN+2H+ 2e^-$	←-------→	FMN_2	-210
$MTT_{ox,} + 2H +2e^-$	←-------→	MTT_{RED}	-110
$Cytochromes_{ox} , + 1e^-$	←-------→	Cytochromes $_{RED}$	+80 to +290
alamarBlue $_{ox} + 2e^-$	←-------→	AlamarBlue	380
$0_2+4H+4e^-$	←-------→	$2H_20$	820

Table 2.5: Oxidation reduction potentials in the electron transport system and alamarBlue. Reduced Flavin mononucleotide ($FMNH_2$), Flavin mononucleotide (FMN), Flavin adenine dinucleotide (FAD), Reduced Flavin adenine dinucleotide ($FADH_2$,), Nicotinamide adenine dinucleotide (NADH), Nicotinamide adenine dinucleotide phosphate (NADPH) and (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The midpoint redox potential (Eo') values were determined at pH 7.0, 25^0C .Table adapted from www.ab-direct.com/alamarBlue.

2.12.1 Rationale for using Alamar Blue in Proliferation Assay

Alamar Blue is advantageous over other redox indicator such as tetrazolium salt (also called MTT assay), in that it does not interfere in any way with the reactions of electron transport chain. This is because the midpoint potential of AB is greater than any of the component of electron transport chain. Other salient advantages of AB includes; non-radioactivity, simplicity, less costly and non labour intense, rapid assessment of proliferation of large number of cell sample, non-toxicity and permits further analysis of proliferating cells by other method (Ahmed et al. 1994)

MTT has a similar oxidation-reduction interaction with the electron chain with a midpoint potential of -110Mv, which is intermediate between that of the electron donors and cytochromes, MTT will not be reduced by cytochromes. Furthermore, following the interaction of MTT with the electron chain, the electrons released by these donors (FMNH2, FADH2 etc) will not be passed to the cytochromes as would normally happen in the electron transport chain, hence the respiratory chain is shut down.

Alamar Blue allows the cells to remain fully functional and healthy, unaffected by the by presence of indicator during experiment. This is property is unique to alamar Blue. AlamarBlue functions by allowing growing cells to cause a chemical reduction, continued growth maintains a reduced environment (fluorescent, red colour) and inhibition of growth maintains an oxidized environment (non-fluorescent, blue colour). Quantifiable data from alamar Blue can be collected by using either fluorescence –based or absorbance –based instrumentation. The fluorescence is monitored at 530-560nm excitation wavelength and 590nm emission wavelength. The limitations of Alamar blue are few, high cell concentrations and prolonged culture times will show reversal of the reduction process. Microbial contaminants will reduce Alamar blueand yield erroneous results, but contamination would adversely affect any assay of the cells. Bovine serum and phenol red may slightly interfere with the colorimetric changes, although we have observed no sign of toxicity in our assays, long term toxicity from Alamar blue cannot be completely ruled out.

2.12.2 Prolifertaion Assay for HDF, HEK and NEB-1 CELL

Cells were trypsinized from sub-confluent cultures by adding 3mL of trypsin solution (100 mg EDTA, 5 mL penicillin– streptomycin, 500 mL gentamicin in 500 mL trypsin) to 50 mL Falcon flasks with confluent cells followed by 10 min incubation at 37^{o}C with regular gentle shaking. The trypsin reaction was stopped by adding 10 ml of appropriate culture medium containing 10 % FCS. The cell suspension was then centrifuged at 750g for 10 min. The cell pellet was suspended in 2 ml of medium with 1% FCS and thoroughly mixed by repeated pipetting. Cells were counted with a Fuchs–Rosenthl hemacytometer and brought first to a concentration of 1x10^{6}cells/ml and subsequently to serial 1:2 dilutions. The resulting different cell suspensions were seeded into triplicate wells of a 12-well plate and incubated at 37^{o}C. After an initial 6hr period to allow cell attachment, 10% of AB solution was directly added to the medium resulting in a final concentration of 10%.

For negative control, AB was added to the medium without cells. The plate was further incubated for 24 h at 37^{o}C. The absorbance/fluorescence of the test and control wells was read at 540 and 630 nm with a standard spectrophotometer. The number of viable cells correlates with the magnitude of dye reduction and was expressed as percentage of AB reduction (Ahmed et al., 1994; Goegan et al., 1995). The values of % AB reduction were corrected for background values of negative controls containing medium without cells. In order to eliminate differences due to medium colour, serum free keratinocyte (K-SF) was used for keratinocyte proliferation and DMEM without phenol red was Fibroblast whilst the same EMG-2 was used for HUVEC proliferation**.**

2.12.3 Proliferation Assay for HUVEC

After culturing HUVEC from the initial clonetic endothelia cell systems, further subculturing and passaging was carried. An initial experiment was performed to determine the optimal seeding density and the cell survival period for proliferation assay. HUVEC proliferation assay was previously described by Segura et.al. HUVEC cells were plated at varying cell density of 2,000, 4,000, 6,000, 8,000 and 10,000 per 24- well plate to optimize the conditions needed for the proliferation assay. The experiment was carried out in triplicate. The first well seeded with full complement of EGM-2, second well contains the reconstituted EGM-2 (1% FCS and without the recombinant growth factor) and the third well-plate contains 1%EGM-2 plus PRP at 50%) this was monitored over 7 days. Day

5 was the critical period were HUVEC survived with 1% EMG-2 media before the apoptosis sets in. Ten thousand was identified as the optimal seeding density for HUVEC proliferation assay.

- HUVEC cultured with full complement of EGM-2, plated at a density of $1x10^4$ in 24 well plate, allowed to attach for 24hrs. The medium was changed after the first 24hrs period The EMG media was reconstituted using the EMG only + heparin+ gentamycin + hydrocortisone +ascorbic acid and 1% FCS as the positive control.
- The media was replaced the following day and media with varying concentration of PRP from healthy volunteer at 50%, 25%, 10% and 5%, PPP and control media respective. The cell was left in the humidified incubator at 37^0C, 5% CO_2, the cells were checked daily for features of cell growth and change in morphology.
- On day 0, day 3 and day 5 of the experiment, growth response was assessed using the Alamar blue dye technique, this was measured by Fluostar optima this works on the principle of Fluorescence intensity- plate mode using 96- well microplate (Nunc-96), which was preset at excitation filter of 544nm and emission filters of 590nm.
- The first experiment was performed with no media change (media + PRP). The plated wells were incubated with Alamar blue over 6hrs period. 10% of the total volume of the cell media was used for the microplate reading. A separate well plate was measured as the untreated control which is the blank that serve as the internal control for the experiment. All experiment were performed over 5 day period in triplicate and repeated to give N= 3.
- Optimization experiment was carried out initially to determine the experimental end point time and the baseline seeding density for the proliferation assay. The experiment was carried out with varying cell density (10,000, 40,000, 80,000, 100,000, 400,000 and 800,000/ well plate) at 4hours and 16hrs **(See result section in Chapter 4)**

2.13 Principles of Wound-Healing Assay

The wound-healing assay is one of the earliest developed methods to study cell migration in vitro (Torado, 1965). This method is based on observation of cell migration into an "artificial wound" that is created on a cell monolayer. Although not an exact duplication of cell migration in vivo, this method mimics to some extent migration of cells in wound healing. Depending on the cell type, cells migrate into the wound as loosely connected populations (e.g.fibroblasts) or as sheets of cells (e.g. keratinocyte and endothelial cells), which also mimic the behaviour of these cells during migration in vivo. In comparison with other popular *in vitro* methods, such as time-lapse microscopy and Boyden chamber assays, the wound-healing assay is particularly suitable for studies of directional cell

migration and its regulation by cell interaction with extracellular matrix (ECM) and cell–cell interactions. Migration of the cells is regulated by both the ECM under the cells and soluble factors (Han, 2001) as well as intercellular interactions in the case of epithelial and endothelial cells (Underwood, 2002). The effects of each of these factors on directional cell migration can be studied using the wound healing method. The basic steps involve creation of a "wound" on monolayer cells, capturing of the images at the beginning and regular intervals during cell migration until closure of the wound, and comparison of the images to determine the rate of cell migration.

2.13.1 In *Vitro* Wound/ Scratch Assay with HEK, NEB-1, HDF

To determine whether there were quantifiable migration rate differences between different HEK, Neb-1 and Fibroblast samples in response to wounding, cell migration assays were performed as described (Zingers, 2003, Spurzem 2002) with slight modifications. The first set consisted of HEK samples derived from the epidermal layer of normal neonatal foreskin/ face lift skin from plastic surgery, were isolated. These were grown to confluence on 12 well plates. Neb -1/ HEK were either treated with mitomycin to eliminate the process of proliferation during the assay or were untreated. Mitomycin treatment was not used for fibroblast proliferation because the rate of cell proliferation is much slower compared to Neb-1 and HEK.

The experiment was carried out in triplicate and cells were treated with varying concentration of PRP (5%, 10%, 25%, 50% and PPP) and the control experiment was with 1% FCS. Scratch wounds were created using a 20µL pipett tip and the artificial wound photographed digitally at six-hour intervals from the start. Photographs were taken with microscope connected with a digital video camera. The images were later analyzed for distance cell migration on Image J. Custom software was then used to calculate the cell-free area, and the cell migration rate was calculated using the changes in this area over time. The cell migration rate was calculated as the distance travelled by the cells over time. Using the cell-free areas measured at the specified time-points, we calculated the change in cell-free area. Because the wound was made in the vertical direction, the cells migrating into the previously cell-free area came from two directions, the top and bottom of the wound area were determined

2.13.2 Materials and Protocol for *In Vitro* Wound Assay in HEK/NEB-1 Cell and HDF

- A 12 well plate was marked on the outer bottom of the plate to be used as reference points during image acquisition. Marking the well plate can be achieved by numerous methods to obtain the same field during the image acquisition. Etching lines lightly with a razor blade on the bottom of the dish provides a good reference under the microscope and can be visualized with

the naked eye for creating the wound itself. Matching the images is essential for quantitative analysis.

- The well plate was coated with either fibronectin for fibroblast (EMD Biosciences, Inc. La Jolla CA 92039-2087) or collagen for keratinocyte. Fibronectin at concentration of 250 µg was dissolved in 250µl of Hank balance salt solution (HBSS) to make 1:1 dilution. Each of the well plate for fibroblast wound assay were coated with fibronectin at concentration of 25 µg/Ml and the well plate for Neb-1 cell and keratinocyte wound assay were coated with type I rat collagen (150mcg/mL) dissolved in Hank's balance salt solution and left overnight in 4^0C or 2hrs in the incubator. The ECM helps to enhance attachment of the monolayer confluent cells to the plate and prevent complete lifting of the cells of the plate. When ready to proceed with the experiment, the unbound ECM substrate (fibronectin and Collagen) were aspirated off and incubated for 1h at 37°C.

- Sub-confluent cells growing in the T-25 flask were washed once with PBS and re- suspended. Trypsin containing versene was added to the re-suspended cells with the complete media, and the desired cell counts were determined. Cells were dispersed gently by pipetting and/or rocking the well plate before incubation so that cells are distributed equally on the plate.

- The plates were washed once with PBS and cells were seeded at the following density; HEK and Neb-1 cell 600,000 in a 12 well plate and for HDF 90,000 in 24 well plate and the cells were allowed to grow to full monolayer confluent. Optimization experiment was previously performed to determine the seeding density and the time it takes the different cell types to reach full monolayer confluent for the different cell types.

- After HEK and Neb-1 cell has reached a full confluent, the cells were either treated with mitomycin (10µg/ml) or not treated. The treated plates were incubated for 1hr at 37C 5% $C0_2$, after incubation the cells were washed thrice with PBS. Mitomycin treatment is believed to inhibit ongoing cellular proliferation during wound assay experiment. For human dermal fibroblast wound assay, the monolayer was not treated with mitomycin.

- A vertical wound was manually created by scraping the cell monolayer with a p20 pipette tip. The cells were once again washed with PBS thrice to remove detached cell debris from the well. The wounds were created relative to the marking/ reference point on the plate. Fresh media were added to the well plates at varying concentrations of PRP (50%, 25%, 10%, 5%) and PPP respectively. The control experiment was treated with 1% of the full complement of the appropriate media for the different cell types.

- Photo-micrographic documentation of the plated cell was taken at time 0 and at 12hrs, 24hrs and 6hrs intervals or until the gaps was fully closed. The migrated cells were measured and the distance migrated were measured by either exporting the image into image J and or Photoshop. The artificial gap created at 0hr was compared with the distance left uncovered at the endpoint, to calculate the migrated distance.

2.14 Transwell Migration Assay with HEK, NEB-1 cells, HDF and HUVEC

2.14.1 Cell Migration Assay with Modified Boyden Chamber

The Boyden chamber assay (Boyden, 1962) was originally introduced by Boyden for the analysis of leukocyte chemotaxis is based on a chamber of two medium-filled compartments separated by a microporous membrane. Random cell motility is generally described as chemokinesis, which is distinguished from directed cell motility toward increasing concentrations of soluble attractants, such as growth factors (chemotaxis), or along a concentration gradient of extracellular matrix (ECM) proteins for haptotaxis (Carter,1967).

In general, cells are placed in the upper compartment and are allowed to migrate through the pores of the membrane into the lower compartment, in which chemotactic agents are present. After an appropriate incubation time, the membrane between the two compartments is fixed and stained, and the number of cells that have migrated to the lower side of the membrane is determined. Therefore, the Boyden chamber-based cell migration assay has also been called filter membrane migration assay, trans-well migration assay, or chemotaxis assay. Alternatively, the assay is left for a long period and cell that migrated to the bottom of the lower chambers were quantify by the method described above. The method described in this research was intended specifically for measuring the migration of HEK/Neb-1, HDF and HUVEC/EA.hys to the bottom of lower chamber using a 24-well transwell insert after a given time interval 24 hrs

2.14.2 Transwell Methodology

The cell types are grown to 70%- 80% confluent (log phase of cell growth) in readiness for the experiment. The protocol previously described by Boyden et.al 1962**,** was slightly modified because of the peculiarity of the different cell type **(Figure 2.2)**. The transwell permeable support from Corning life science (900, Chelmsford St. Lowell, MA 01851) was used, which has 24 well plates with 12 transwell insert, with 8.0 μm micropore, growth surface area of 0.33cm^2 and membrane diameter 6.mm, made with polycarbonate material.

The cells were seeded at 100,000/well for HEK and Neb-1 cell line, fibroblast 50,000/well and HUVEC/ EA. Hys at 10,000/ well. The cells were trypisinzed with trypsin-EDTA to allow the cells to remain unbound during the migration process and prevent the cells clumping together. The cells were later re-suspended in a media appropriate for the cell type in ration 1:1. Cells seeded at a volume of 100 - 200µl per insert. A separate well plate without filter was used as the control experiment with equal density/volume of cell in the well treated with DMEM with FCS 1%. Varying concentration of PRP (PRP 50%, 25%, 10%, PRP), PPP and control were used for the repeat experiments and as appropriate for the different cell types.

The cells were left to migrate in the incubator over varying period of 6-24hrs. An early experiment performed to optimize the protocol with varying cell density (40,000-400,000 cells) and migrating time showed the following results; the maximum migration time was 24hrs and the cell density for HEK and Neb-1 (100,000/insert), HDF (50,000/insert) and HUVEC/ EA.hys cell (10,000/insert).All experiments were performance in triplicate.

After the end of experiment, the insert was removed; the non- migrated cells left on the membrane were removed with moist cotton wool. The cells that have migrated to the bottom of the well plate and the well-plate without the filter (positive control) were treated with Alamar blue which is 10% of the total volume of the treatment concentration. This was incubated for 16hrs and the 10% of the total volume with Alamar blue was measured in a 96 well plate (VWR, international) reader at 570nm. Experimental repeats were carried out in triplicate.

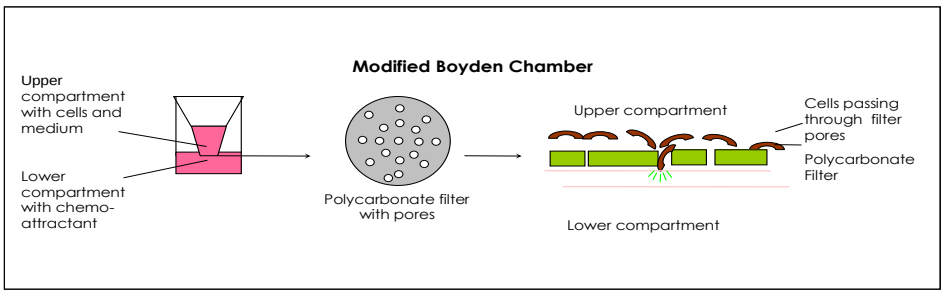


Figure 2.1 A diagrammatic illustration of the modified Boyden Chamber used in this experiment for transwell migration assay. The far left showed the insert seeded with cells (upper chamber) and the lower chamber containing the specific media. The far right showed the mode of cells transmigration through the membrane pores.

A separate well-plate was seeded with the same initial cell density that was seeded into the upper chamber insert, which serve as the positive control, the cells in this well-plate was equally measured with AB technique.

$$\% \text{ Cell migrated per well} = \frac{\text{Average fluorescence/ absorbance in treated well with filter}}{\text{Average Fluorescence/ absorbance in well without the filter}} \times 100$$

Besides measuring the metabolic cell activity through AB technique, the cells that migrated to bottom of the well-plate were stained and counted manually under HPF with light microscope **(Figure 2.4)** . To find the % of the migrated cell, the total migrated stained cell at the bottom of well-plate was divided by the total original cell density which was represented by the cells in the positive control well plate.

$$\text{Percentage Cell migration} = \frac{\text{Total number of stained migrated cell}}{\text{Total original cell density (positive control)}} \times 100$$

2.14.3 Preparation of PFA and Methyl blue for Staining

Eight gram of sterile of Para formaldehyde (PFA) 4% (manufacturer) was weighed and 80mL of 2Molar of Sodium hydroxide was prepared. The Para formaldehyde (PFA) was stored in -4^0C after preparation. Distil water (180ml) was boiled to boiling point, and the prepared PFA was mixed with the boiled distil water in the fume hood, which was followed by the addition of 50mL of 10X PBS. The pH of the mixture was adjusted until a pH of 7.4 was obtained by using the pH reader. 0. 2% (weight for volume) of methyl blue (Sigma, UK) (0.2g) was weighed in a sterile condition. 3.23g of sodium acetate (0.4M) was dissolved in 100mL of distil water, 0.4M of sodium acetic acid (2.3mL was dissolved in 97.7mL of distil water) Sodium acetate and sodium acetic acid were prepared in 100mL solution. 50mL from each solution was mixed together with 0.2g of methyl blue; methyl blue was stored in room temperature after preparation.

PFA (400µl) was added to the migrated cells at the bottom of the well or the membrane in fresh well well-plate; the well-plate was left in the incubator for 10-12minutes and was washed 3 times with PBS. After incubating, 500µl of methyl blue was added to the well after washing and left on the bench for another 4-6minute and the methyl blue was washed off gently under running tape. The

well-plate was left to air dry and then examined under an inverted microscope at x20 high power field.

2.14.3.1 Staining Technique for Membrane and Cells in Well-Plate

- A damp cotton swab was used to wipe the un-migrated cells from the top of the membrane. The insert with the membrane was dipped into the freshly prepared 4% Para formaldhyde for 15-20 min to fix the cell, and washed thrice in x1 PBS, with the side of the membrane with the migrated cells facing down. The insert with the fixed membrane was dipped in methyl blue (the side with migrated cells facing down) for another 5 min. The membrane de-stained by briefly rinsing it in x1 PBS. The excess PBS is drained from the membrane and place (the side with migrated cells facing down) on the Petri dish cover.

- The membrane was cut off from the insert and attached to the slide with a little amount of nail polish to a glass slide (super frost, VW international, UK) and was covered with a cover slip (32 × 24 mm). Another method employed in analysing the migrated cells, was to stain the migrated cells at the bottom of the lower chamber with the same method described above.

- Surveying the stained membrane under a light microscope at 20x and 40x magnification and selecting at least three wells for each experimental group on which the migrated cells are well stained and evenly distributed was an option. Counting the individual cells at the bottom of the lower chambers was easier and easily quantifiable under high power field of x10 or x20. There was no mistaking membrane pores for migrated cells, which was a common mistake with keratinocyte and Neb-1 cell. Micrographs are taken using a digital camera (Nikon COOLPIX995) connected to the microscope at 20x magnification and the cells were counted by moving the microscope in 5 times randomly and the average was calculated.

2.14. 4 Quantification of Transwell Assay

Another method employed in quantifying the amount of migrated cell was based on principle that not all the cells will transmigrate to the bottom of the well, because of the attachment property of the polycarbonated membrane. Some of the cell will attach to the under surface of the membrane and these cells could potentially be fixed, stained and counted. At the end of the transmigration period of 24hrs, the upper chamber (surface) of the transwell insert was cleaned with moist cotton wool, which helps to remove non-migrated cells. The membrane was turned over so that the under surface was now facing upwards and membrane was fixed and stained as per the cell fixing and staining protocol **(See section 2.14.3.1)**. After fixing and staining the membrane, the membrane was then gently placed unto a super frost plus slide (VWR international); a drop of dePex mounting medium (VWR

international) was applied to fix the membrane. The slide was reviewed under HPF light microscope and the stained cells were calculated randomly in 5 places to determine the average cell migrated. The cellular clusters formed makes counting of individual cell practically difficult **(Figure 2.3).** For the purpose of this research, the method of quantifying the cell at the bottom of the well plate through AB technique was favoured over the other 2 above described methods. This was because the AB method provides a more reliable, accurate and reproducible quantifying method and more importantly the chance of human error in counting is completely eliminated.

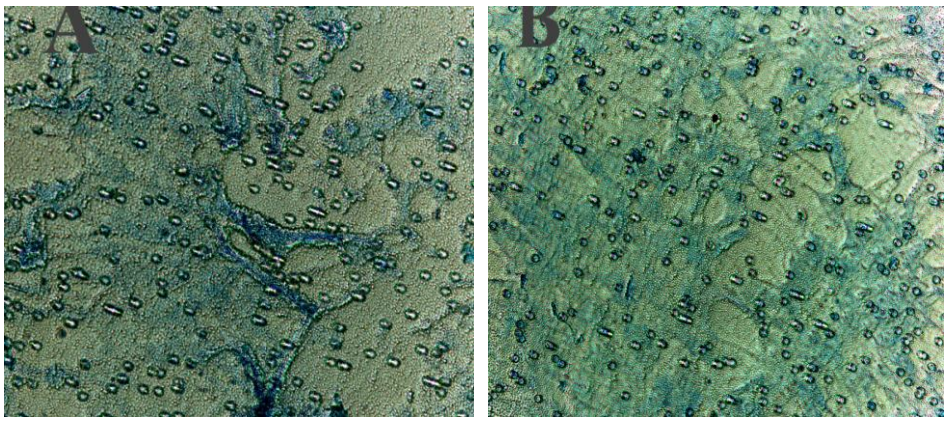


Fig. 2.3 Photomicrograph of stained migrated cells on the undersurfaced of transwell membrane at x20 **(A)** HDF cells formed clusters of cell **(B)** HUVEC also formed complex cellular network. Cells are in densed network, distinct from the uniformed size membrane pores. Manual counting of individual cell was not possible because of cell clustering

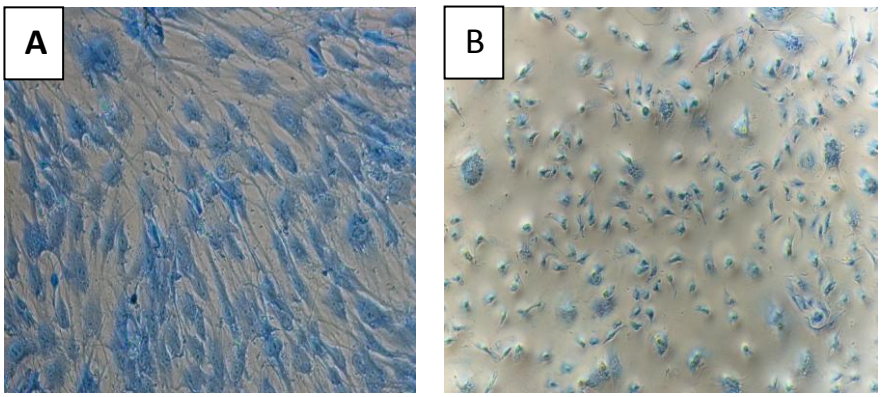


Figure 2.4 Photomicrograph of cells fixed with PFA and methylblue at the bottom of well-plate. (A) HDF at the bottom of the lower chamber (not transwell migrated) . (B) Transwell migrated HDF with a change in morphology.

2.15. Endothelial Angiogenesis

Angiogenesis, or neo-vascularization, is the process of generating new blood vessels derived as extensions from the existing vasculature (Auerbach and Auerbach, 1994). The principal cells involved are endothelial cells, which line all blood vessels and constitute virtually the entirety of capillaries. New blood vessels are formed by vasculogenesis or angiogenesis; vasculogenesis is the process of vessel formation from mesodermal-derived endothelial cells precursors called angioblast, and angiogenesis is remodelling from the primary plexus in the embryo or from pre-existing vasculature (Risau, 1997). Remodelling and pruning process are common to angiogenesis and vasculaogensis during embryonic development; both events are poorly understood but involved the growth of new vessels and regression of others. In adult mammals, physiological angiogenesis takes place only during wound healing and in the female reproductive cycle; its nonetheless has a crucial role in pathological processes, such malignant tumour growth (Meier et al., 2000).

To achieve new blood vessel formation, endothelial cells must first escape from their stable location by breaking through the basement membrane. Once this is achieved, endothelial cells migrate toward an angiogenic stimulus such as might be released from tumour cells, activated lymphocytes, or wound-associated macrophages. Behind this migrating front, endothelial cells proliferate to provide the necessary number of cells for making a new vessel. Subsequent to this proliferation, the new outgrowth of endothelial cells needs to reorganize into a patent three-dimensionally tubular structure. Each of these elements, basement membrane disruption, cell migration, cell proliferation, and tube formation, can be a target for intervention, and each can be tested in vitro. ((Auerbach and Auerbach, 1994).

The central mechanism of angiogenesis involves migration of endothelial cell, vascular tube formation, proteolytic digestion of extracellular matrix, cellular proliferation and connecting of newly formed channels with resultant new vessel formation (Risau, 1997). New insight into specific steps involved in angiogenesis has fostered the development of variety of models in *in vivo* and *in vitro* assay, this tools have been instrumental in the development of quantitative methods of assessing efficacy of therapeutic agents that either up-regulate or down-regulate specific angiogenic mechanism (Auerbach et al., 2003). Angiogenesis needs to be looked at from the view point of intercellular adhesions of endothelial cells, proliferation and endothelial cellular migration (Nehls and Drenckhahn, 1995). Reconstituted basement membrane provides a quick and effective means of studying angiogenesis, thereby circumventing the difficulty faced with the use of fibrin and collagens. Matrigel induces endothelial cells to differentiate as evidence by both the morphogical changes and by reduction in proliferation.

2.15.1 Protocol for Angiogenesis using BD Matrigel™

BD Matrigel™ Matrix Growth Factor Reduced is a solubilised basement membrane preparation extracted from Engelbreth-Holm Swarm (EHS) muse sarcoma, a tumour rich in extracellular matrix protein, purchased from BD Bioscience (OakPark, Bedford, MA 01730). Its major component is laminin, collagen IV, heparin sulphate proteoglycans, entacin nidogen (Kleinman et.al 1982). BD Matrigel Matrix Growth Factor Reduced also contains TGF-Beta, epidermal growth factor (EGF), insulin-growth factor (IGF), fibroblast growth factor (FGF), tissue plasminogen activator (Kleinman et.al 1986) and other growth factors which occurred naturally in EHS tumours. BD Matrigel reduced growth factor provides the substrate necessary for the study of angiogenesis both in vivo and in vitro (Vukicevic, 1992). BD Matrigel Matrix Growth factor Reduced was developed to enable reconstitution of basement membrane preparation purified and characterized to a greater extent than BD Matrigel Matrix. The method of preparation effectively reduced the level of a variety of growth factors except for TGF-beta which may be bound to collagen IV. The major components: laminin, collagen IV and entactin are conserved by the process whilst heparin sulphate proteoglycan is reduced by 40-50%. The table below compares the level of growth factors in the reduced BD Matrigel Matrix to BD Matrigel Matrix.

- BD Matrigel Matrix Growth Factor Reduced may show colour variation, in frozen state (straw yellow colour) or thaw (dark red) , this is due to the interaction of carbon dioxide with the bicarbonate buffer and phenol red , this variation does not affect product efficacy and it disappear upon equilibration with 5% CO^2 .

- BD matrigel was allowed to thaw overnight on 4^0C ice. After thawing, the vial was kept on ice in a sterile tissue culture hood. For the purpose of my experiment, the original BD Matrigel (10mL) was diluted with serum free DMEM media in a ratio 1: 3, to make a total of 30ml of BD Matrigel. Two millimetre of reconstituted BD Matrigel was aliquot into pre-cooled eppendoff, using a pre-cooled pipette and stored in -20^0C to avoid repeated process of thawing.

- A 24-well plate was pre-cooled for optimisation at -20^0c and the plate was kept on ice during the coating process. Wells required for experiment were coated with BD matrigel whilst the plate was on ice. The BD matrigel was mixed to homogeneity and suspended gently into the plate to prevent bubble formation, 175µL of BD Matrigel / cm^2 of growth surface; for example a 24 well-plate has a growth surface area of is $2cm^2$, which makes a total of

350µL/ well. The well plate was placed in the incubator at 37^0C for 40minute, which allows for gel polymerization.

- Whilst the matrigel coated plate incubates, cultured HUVEC were trypsinized and counted according to earlier described protocol. An initial experiment was initially carried out to determine the optimal seeding density for subsequent experiments. HUVEC were seeded at varying densities of 50,000, 100,000,150,000 and 200,000 / well-plate.

- The cells were suspended in 400 µL of media and additional 400 µL of EGM-2 was added to each well. The experiment was carried out in triplicate. The plate was then incubated 37^0 C with 5% CO_2. The plates were examined at 6hrs, 12hrs, and 24hrs and 36hrs interval respectively to determine the optimal time for the experiment. Photographs were taken at this time point for documentation and a comparison of the morphological changes at different time point was made.

- After optimization, the best result was obtained with 200,000 cells per well-plate after 36hrs. Subsequent repeat experiments were repeated in triplicates using full complement 1% EGM-2 as the positive control (which basically represents reconstituted EGM-2; which means a reduction in the recommended 10ml fetal calf serum(FCS) to 1ml and all the growth factors supplement which form part of a full EGM-2 complement were added (VEGF, R3-IGF-1, hFGF-B and h-EGF). This represents reconstituted 1% EGM-2. The 1% EMG-2 has been previously tested with HUVEC proliferation and the HUVEC cells survived in this media for 5 days, before apoptosis sets in.

- Other experimental repeats were conducted with PRP 50%. PRP 25%, PRP 10%, PRP 5% and PPP respectively. In this case, only the endothelia cell basal medium was used as the tissue culture media without the growth factor supplement. The PRP was reconstituted at 50%, 25%, 10% and 5% volume/ volume of the endothelia cell basal medium. On the other hand PPP was reconstituted has 50% (v/v) of the endothelial cell basal medium.

- The end point was set at 36hrs, after the time, the media and the gel in the well were aspirated off and the HUVEC which have been confirmed to form angiogenetic pattern were stained with method using paraformaldehyde and methlyene-blue (**see 2.14.3 for the details of the staining technique**). This allows for easy visualization of the cellular network pattern and more appropriate quantification.

Parameter	BD Matrigel Matrix	BD Matrigel Matrix Growth Factor Reduced
bFGF(pg/ml)[4]	0-0.1	0-0.1
EGF (ng/ml)	0.5-1.3	<0.5
IGF-1(ng/ml)	15.6	5
PDGF(pg/ml	12	<5
NGF(ng/ml)	<0.2	<0.2
TGF-beta (ng/ml)	2.3	1.7
%Protein that gels	80	83

Table 2. 4. Comparing the levels of growth factors available in BD matrigel with BD matrix growth factor reduced. Table excerpt from the BD science

2.15.2 Angiogenesis Assay using Collagen

Collagen is a fibrous protein that consists of three α-chains which can combine to form a rope like triple helix, proving tensile strength to the extracellular matrix. The chains contain GXY repeats: glycine (G) is a small amino acid that fits well with the triple helix. X and the Y are typically proline and hydroxyproline, which is critical for collagen stability. Type I (most common fibrillary collagen 90%) is found in most tissue and organs, but most abundant in dermis, tendon and bones other connective tissue. Type 1 molecule is heterotrimer ($alpha_1$ and $alpha_2$) of 300nm length being composed of two alpha1 chains and one alpha 2 chains. Collagen binding intergrin receptors are $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$. Collagen facilitates in vitro cell culture adaptation and enhances expression of cell-specific morphology and function, when used as a gel. Other use includes promotion of attachment, tumour cell invasion and migration, fibrillogeneis studies and autographic studies of granulocytes and macrophages.

2.15.2.1 Preparation of Collagen Gel for Angiogenesis Assay

The angiogenesis model based on rat tail collagen type I (BD Biosciences Two Oak Park, Bedford, MA) was used after optimisation of the protocol. The original total concentration of the collagen was 2.14mg/ml (Volume of 100ml). To determine the volume of collagen 1 needed, a sterile 10X phosphate buffer (PBS), sterile distilled water (dH_2O) and sterile 1 molar of $NaOH_2$ on ice were equally prepared. The recommended volume of collagen to produce a firm gel is 4mg/ml. The Following equation was employed to determine the concentrations of dH_2O, PBS, NaOH and collagen required for the experiment.

Volume of dH_2O needed (V4) = Total volume (V) - (V1+V2+V3)

Volume of collagen needed (V1) = $\frac{\text{Final concentration of collagen x total volume}}{\text{Initial concentration of collagen}}$

Volume of 10xPBS needed (V2) = $\frac{\text{Total Volume (V)}}{}$

10

Volume of 1M NaOH needed (V3) = Final volume of collagen needed (V1) x 0.0025

- After determining the individual volume needed for dH_2O, the 1 molar $NaOH_2$ and PBS x10 is mixed in a sterile tube. Collagen is slowly pipette into this mixture and mixed well, pH of 7 was the optimal pH desired to produce the perfect gel, this was ensued by checking with a pH meter, this was titrated with a base or acid to achieve the desired pH.

- After sub-culturing and determining the needed cell density, $2x10^5$ of HUVEC were suspended in 100 µL of EGM-2 and 212µL collagen on a 24 well- plate, this was allowed to polymerize in an incubator for 40minute at 37^0C of 5% CO_2. After the incubation period, 1% EPES –BSS (6 µL) was added to 282µL of EGM-2, which was then added to the mixture of HUVEC and collagen gel.

- The positive control for the experiment was carried out using full complement EMG-2; the negative control was done using varying concentration of DMSO at 1% and 5% respectively as substitute for HEPES-BSS. The assay was also tested with varying concentration of PRP at 10%, 25% and50% respectively, 1% reconstituted EMG-2 for the control experiment (a similar concentration was used as earlier described for matrigel angiogenesis assay).

- The experiment was monitored over 7 days, but the plates were inspected daily for the formation of capillary network formation. Once satisfactory capillary network was formed the experiment was discontinued and the same protocol employed for fixing and staining the capillary network in matrigel BD was employed.

2.15.3 Analysis of Angiogenesis Assay based on Morphometeric and Topological Parameters

Following the completion of the angiogenesis assay, the cultured cells where fixed in araformaldhyde (PFA) and stained with methylene blue and which allows for a contrasting back ground. The phase-contrast images of the endothelial cell cultures were captured using a reverted phase –contract light microscope connected to a KS-300 system (Zeiss) at x10, and saved as JPEG files. This software allowed a control of the bright field illumination of the microscope to acquire images of comparable mean brightness. These images were processed and analyzed using computer-assisted image analysis software KS400 (Zeiss), on a customized written macro designed program specifically for this study. The steps involved in processing the captured images are as follows:

1. A gradient detection algorithm was applied to the image; this was for enhancing the edges in an image. It produces a black & white image that has dark areas where the original image has smooth areas, and light areas where the original image has areas of rapidly varying intensity (particularly the near edges).

2. Binary thresholding was achieved by selecting pixels of a given intensity in each of the three (Red, Green and Blue) RGB color components. Thresholding was intended to reduce the vast information content of a coloured or gray-scale image, while at the same time ensuring that the features of interest remain recognizable. The threshold levels are set in such a way that the features of interest are assigned to "on" (or white) pixels in a binary image, while assigning all other pixels (the background) that are deemed to lack specimen information to the "off" (or black) state. It allowed a good identification of the cell profiles present in the field. A single set of threshold levels was selected interactively and then applied to all other images because they were captured under identical conditions. In order to remove artefacts from the resulting binary images as well as profiles corresponding to isolate inactivated cells, a geometric filter was applied, selecting only thresholded regions with an area greater than 400 pixels and circularity (Russ, 1995) less than 0.6. This process produced a network of tubules and interconnected cell clusters.

3. Furthermore, with the use of binary thinning procedures, the networks were skeletonised and pruned to remove small artefactual branches (Seul, et al., 2002). Skeletonization is a process for reducing foreground regions in a binary image to a skeletal remnant that largely preserves the extent and connectivity of the original region while throwing away most of the original foreground pixels. Skeletonization is the morphological thinning that successively erodes away pixels from the boundary until all that remains is line segments one pixel in thickness (**Figure 2.5).**

4. The endothelial cell organisation was characterised by measuring the following variables; percentage area covered and the total length of the network. Size parameters are insufficient to fully characterize the complex pattern of endothelial cell network formation. The binary profiles of very similar size can be structurally quite different and create a different spatial texture.

5. Additional topological analysis of the skeletonised binary images was performed to identify branching points (nodes) and cellular area in the zones image surrounded on all sides by skeletonised lines. Nodes are located by simply locating pixels of the skeleton with more than two neighbours (Russ, 1995). The meshes are identified by algorithms which eliminates area touching

one or more sides of the image (Seul et al, 2000). The number of nodes and meshes per field were determined.

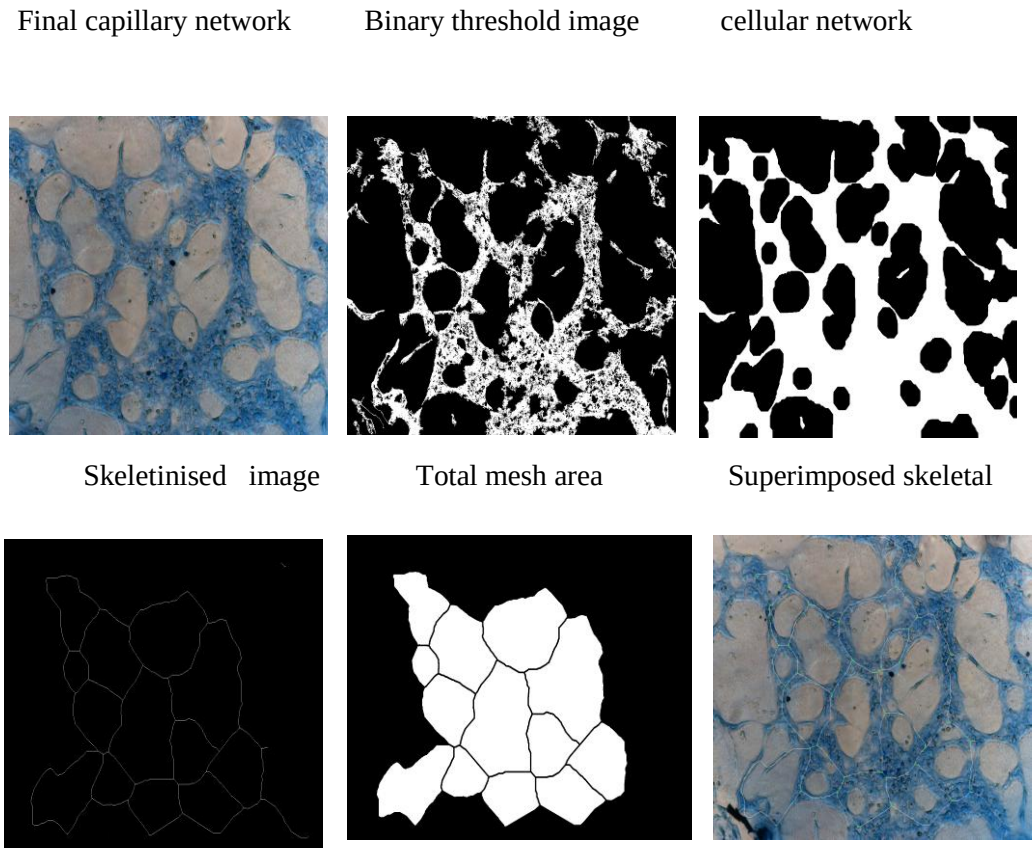


Figure 2.5 Steps involved in the processing of a final photodocumented HUVEC angiogenesis assay image. The image was processed by the KS400 Marco soft programme, which seperates the image into different components and allows for easy quantification.

2.16 Immunoassay Quantification

Growths factors can be quantified by using bio-immunoassay techniques that allow for accurate measurement of the specific growth factor. In this study we employed the use of solid phase immunoassay technique (Luminex Xmap) and ELISA (Enzyme linked immunoabsorbent assay technique) in measuring growths from PRP/ PPP. The two techniques operates on very similar principles, however Luminex has an advantage over the traditional ELISA method. Luminex has the capacity of performing a variety of bioassays on the surface of fluorescent-coded beads known as microspheres. It also has the advantage of reducing time, labour and costs over the traditional method.

2.16. 1 Principle of ELISA Assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for the growth factor of interest has been pre-coated onto a micro-plate. Standards and samples diluent are pipetted into the wells and any proportion of the specific growth factor present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for the growth factor of interest was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, substrate solutions were added to the wells and a colour develops in proportion to the amount of the specific growth factor bound in the initial step. The colour development was stopped and the intensity of the colour was measured through a microplate reader, the optical density obtained by measuring the wave length of the colour change was used in plotting the standard curve which helps in the determining the final concentrations.

2.16. 2 ELISA Methodology

1. All reagents are brought to room temperature before use. All samples, standards and controls are assayed in duplicate. All reagents, working standards, wash buffers and samples were prepared according to manufacturers' instructions before this stage. The procedure described in this thesis was according to the manufacturer instructions- (R&D Systems, 614 McKinley Place NE Minneapolis, MN 55413 United States of America).

2. After mixing the diluents properly, 100 µL was added to each. This was followed by the addition of 100 µL of Standard, control, or sample to each well per well and the well was covered with adhesive strip. The well was incubated for between 2-3 hours depending on the manufacturer instruction for the particular growth factor.

3. Each well was aspirated and washed, this process was repeated thrice for a total of four washes. Each well was washed with 400 µL wash buffer using a squirt bottle. Complete removal of liquid at each step is essential to good performance. After the last wash, any remaining wash buffer was removed by aspirating or decanting. The plate was later inverted and blotted against a clean paper towels.

4. Detection antibody (specific growth factor conjugate) at 200µL was added to each well. The well was covered with a new adhesive strip and incubated for 1 hour at room temperature. After incubation aspiration/wash process was repeated again as described above.

5. Substrate solution of 200µL was added to each well, at this stage the wells are photosensitive, and hence they are protected from light with aluminium foil, the well was further incubated yet another 30 minutes at room temperature. Furthermore, a 50µL of stop solution was added to each well, at this stage a colour change was noticed in the well, this colour depends on the specific growth factor been assay. If no uniform colour occurs, the plate was tapped gently to ensure thorough mixing.

6. The optical density of each well was determined within 30 minutes, using a microplate reader set to 540 nm and 450 nm. By subtracting the readings at 450 nm from 540 nm, the optical imperfection in the plate was corrected. The optical density was sued to plot the standard curves from which the concentration of the growth factor was derived.

2.17 Principle of Luminex ® Xmap

Milliplex is based on the principles of luminex technology. Luminex uses proprietary techniques to internally colour code microspheres with two fluorescent dyes. Through the precise concentration of these dyes, 100 distinctly coloured bead sets can be created, each of which is coated with a specific capture antibody. The capability of adding multiple conjugated beads to each sample, results in the ability to obtain multiple results from each sample. This technique enables multiplexing of many types of bioassays, reduces time, labour and cost over the traditional methods of immunoassay.

After an analyte from a test sample is captured by the bead, a biotinlylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. The microspheres pass rapidly through a laser which excites the internal dyes marking the microspheres set. A second laser excited PE, the florescent dye on the reporter molecule. Finally, high speed digital-signal processors identify each individual microsphere and quantify the results of its bioassay based on fluorescent reporter signals (www.millpore.com/techilibrary/index.do)

Briefly standards of known concentration and PRP/PPP samples were added to a microwell plate with an antibody against each factor. Any growth factor present was bound by the immobilized receptor. After any unbound substances were rinsed away, an enzyme-linked polyclonal antibody specific for each growth factor was added to the wells. After a second wash, a substrate solution was added, and the colour developed in proportion to the amount of bound growth factor in the first step.

The colour development was stopped, and the intensity of the colour was measured by running the plates on the Luminex 100™ and the median fluorescent intensity data obtained was analysed. Samples were initially diluted 1:10 in assay buffer, and retested at higher dilutions if the median fluorescent intensity (MFI) was higher than the value of the top standard. Samples were diluted 1:100 for these assays in the kit diluents. The concentrations of TGF-β1, EGF, VEGF, FGF-2, PDGF-AA and TGF-β1 were measured with the MILLPLEX ™ MAP kit. All samples were tested in duplicate. Manufacturer's control was available and run and was found to fall in the range indicated for the kit. Standards and samples were assayed in duplicate, and mean values were calculated. The results were multiplied by the dilution factor applied to the samples.

2.18 Study Design for the Clinical Pilot Study

2.18.1 Study Design

The study was conducted at the Royal London Hospital between May 2008 and May 2009, in accordance with the Declaration of Helsinki. Ethical approval was obtained from the City and East London research and ethic committee, with 08/H0704/17. Informed written consent was obtained from each patient before entry to the study.

2.18.2 Patient Recruitment Process

Diabetic patients were recruited from diabetic and vascular clinics at the Royal London Hospital, Mile End Diabetic Clinic, and Newham Healthcare Services, as approved by a central office for National Research Ethic Service (NRES). Patients were provided with a full explanation of the nature, purpose and requirement of the study including information sheets and informed consents forms. Patients were invited to participate in a screening evaluation, which will include a medical history, physical examination of the ulcer and the affected limb, and blood tests. Results of the screening evaluation will determine eligibility for entry into the study. Patients with active wound infection were treated by surgical debridement. Surgical debridement helps to reduce the bacterial load, remove necrotic tissue and ultimately convert chronic wound to acute wound and provide an environment were autologous derived platelet rich plasma gel could be effectively apply.

2.18.3 Pilot Study Design

In the pilot study, five patients were to be recruited into the treatment and control group alike, which should be sufficient to establish our hypothesis. The patients will be randomly assigned to either Active or Placebo treatment. The active agent was platelet rich plasma and the placebo is platelet poor plasma. The platelet gels are topically applied to the wounds, which will be followed by the trust conventional dressing protocol, moist dressing is preferred to prevent the gel from been absorbed into the dressing material **(see Appendix for wound dressing protocol)**. At the end of the

study, patients with non-healing wounds were returned to routine clinical care and best wound care practice. However, we could not recruit sufficient patients into different arms as planned, 7 patients were recruited in total, 1 dropped out at the end of week 3 week and the other dropped out at 5 week because of wound sepsis and a definitive treatment to save the patient's life was implemented. After the initial surgical wound debridement, punch wound biopsy (6mm) was taken before treatment. Accurate wound measurement was taken with the aid of a tracing planimetry and also a photographic documentation of the wound size before commencing treatment. Photo-documentation to determine the rate of wound closure was taken during each clinic visit.

2.18.3.1 Inclusion Criteria

In order to be eligible to enter the study, patients must meet the following criteria.

- A patient with documented Type I or Type II diabetes mellitus of not less than 2 years duration.
- Only patients that are on pharmacotherapy treatment for diabetes mellitus
- Only one ulcer on the foot of the Lower limb
- The ulcer should have a surface area of between $2cm^2 – 40cm^2$.
- The treatment is specific to clinically non-infected full thickness wounds
- Ulcer present for a minimum of 4 weeks or more
- Wounds extending through the dermis and may involve tendon, muscle or bone
- The wound must have failed to respond to intensive conventional treatment.
- The patient should be competent to give consent and have an appropriate support system to facilitate his/her full participation in the follow-up care associated with the treatment of the wound.
- An ankle brachial pressure index value of ≥ 0.8

2.18.3.2 Exclusion Criteria

- Patients with clinical evidence of platelet dysfunction and thrombocytopenia < 100,000.
- Patients with documented malignant disease and connective tissues disease.
- Patients with who are not ambulant from other reason other than the poor mobility due to the ulcer, except inpatients, will not be included.
- Any other significant pathology, which might impair healing other than diabetes such as end stage renal impairment.

- Patients with clinically documented Hepatitis B & C infection and HIV.
- Patients with documented MRSA in wound infections.
- Patients with poor prognosis associated with other disease processes.
- Ulcers from any cause other than diabetes will be excluded such as pressure sore.

2. 18.3 3 Patients

Patients with one ulcer on the foot were enrolled into the study from recruitment from vascular clinic or diabetic foot health clinic. The diabetic foot ulcers were classified according to the American Diabetic Association. Patients who fit with entry and exclusion criteria were treated. All patients had surgical wound debridement before been treated. Patient that require admission were admitted on the ward and input of the diabetologist were employed in glycaemia control. Concomitant medications for other illnesses were continued. Patients were followed up in vascular clinic after the first treatment at regular interval. During follow up digital photograph of the wound and computerised planimetry (Smithkline, see section on material and methodology for details on computerised planimetry) measurement were taken at every clinic visit.

Patients were initially followed up weekly for the first one month and then bi-weekly until complete wound healing is achieved or until 16 weeks. Wound biopsy was taken from the wound margin after the initial debridement on day 0 and subsequently at day 8, week 4 and week 8. The biopsy samples are preserved in formalin saline or snap frozen in liquid nitrogen. The inclusion and exclusion criteria have been discussed under materials and methodology.

2.18.3.4 Ulcers

The ulcers were adequately described and characterised with respect to its size, depth, appearance, and location, which helps in mapping wound healing progress. After describing the dimensions and appearance of the ulcer, the research doctor examined the ulcer by gently probing to detect sinus tract formation, undermining of ulcer margins, and involvement of tendon sheaths, bone, or joints. In addition, the existence of odour and exudates, and extent of cellulitis are noted and documented. This detailed examination forms the bases for clinical classification of the ulcer. The vascular status was assessed by measuring ankle brachial pressure index (ABPI), which is expected to be between 0.8-1 in non-ischemic ulcers. However some of the patients had an element of microvascular disease as well as calcification of the vessel, which may give a false ABPI reading.

The presence of lower limb pulses and capillary refill are routinely documented. However, the absence of pedal pulses in the presence of a palpable popliteal pulse is a classic finding in diabetic

arterial disease because of the selective involvement of the tibial arteries below the knee in peripheral vascular disease. Non-invasive Doppler signals were also used to augment the clinical examination. Cultures are taken from purulent drainage or curetted material from the ulcer base. Classification of ulcerations can facilitate a logical approach to treatment and aid in the prediction of outcome. In this study a standardized Wagner classification was employed.

2.18.3.5 Classification of Ulcer

Several wound classification systems have been described, based on parameters such as extent of infection, neuropathy, ischemia, depth or extent of tissue loss, and location. The most widely accepted classification system for diabetic foot ulcers is the Wagner ulcer classification system, which is based on the depth of penetration, the presence of osteomyelitis or gangrene, and the extent of tissue necrosis. The main drawback of this classification is that, the severity of arterial insufficiency can be underestimated. Wagner classified diabetic ulcers as follows: Grade 0 -No open lesions; may have deformity or cellulitis; Grade 1- Superficial diabetic ulcer (partial or full thickness); Grade 2- Ulcer extension to ligament, tendon, joint capsule, or deep fascia without abscess or osteomyelitis; Grade 3- Deep ulcer with abscess, osteomyelitis, or joint sepsis; Grade 4- Gangrene localized to a portion of forefoot or heel and Grade 5- Extensive gangrenous involvement of the entire foot (**Wagner, 1987**). Most of the patients recruited into the study had a minimum of grade 3 ulcers, which meant that these patients required wound surgical debridement.

2.18.3.6 Wound Debridement

Wound debridement was carried out under general anaesthesia by the vascular surgeon in charge of the patient overall care. A mainstay of ulcer management is debridement of all necrotic, callus, and fibrous tissue. Certain evidence suggests that the best results are obtained with aggressive wound debridement, a process which removes senescent fibroblast and pathogens (Steed DL, 1996). Necrotic tissues are removed by sharply debriding back to bleeding tissue to allow full visualization of the extent of the ulcer and detect underlying abscesses or sinuses. In this study, patients wound were debrided and a digital amputation was performed in the presence of gangrene and spreading infection. Patients with grade 2 ulcers had non- surgical wound debridement; this is a form of sharp debridement on the non-sensate foot carried in the outpatient clinic. After the debridement, the wound was dried with gauze and ready for platelet gel therapy application.

2.18.4 Technique for Wound Measurement

The patients that were recruited for the research had chronic and complex wound. The process or rate of wound healing was monitored by physiological and biochemical means but also geometrical wound measurement was also employed.

VISITRAK (Smith &nephew, Healthcare house, Hull, U.K) is a portable digital device that provides accurate, reproducible data for tracking wound progress. By providing a standardised approach to wound measurement, it promotes best practice in wound management. This device has 3 components.

2.18.4.1 Steps Involved in Measuring Wound with Visitrak.

- Following wound debridement, the wound was cleaned to dryness, to allow its edges to be visible. The white backing layer was removed from the visitrak and the sterile grid was placed over the wound.

- The wound edges were traced with the use of a permanent maker pen. Care was taken to ensure that the area remain within the grid area d the visitrak grid film. The perimeter of the viable (areas of epithelisation or granulation) or non viable tissue (necrotic or sloughy areas) was traced out. The traced out shapes of the wounds are often complex shapes.

- The contaminated layer of the grid was removed and disposed off. The transparent grid was then transfer to the visitrak digital for reading. Patient identification was inputted unto the visitark digital for identification purposes and the area of the wound was displaced as a number in centimetre square.

2.18.4.2VISITRAK Digital

This is a convenient and portable digital tablet which is easy to handle, provides a quick, convenient and accurate area measurement. Once the wound tracing is completed, the device emits an audible beep. The immediate default result is the wound area, but other functions are also provided to allow calculation of percentage wound area that is necrotic (percentage of the wound that is non viable) and width and length measurements.

2.18.4.3 VISITRAK Grid

A tracing film (visitrak grid) has been specially designed as a three-layer pack to minimise the risk of cross-contamination and secondary infection. The patient contact layer is sterile, and the clean layer was used to record wound measurements which can be stored in patient records. The conformable film is easy to draw on and transparent, which makes it easy to see the wound through the film.

2.18.4.3 VISITRAK Depth

The disposable depth indicator is a sterile probe, which allows for easy measurement of wound dept in millimetres.

Visitrak Grid Visitrak digital device Visitrak depth indicator

Figure 2.6 visitrak devices for measuring wound area

2.18.4.4 Advantages of Routine Wound Measurement

Systematic assessment of a patient's wound is one of the most important means of optimising their chances of wound closure. By gathering information about the wound over time, a realistic and clearly defined management plan can be agreed upon. It has been shown that early identification of wounds that are not responding to treatment ensures the best possible outcome. This is because as the treatment regime is re-evaluated, more appropriate interventions can be initiated as necessary. This is particularly relevant to diabetic patient were glycaemic control could be closely monitored, treating stenotic lesion in the lower limb vessel runs The more accurate the rate of wound healing is documented, the faster non-responsive wounds will be identified. There is now considerable published evidence that suggest that accurate percentage reduction in wound area is a useful measurement within the first few weeks of treatment, specifically, a percentage area reduction of less

than 20-40% over the first two to four weeks is a reasonable indicator that the wound is showing a poor response to the treatment.

Visitrak has a number of benefits, including accuracy, flexibility, simplicity and reassurance. There are many methods of tracking wound progress. These include length and width measurement and counting grid squares. However, the most accurate method is to measure changes in precise wound area (www.Smith-nephew.com).

2.18. 5 Protocol for Wound Care

After treating the patient's ulcer according to APG treatment protocol **(See appendix and chapter 6)** the patients were encouraged to wear an offloading orthosis walker (medis shoe) to enhance easy mobility and pressure offloading after treatment. Some of the patients were followed up in the podiatry foot clinic; this was to ensure patient compliance and to allow for repeated non-surgical debridement.

For the purpose of this research, biological wound dressing agents or devices that could aid wound healing were not used for wound dressing **(See wound dressing protocol in Appendix 5).** Wounds were initially inspected weekly for the first month and then bi-weekly until complete wound closure was achieved. Wounds with poor clinical signs of healing might require early re-application of platelet gel as recommended by the research doctor. Wound dressing will only be changed 5 days after the first application of the treatment except when otherwise stated. The second treatment will only be done 14 days after initial treatment when indicated. The second course of treatment may be carried out before day 14 only when otherwise stated by the research clinician. The size of the wound was serially monitored until complete wound closure or treatment is deemed to have failed (end of treatment which was stipulated as 16 weeks of follow up). The decision to perform further wound dedridement and platelet gel application after the first treatment depends on the clinical response elicited from the wound and this is decision was only made the research team.

2.18.6 Procotol for Growth factor Measurement

Six male diabetic patients with chronic foot ulcers, age ranging from 45 to 68 years as well as fourteen healthy volunteers (5 female and 9 male), age range of 18- 40 years were recruited. Ethic approval was obtained from the East and City of London Research Ethic Committee and informed consent was obtained from the volunteers and patient. Platelet count from the whole blood and autologous derived platelet concentrate from the diabetic patients was analyzed. However platelet count was not measured in the healthy donors this was because the result obtained from the diabetic

group provided a template needed. The initial research design was to measure platelet count from both the diabetic patients and health volunteers. After the initially analysis of the result from the diabetic group, the result showed that the diabetic patient in spite of their other medical co morbidity, they have sufficient thrombocyte count (100,000-300,000/μl), which generated good quality platelet gel. In addition, the blood samples collected from the diabetic patients were used to validate the concentrating capacity of the point-of-care device used in this study (Angle Sorin). The concentrating ability was found to be adequate and within the manufacturer specific, hence we desired not to measure platelets count from the healthy volunteer group.

Growth factor levels from PRP and PPP in both diabetic patients and healthy donors were measured. The following growth factors were analyzed using the traditional ELISA technique; P-seletin, Platelet factor 4 and thrombospodin -1 and IGF whilst the remaining GFs were measured using multiplex fluorescent immunoassay technique (MFI). MFI has a few advantages over ELISA in that it has increased sensitivity, specificity, better reproducibility. The GFs measured with ELISA were not available on the commercial multiplex kit. However, in a study conducted by research animal diagnostic laboratory (RADIL), they showed a correlation of greater than 99.5% existed between MFI and ELISA techniques.

2.18.6.1 Protocol Amendment for Voluntary Donation of Blood

The purpose of this amendment is principally to enable compliance with the recent developments and guidance of the Human Tissue Act 2004; which has recently come into force. Normally the collection of blood for diagnostic, validation and calibration purposes was viewed as outside of the Research Ethics Process. Now that assumption is questionable. To ensure compliance we request that we are able to recruit a further maximum of twenty Healthy Volunteers (both male and female) which comprise of a "NON-TREATMENT" group. They have no ulcer or wound, they will not enter the main study and their participation is restricted to the provision of a raw material – fresh blood. A stand-alone consent form was prepared for this purpose. Volunteers that have participated or planning to participate in another clinical research study judged by the research team as conflicting or indicating "over-volunteering" were not recruited into the study.

2.18.6.2 Inclusion/Exclusion Criteria for Voluntary Blood Donation:

- Maximum volunteer needed **20**
- Duration of extended cohort and APG study – Completion on 1yr
- Male and Female

- Adult – Aged between 18 and 40 years.
- Healthy Volunteer.
- Not on any medication known to have a vasoactive or anti-platelet effect.
- Volume of blood collected **300ml of one occasion.**

2.19 Blood collection and Preparation of PRP

This study was approved by the City and East of London Ethic Research committee **(08/H0704/17)**. Fourteen healthy volunteers aged 20-45 (mean age, ration of Male: Female) and 6 diabetic patients were recruited into the study. The volunteer were informed of the purpose and procedure of the study and they were provided with written informed consent. One hundred and fifty millilitre of venous blood was taken from each person and was immediately mixed with 30mL of acid-citrate –dextrose (Sigma-Aldrich Co. St.Loius MO). The whole blood was processed according to the procedure described in **Appendix 4** using the Sorin Angel platelet separating system. At the end of the process the platelet separating system, separates the whole blood into plasma (platelet poor plasma, the red cell and the platelet rich concentrate. Before and after the preparation of PRP, an aliquot was removed and the platelets in the whole blood and PRP were counted (using Beckman and Coulter). 150ml whole blood will yield a harvest of 12-15ml of platelet rich concentrate.

2.19.1 Preparation of Platelet –Rich Plasma

The PRP was processed in a sterile environment. Six millimetres anticoagulant citrate dextrose–A (ACD-A) solution was drawn into 60-cc syringe, followed by 54 cubic centimetre of whole blood drawn from a large antecubital vein. The acid citrate dextrose serves to preserve the integrity of the platelet membrane. The blood was gently agitated by mixing the anticoagulant with the blood. The 60 ml of blood was injected into the angel system and centrifuged for 25 minute (first hard spin) at 5200rpm, followed by a soft spin at 3200rpm for another 45 minute. The whole blood was sequestered into a semiautomatic controlled operation mode by centrifugation, through the whole blood separation processing system (Angel; Sorin Group, Mirandola, Italy), which separates the blood into PRP which contains platelet concentrate and leukocyte, PPP and erythrocytes. The desired end product (PRP) is usually 10% of the starting volume of the whole blood.

2.19.2 PRP and PPP Activation

To initiate the release of the GFs from PRP, the platelets have to be activated, using the potent platelet activator thrombin. Human thrombin was generated from a commercial thermogenesis which consists of [66% volume for volume of Ethyl Alcohol, U.SP.25 Mm Calcium Chloride U.S.P], stored at between 15– 30℃, as a kit called "Activat" [Sorin Group, Mirandola Modena, Italy 41037]. Thrombin was generated after 12ml of PPP was mixed with the Activat beaded material in a pressurized syringe for about 20-25minutes and the thrombin was squeezed out of the syringe, which generates a total of 4-6ml. Autologous thrombin is preferred in the United Kingdom to pre-prepared bovine thrombin, which has been implicated in the development of antibodies to clotting factor Va, XI and thrombin. The harvested PRP was combined with thrombin for platelet activation to produce the gelatinous material in ratio of 1: 10 [volume/volume]. Similarly, the PPP was activated using the thrombin in the same ratio. Following activation the formed gelatinous material was left in a 15ml falcon tube over night at 4℃, this allow for maximum clot retraction **(Figure 2.7)**. The supernatant from the gelatinous coagulum is removed after centrifuging for 10min at 3000rmp and was stored at -80℃, until GF analysis. The supernatant is much easier to handle when performing the ELISA as compared with gelatinous coagulum, hence in our research supernatant was the preferred serum in performing immunoassay and in tissue culture assay.

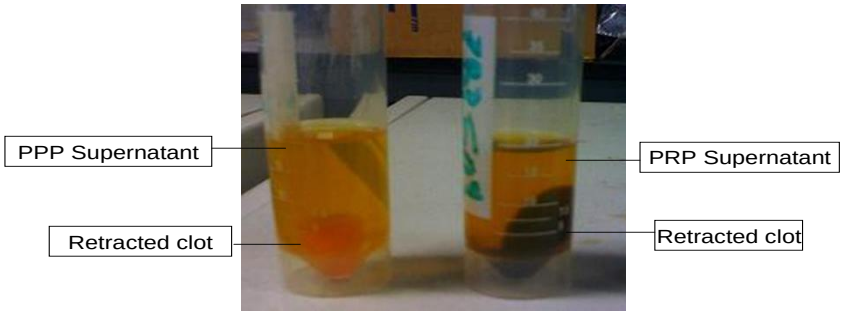


Figure 2.7 The separation of the supernatant and retracted clot from the PRP and PPP was achieved by leaving the PRP and PPP to stand overnight in a 0-4^{0}C refrigerator, followed by centrifuging for 3000g for10min before harvesting the supernatant.

2.19.3 Haematology Analysis of Whole blood and Platelet –Rich Plasma

Blood samples from diabetic patients (5ml of whole blood and or 3-5ml of inactivated PRP) were retained in EDTA bottles and allow mixing properly. Each sample was counted in triplicate and then average (mean +/-SD). Complete blood count was carried out with the Beckman Coulter LH750 analyzer by the Haematology department of Barts and the London London Hospitals NHS Trust. This step was repeated for the platelet-rich plasma samples from the diabetic patient.

2.19.4 Pitfalls in Autologous Derived Platelet Rich Plasma Gel Production

Centrifugation forms the basis of the current methods for producing platelet-rich plasma. From the drawn whole blood, platelet fragmentation should be avoided during this process, which could result in the untimely release of high levels of proteins with compromised bioactivity. A large bore cannular (17G) is recommended for drawing the blood and possibly from a large anticubital vein under no tension. Veno-puncture done under tension could activate the platelet before the centrifugation, which may negate the yield. Low gravity force during centrifugation and minimal platelet activation should be used.

Despite all the efforts to prevent activation, bioactive secretory proteins could still be produced properly, but lost during transferring to the surgical bed. This failure is largely due to the delivery technique used. It is important to achieve a good quality gel; the gel serves as a vehicle that delivers the secreted proteins to the wound site. It is imperative to safely transfer the gel to the surgical site and use it within 10 minutes of its production. Otherwise, the gel will retract and lose the originally expressed growth factors (Akingboye et .al, 2010). It has been reported that the quality and quantity of the PRP produced is a function of the quality of the platelet. However, there are other conditions that are relatively contraindicated to the use of PRP, such as pre-existing coagulation defects (thrombocytopenia, hypofibrinogenemia) or potential hypersensitivity to bovine products (Grant et. al, 2005).

CHAPTER 3

Growth Factor Levels in Autologous Derived Platelet–Rich &Plasma and Platelet-Poor Plasma; Implication for Tissue Reparation and Wound Healing

3.1 Introduction

Growth factors (GFs) released from activated platelets is believed to initiate and modulate wound healing in both soft and hard tissues. A recent strategy to promote wound-healing is in the application of autologous derived platelet concentrate (APC) suspended in plasma, known as platelet–rich plasma. Platelet-rich plasma (PRP) acts as storage vehicles for GFs. GFs such as PDGF [AA, AB, and BB], *TGF*-β 1 &2, FGF- acid and basic, EGF, IGF and VEGF etc. have been identified in the wound healing cascade and are known to influence the process of tissue regeneration (Eppley,2004). PRP has been most used in acute surgical conditions and in the management of chronic non- healing wounds (Margolis et.al 2001, Borizin, 2007).

Point-of-care devices have the ability to fractionate the blood into platelet –poor plasma (PPP), PRP, and red blood cells (Weibrich, 2002). The PRP fraction, which is a mixture of concentrated platelets and leukocytes, can be activated by autologous thrombin to create a viscous solution known as PRP. PRP can be exogenously applied to soft tissue wound, bone or synthetic prosthesis as a spray-on or a solid gelatinous mass.

3.1.1 Biological Effect of Growth Factors in Wound Healing

PRP is believed to promote cell proliferation, differentiation, angiogenesis, chemotaxis, formation of extracellular matrix, and the migration of various cells involved in both wound healing and bone growth (Everts et. al 2007). APC offers an easy, cost-effective way to obtain high concentration of growth factors for tissue healing and regeneration. Wounding healing is a well –orchestrated and complex series of events involving cell-cell and cell-matrix interactions, in which platelet growth factors serves as messengers to regulate various processes. Tissue repair begins with platelet clot formation, activation of coagulation cascade and platelet degranulation (Werner, 2003). The GFs released bind to specific tyrosine growth factor receptors, which subsequently activate intracellular signal transduction pathways (Tabata, 2003). Beyond maintaining haemostasis, the fibrin clot then provides a matrix for the migration of tissue- forming cells, notably the fibroblast; responsible for collagen synthesis and endothelial cells involved in angiogenesis (Eppley, 2004). Tractional forces generated by these migrating cells on the fibrin clot can aid in contraction. These same migrating cells are also responsible for remodelling the clot into repair tissue.

During normal wound healing, trapped platelets become activated and degranulate, resulting in the release of α-granule content. Release of PDGF into wound bed can have a chemotactic effect on monocytes, neutrophils, fibroblast, mesenchymal stem cells and smooth muscle and osteoblast. PDGF is a powerful mitogen for fibroblast and smooth muscle cells and is involved in all three phases of wound healing, including angiogenesis, collagen formation and wound re-epithelization (Eppley, Borzini, 2007) TGF–β is member of the super-family of growth and differentiating factors, including bone morphogenetic proteins. TGF- β is a mitogen for fibroblast, smooth muscle cells, a potent mediator of collagen matrix formation and it promotes angiogenesis and extracellular matrix production. Another critical component of wound repair is neovascularization- granulation tissue formation.

Presence of VEGF stimulates endothelia cell proliferation and capillary tube formation. VEGF production is amplified in the presence of nitrous oxide, which has an important vasodilatory effect on microcirculation (Goldman, 2004). EGF acts as a mitogen for fibroblast, endothelia cells, and keratinocyte and is useful in healing chronic wounds. IGF is another platelet-contained growth factor, regulating bone homeostasis and modulating of cell apoptosis (Goldman, 2004). Other studies have found beneficial clinical outcomes from the use of PRP particular in maxillofacial, periodontal and oral surgery (Tawes, 1994; Seikdo, 1987). Platelet activation during preparation of the platelet concentrate can result in early degranulation and loss of GF during the collection process. It is therefore critical to recognize that each PRP processing method may differ in regards to platelet number, activation and GF expression profile (Kevy, 2001).

3.1.2 Surgical Applications of PRP/PPP

Clinical trials coming from the use of Recombinant human- PDGF-BB (Beclapermin) reports a degree of success following it use in treating diabetic neuropathic ulcers. Following several clinical trials on the efficacy of PDGF-BB, evidence suggests that best result are obtained with PDGF-BB in combination with aggressive debridement, a process which removes senescent fibroblast and pathogens from chronic wounds (Steed, 2006; Alsousou 2009; Steed, 1996). Autologus derived Platelet –rich Plasma has advantage over single recombinant growth factor, in that, the combined GFs has synergistic effect on each other and promotes mitogenesis of mesenchymal stem cells at the wound site (Goldman, 2004). PRP in orthopaedics surgery has yielded positive results in areas of cruciate ligament reconstruction, and in trauma involving soft tissue, tendon and ligament injury (Everts, 2007 and Alsousou 2009).

Recently, a novel PRP application has emerged for morbidly obese patients undergoing bariatric surgery. Brady et al., 2006 used PRP via an endoscopic delivery system, after laparoscopic Roux-en Y gastric bypass procedure to avoid haemorrhage, infection and anastomotic leaks. He concluded that the use of PRP contributes significantly to wound healing and improved the overall clinical outcome. Autologous fibrin derived from PPP, has been used as a biological sealant during aortic, thoracoabdominal- surgery and thoracic surgery as well as patch graft angioplasty. This material mimics the final stage in the coagulation cascade (Zhu, 2006). Saratzis et al, 2008; reported the use of PRP in endovascular repair of abdominal aortic aneurysm. He conducted a patient- assessor-blinded controlled trial involving 100 patients, with 50 patients in the treatment group. He reported a significantly lower postoperative hospital stay and lower inguinal wound-related complication (Tawes, 1994). However, it is unknown whether autologous PRP (or PPP) from patients requiring GFs contains level similar to those of the healthy controls. The purpose of this study was to compare the levels of growth factors released from PRP from diabetic patients with chronic ulcers as compared with healthy donor.

3.1.3 Physiology of Platelets as it Relates to Wound Healing

Platelets are formed from megakaryocytes and are synthesized from bone marrow by pinching off pieces of the cytoplasma. Platelets are small discoid blood cells, with the average platelet count ranging from 1.5–3.0 × 10 −5 /mL of the circulating blood, and with an average half-life of about 7 days. They have a trilaminar cell membrane with a glycoprotein receptor surface overlying and are partially interspersed with a penetrating bilayer of phospholipids and cholesterol. Platelets lack nuclei but contain other organelles and structures such as mitochondria, microtubules, lysosomes, and granules (α, δ, λ). The cytoplasma contains an open canalicular system that increases the effective surface area for intake of stimulatory agonist and the discharge of effectors' secretions. The dense granules contain adenosine diphosphate adenosine triphosphate, serotonin, and calcium. The α-granule contains clotting factors, growth factors, and other proteins. Platelets are normo-thrombogenic and require a trigger, before they become potent and active in haemostasis and wound healing. They are found intravascularly and are concentrated in the spleen.

Hemostasis is the balanced interaction of platelets, endothelia cells, and plasma clotting proteins. Platelets are responsible for initiating the immediate and most important step in coagulation. Platelet adhesion occurs through the interaction of the damaged vessel wall and exposes the subendothetial collagen, which binds with von Willebrand factor, with a resultant change in the platelet structure,

thus, making it possible for its adherence to blood vessels. This effect is accomplished through the activities of glycoprotein 1b, and 11b/111a receptors on the platelet membrane. After aggregation, the granular contents are then released; serotonin probably assists with vasoconstriction. Adenosine diphosphate (ADP) promotes release of granules, which further encourages platelets aggregation. Phospholipase A2, when activated, results in release of arachidonic acid, which is converted into thromboxane A2, resulting in further platelet aggregation and growth factor release. The process described attempts to produce a platelet plug—the primary haemostasis.

The platelet plug acts as a barrier to micro-organism invasion of wounds, with the help of highly concentrated leukocyte buffer. The clotting cascade is also activated along with the complement system, producing the secondary haemostasis with a resultant fibrin network formation. Degranulation of α-granules results in the fusion of platelet cell membrane during which some of the secretory proteins (e.g., PDGF) are transformed to a bioactive state by the addition of histones and carbohydrate side chain (Marx, 2001). These active proteins then bind to the transmembrane receptors of target cells such as mesenchymal stem cells, osetoblast, and fibroblast, endothelial and epidermal cells. Once bound to the transmembrane receptors, intracellular signal proteins are activated, resulting in expression of gene sequence that directs cellular proliferation, matrix formation, and initiation of collagen synthesis (Marx, 2001). These proteins are secreted within 10 minutes of clotting, with more than 95% of the pre-synthesized growth factors secreted within 1 hour (Akingboye et.al, 2010).

3.2 Materials and Methods.

The process of PRP and PPP preparation has been described in details in **section 2.19.**

3. 2.1 Quantification of Growth Factors in PRP and PPP

The supernatant (post activation) obtained from PRP and PPP that was allowed to thaw at room temperature after been in storage (-80℃.) The milliplex is based on the luminex Xmap technology, which performs immunoassays on the surface of fluorescent-coded beads known as microspheres [Millipore Corporation 290 Concord rd. Billerica]. The following growth factors were measured using the Milliplex ^{TM} map kit; PDGF-AA, TGF-β1, FGF-2, EGF and VEGF whereas IGF and P-Seletin ELISA kits were measured by ELISA (R&D Minneapolis Minn) according to manufacture's instructions. The optical reading obtained for both the control and tested samples the experiments were fed into an automated analyser that generated the concentrations of the growth factors. Furthermore, PF4 and TSP-1 were also measured using the ELISA kit (Quantikine, R&D Systems,

Minneapolis, MN) but the automated analyser was not available to be used for this 2 immunoassay hence, the final concentration were derived from the plotted standard curves **(Figure 3.2).**

3. 3 Statistic Analysis

Statistical analysis was carried out using a linear model based on log transformed values of the growth factors. PRP and PPP groups for each of the diabetic and healthy groups separately. In addition, the mean difference, 95% confidence interval and the n-fold increase of PRP to PPP was derived. The relationships between platelet count and growth factors were investigated using a linear regression model. Correlations between growth factors and platelet count were assessed by Pearson's correlations. Non parametric student t-test was showed when comparing individual growth factors within a group and one way- ANOVA (univariate analysis) when the growth factors expression was compared between the groups. All analysis was carried out at the 5% (2 –tail) level of significance.

3.4 RESULT

3.4.1 Haematology Analysis of Platelet-Rich Plasma

Complete blood count analysis was performed on the whole blood and platelet-rich plasma samples from the six diabetic patients. Thrombocyte baseline value ranged from 130,000 to 500,000 platelet /µL. The platelet separation system (PSS) increased the platelet count on the average from 334,000 ± 117, 000 platelet/ µl to 1,995,000±805,000 platelet/µl, this an average 6-fold increase in platelet concentration. The platelet rich plasma group was significantly higher in platelet number than the baseline whole group, with a value of $p > 0.0005$. Thereby, confirming the concentration ability of the platelet separation system.

3.4.2 Quantification of Growth Factors

The quantification of the GFs from the PRP was determined in both the healthy volunteers and diabetic patients. Values of the GFs in both groups appear comparable. On the other hand when the values of GFs present in PRP were compared with that of PPP; TGF-b1, EGF, PDGF-AA, and VEGF were significantly greater in the PRP from both the diabetic patient and the healthy volunteers. Furthermore, the only statistic significance observed was the comparison of PRP/PP for TSP-1 in the diabetic group, with p value of 0.015; on the other hand the healthy volunteer group did not reach statistical significance.

3.4.3 Comparison of Growth Factor Levels in Diabetic Patients (PRP vs. PPP)

In this study PPP was used as the benchmark in which the n-fold increase for the GFs from PRP was estimated. The mean ± standard deviation (SD) of PRP versus PPP in EGF was 234.9± 193.8 pg/mL *vs* 31.9 pg/mL with 5 fold increase, FGF-2; 49.2 ±33.64pg/ml *vs.* 49.2 ± 42.21pg/mL with no

increase, PDGF-AA; 104.8 ± 101.ng/mL *vs* 1.125 ± 0.674ng/mL had 68 fold increase; VEGF; 888 ± 970ng/ml *vs* 167 ± 180ng/mL with a 4 fold increase, TGF-β1; 28.384 ± 14.63ng/mL *vs* 1.96 ± 2.464ng/mL with a 27 fold increase. No significant increase was found in IGF in this study, IGF in PRP was 115.1± 32.54ng/mL *vs* 106.1 ± 102.2 ng/ml in PPP. The level of platelet activation was estimated by the concentration of the P-seletin, there was two-fold increase in the value of the P–Seletin in PRP (161.5 ± 113.6 ng/ml) compared to PPP(65.2 ± 17.67ng/mL), which is statistically significant <0.0001.

3.4.4 Correlation of Platelet Concentrate with Expression of Growth Factors

The correlation was done for the four most highly expressed GFs (VEGF, TGF, PDGF and EGF). **Fig 2** is the graphic illustration of the correlation. None of the parameters was statistically significant predictor of PRP count; this might be due to the small sample size or to apparently paradoxically low secretion of the GFs in patients with very high platelet concentration. Overall there is no evidence of a linear relationship between platelet count and growth factor. Pearson correlation test revealed that none of the growth factors has a positive linear correlation with the platelet concentration (R^2 for TGF, EGF, VEGF and PDGF-AA are 0.029, 0.008, 0.017 and 0.011 respectively).

GF expression in diabetics did not correlate with the platelet count nor with PRP on the Pearson's correlation coefficients analysis (rp) (r (p) - >0.2 ≤0.9), **Table 3.2** showed all the correlation for each of the GFs. Relationships between platelet count and growth factors were investigated using a linear regression model, was only performed for the four most expressed GFs and the p value results are as follows; PDGF-AA- 0.7157, VEGF-0.6616, TGF-1-0.3740, EGF- 0.6489.

3.4. 5 Comparison of Growth Factor Levels in Healthy Donor (PRP vs PPP)

A similar trend was noticed in the growth factor expression in the healthy group but the mean value appears higher in the healthy donor. The exception is the expression of VEGF and TGF- β1 which are higher in the diabetic group. As explained above PPP was used as the benchmark in this study. EGF in PRP to PPP was (463 ± 259pg/mL *vs* 31.9pg/mL) which is an fold increase; FGF (136 ± 110.6pg/ml *vs* 59.8± 48.98 pg/ml), a 2 fold increase, PDGF-AA (123.6 ± 8.30ng/ml *vs* 1.51± 1.04ng/ml) a 70 fold increase, VEGF (688.3 ± 808.4ng/mL *vs* 136 ± 132ng/mL) a 4 fold increase, TGF- β1(27.245 ± 31. 074ng/mL *vs* 0.833± 1.004ng/mL) a 40 fold increase and IGF (122.7 ± 20.89ng/mL *vs* 128.4± 21.57ng/mL), no increase. The activities of P-Seletin estimating level of platelet activation of PRP (275.1 ± 115.2ng/mL) are again two-fold higher compared with PPP (117.9 ± 129.8ng/mL). Table 1 showed the n-fold increase in the expression of the growth factors in both groups tested as it relates to the chosen confidence interval of P= 0.05. Figure 1 also shows the mean expression of the growth factors in the diabetic and healthy donor.

3.4.6 Quantification and Comparison of PF-4 and TSP-1 in the Diabetic and Healthy Group

The mean ± standard deviation (SD) for PRP for PF-4 was 91.2± 7.54 gn/mL and PPP was 89.0 ± 20.50 ng/mL p value of 0.33 on analysis of variance. The mean ± standard deviation value for TSP-1 for PRP was 2408.60± 458.50ng/mL and PPP was 808.60±563.0ng/mL with a p value of 0.03 . **Table 3.1** the expression of the 2 distinct growth factors, one way ANOVA (variance of analysis) was used to test the significance, when comparing PRP/PPP within the diabetic and healthy group a P value of< 0.0001 and 0.0055 was detected.

Furthermore, the mean and standard deviation of TSP-1 in the healthy volunteer group was 2679.80 ±327.0 and PPP was 1326.40 ± 458.50 ng/mL with a p value of 0.07 when PRP/PPP was compared. On other hand, PF-4 for PRP was 105.2 ± 13.10ng/mL and PPP of 108.50± 8.25ng/mL and a p value of 0.354. Analysis of variance for TSF for PRP/PPP in diabetic and healthy volunteer group showed p values of 0.0001and 0.015 respectively.

Tested group	**Growth factor**	**Mean ±SD (ng/ml)**	**P value**	**Anova Analysis**
Diabetic patient	PF4- PRP	**91.2± 7.54**	**0.33**	
	PF4- PPP	**89.0 ± 20.50**		**<0.0001**
Healthy Volunteer	PF4- PRP	**105.2 ± 13.10**	**0.354**	
	PF4-PPP	**108.50± 8.25**		**<0.0055**
Diabetic Patient	TSP-1 -PRP	**2408.60 ±458.50**	**<0.03**	
	TSP-1 -PPP	**808.60 ± 563.0**		**<0.0001**
Healthy Volunteer	TSP-1-PRP	**2679.80 ±327.0**	**0.07**	
	TSP-1- PPP	**1326.40± 458.50**		**<0.015**

Table 3.1 Represents the expression of PF-4 and TSP-1 from the diabetic patient and health volunteer group with mean and standard deviation (±SD) expressed. A significant difference of P value< 0.05 was considered when comparing PRP/PPP for the 2 different GFs in each group and the overall univariate analysis for the 2 separate groups was analysed.

Groups Tested	**Growth Factors**	**Comparison**	**n-Fold increases**	**P- value**
Diabetic Patient	EGF	PRP vs PPP	**4.5698**	**<0.003**
	FGF-2		**1.0414**	**0.8480**
	PDGF-AA		**68.6203**	**<0.0001**
	VEGF		**4.2307**	**0.008**
	TGF-β1		**27.3338**	**<0.0001**

	IGF		**1.0285**	**0.021**
	P-Seletin		**2.2171**	**<0.0001**
Healthy Volunteer	EGF	PRP vs. PPP	**11.0756**	**<0.0001**
	FGF-2		**1.9372**	**0.0002**
	PDGF-AA		**69.9147**	**<0.0001**
	VEGF		**4.4117**	**<0.0001**
	TGF-β1		**38.9119**	**<0.0001**
	IGF		**0.9553**	**0.1272**
	P-Seletin		**3.1759**	**<0.001**

Table 3.2 Comparison of PRP and PPP between the Diabetic and Healthy donor. The difference with 95% confidence intervals for mean differences between PRP vs. PPP were derived and back transformed to provide an estimate of the n- fold increase of PRP to PPP

Super-Family	Member	Role
Platelet Derived Growth Factor	PDGF	PDGF has three isoforms; PDGF-AA, AB, BB. PDGF affects cells of mesodermal origin and has its primary effect on these cells and could also be secreted from other polymorph nuclear cells, fibroblast, and smooth muscle cells. Its roles include stimulation of chemotaxis, proliferation, and new gene expression in these cells. Other reported activities for PDGF includes stimulation of granule release by neutrophils and monocytes, stimulation of neutrophil phagocytosis, inhibition of natural killer (NK) cell activity, stimulation of collagen synthesis modulation of thrombospondin expression and secretion and stimulation of collagenase activity (Everts,2006) .
	(VEGF)	VEGF also known as vascular permeability factor or vasculotropin is a homodimeric 34 – 42 kDa, heparin-binding glycoprotein with potent angiogenic, mitogenic and vascular permeability enhancing activities specific for endothelial cells. In normal tissues, VEGF expression has been found in activated macrophages, keratinocytes, renal glomerular visceral epithelium and mesangial cells, hepatocytes and smooth muscle cells. Its receptors are exclusive to the endothelial cells and it acts as an effective mitogen during angiogenesis. FGF-4, PDGF, tumour necrosis factor (TNF)-α, Insulin-like growth factor (IGF) and some interleukins stimulate VEGF production and others inhibit it (Neufeld, 1999).
Epidermal Growth Factor	EGF	EGF binds to the same 170 kDa receptor, TGF-α, EGF is initially synthesized as a large (130 kDa) precursor molecule in which the mature, soluble EGF sequence (6 kDa) is located. EGF is released on degranulation of

		platelets. Most cells have receptors for EGF but epithelial cells have the largest number of receptors. Other sites for the receptors include endothelial cells, fibroblasts and smooth muscle cells. In vitro, EGF is a mitogen for fibroblasts, endothelial cells and promotes colony formation of epidermal cells in culture. EGF is also chemotactic in nature, stimulates angiogenesis and collagenase activity (Lawrence, 1994 and Grotendorst et.al, 1989).
	TGF-α	TGF-α has 30% structural homology as EGF. TGF- α expression is characterized by a relatively widespread distribution. Soluble TGF- acts in autocrine and paracrine fashions with a high affinity binding, via the EGF-like motif, which is critical to the achievement of high affinity binding to its receptor and is common to all members of the EGF family. TGF- α expression is exaggerated in transformed cells and tumours. It is produced by activation of macrophages, platelets, and keratinocytes. It stimulates mesenchymal, epithelial, and endothelial cells via chemotactic effect. Although TGF- α is most often associated with its proliferation, differentiation, and transformation-promoting effects, it is also involved in angiogenesis, cell metabolism, cell migration and wound healing (Lawrence, 1994 and Grotendorst et.al, 1989).
Fibroblast Growth Factor	FGF (a&b)	FGF has 2 different forms; the acidic and basic, both have 50% homology. Cells known to express FGF acidic include skeletal and smooth muscle cells endothelial cells, macrophages keratinocytes and fibroblasts. It is suggested that both membrane receptor activation and internalization is necessary for a full mitogen response. FGF acidic is best known for its mitogenic activity on endothelial cell. Both forms stimulate endothelial cell proliferation and motility, thereby contributing to wound angiogenesis. FGF- basic is 10 times more potent as an angiogenic stimulator. FGF basic is chemotactic and mitogenic for endothelial cells in vitro, inducing endothelial cell production of factors involved in the breakdown of the basement membrane

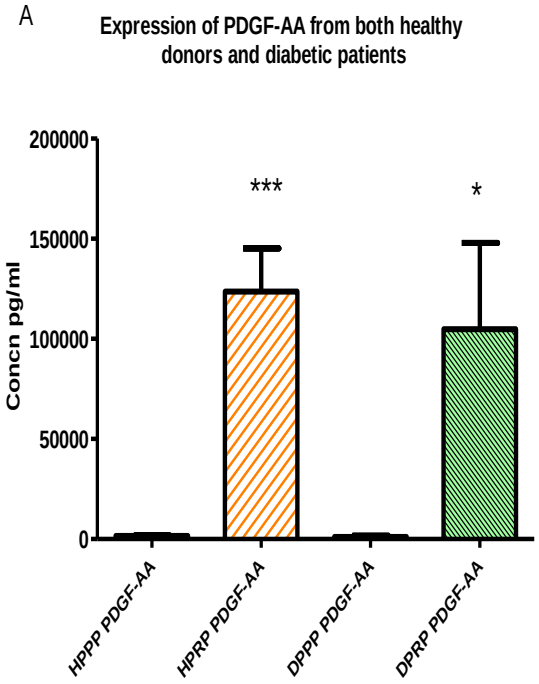
		and the migration of capillary endothelial cells into collagen matrices to form capillary-like tubes. Other stimulatory effects include collagen synthesis, wound contraction, epithelialization, and fibronectin and proteoglycan synthesis (Lawrence, 1994 and McGee et.al, 1998).
	KGF I &II	Within the FGF family, KGF shows 29% amino acid sequence identity to FGF-2. The number of cells expressing KGF receptors is few and limited to epithelial cell types such as keratinocytes, transitional epithelium, embryonic lung epithelium and hepatocytes. In addition, KGF also binds to heparan sulfate proteoglycans; the biologoical role of heparin binding is unclear. High quantity of KGF is produced after tissue damage, which is mainly produced by fibroblast. KGF-I is the most potent mediator of keratinocyte proliferation and motility. KGF-2 has been shown to increase granulation tissue formation by directly stimulating the migration of fibroblasts in wounds. KGF is synthesized by dermal or lamina propria fibroblasts, it is proposed to act locally on the overlying epithelial sheet. In addition to its ability to induce cell proliferation, it may also promote epithelial differentiation. During wound healing, KGF's role as a re-epithelializing agent is complemented by the presence of proinflammatory molecules which appear as a result of tissue damage (Cross K, 2003 and Beer, 2000).
Transforming Growth	TGF (β1, β2, β3)	TGF-β has been isolated from platelets, macrophages, lymphocytes, bone, and kidneys. TGF are also released after platelet degranulation. It stimulates monocytes to secrete other growth factors (FGF, PDGF, TNF- α, and IL-1). TGF is chemotactic for macrophages and regulates its own production within the macrophages in an autocrine fashion. It also stimulates fibroblast chemotaxis and proliferation. The presence of other growth factors and the variability in concentration of TGF-b could modulate the role of TGF-b. This is either an inhibitory or stimulatory effect on cellular proliferation. TGF-b stimulates fibronectin, proteoglycan synthesis

		by fibroblast. It organizes extracellular matrix and may be involved in scar remodelling and contracture. It also stimulates epithelial cell proliferation and inhibits endothelial cell proliferation, but with a co-factor it will stimulate angiogenesis (Lawrence, 1994; Massague,1987;Wahl et.al 1987 and Fukamizu, 1990).
Insulin Growth Factor	IGF (I&II)	IGF-II is most prominent during fetal development, where-as IGF-I persists throughout life and is synthesized in the liver, heart, lung, kidney, pancreas, brain, and muscle. IGF is stimulated by human growth hormone (especially in the liver) and the two together stimulate skeletal cartilage and bone growth. Degranulation of platelets will also produce IGF-I and the stimulation of fibroblasts. IGF-I is a potent chemotactic agent for endothelial cells, and is also involved in neo- vascularization. It may also act with PDGF to enhance epidermal and dermal growth. The effect of IGF-I on wound healing depends on the amount of available free IGF-I (Guler,1988 and Grant,2004).
Platelet Factor 4	PF-4	PF-4 is a very abundant platelet α-granule CXC chemokine that is released during platelet activation. Chemokines are a subfamily of the larger chemokine family of small proteins. Mature human PF4 is a 7.8 kDa in size. The biosynthesis of PF4 is almost exclusively limited to megakaryocytes from which mature circulating platelets are derived. PF4 binds with high affinity to heparin and to such negatively charged cell surface gylcos -aminoglycans (GAGs) as heparan sulfate (HS) and dermatan sulphate. PF4 may also affect hemostasis and thrombosis through inflammatory and vascular pathways and these possibilities have been explored in vitro. In the presence of a co- stimulus, such as tumour necrosis factor (TNF)-α, PF4 can activate neutrophils, cause exocytosis and promotE adhesion to protein matrices or endothelium. PF4 was found to have a number of different effects on circulating monocytes, preventing apoptosis and facilitating macrophage differentiation during the inflammatory process. PF4 also appears to augment monocytE

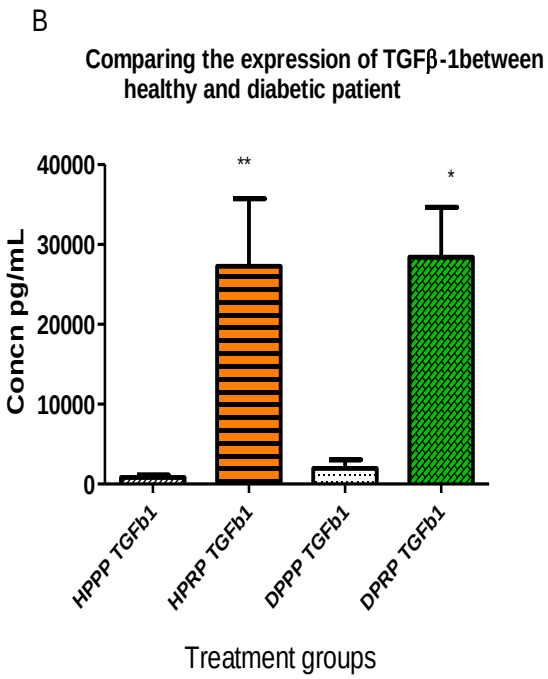
		phagocytosis and the subsequent respiratory burst and to induce cytokine secretion (Pervushin et .al, 2004. Rucinski et al , 1990, Petersen et.al 1999).
Thrombospodin	(TSP 1)	TSP-1 is a 150 KDa secreted member of the thrombospondin gene family of proteins. There are 5 structurally related extracellular matrice protein; TSP 1-5 and only TSP 1-2 are functionally and structurally related. TSP-1 is produced by megakaryocytes/ platelets, endothelial cells, vascular smooth muscle cells and fibroblasts. In conjunction with von-Willebrand factor (VWF) and fibrinogen, TSP-1 contributes to clot function. Following vascular injury, platelets bind to exposed vessel wall collagen via membrane GP- VI. This results in platelet activation with VWF and TSP-1, and subsequent VWF binding to platelet membrane. TSP-1 also prevent proteolysis by binding to VWF at site targeted by proteases. TSP-1 is co-expressed with SDF-1 and MMP-9 in platelet α-granules. The expression of pro- angiogenic – MMP-9 and SDF-1 are regulated by TSP-1. It has also been shown that TSP-1 minimizes new vessel growth which may be through its ability to regulate nitrous oxide (NO). NO at low doses promote endothelial cell proliferation and migration. These findings suggest that TSP inhibit angiogenesis by acting as sponges that absorb angiogenesis-stimulating molecules. Therefore, thrombopoietic cell TSP is well established by these findings as an angiogenesis suppressing switch (Armstrong and Bornstein 2003 and Legrand et.al 1997).

Table 3.4 is a summary of growth factor super families and individual growth factors with there respective functions, structural morphology and site of production.

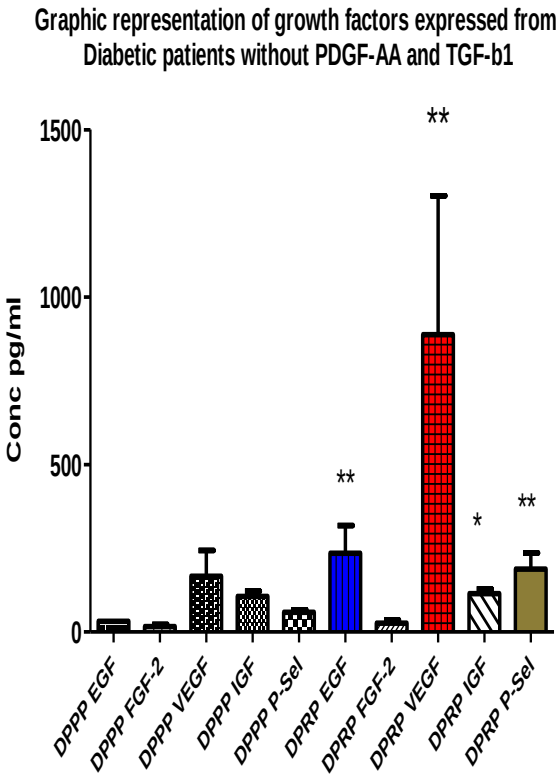
A


Expression of PDGF-AA from both healthy donors and diabetic patients
Concn pg/ml
200000
150000
100000
50000
0
***
*
HPPP PDGF-AA
HPRP PDGF-AA
DPPP PDGF-AA
DPRP PDGF-AA

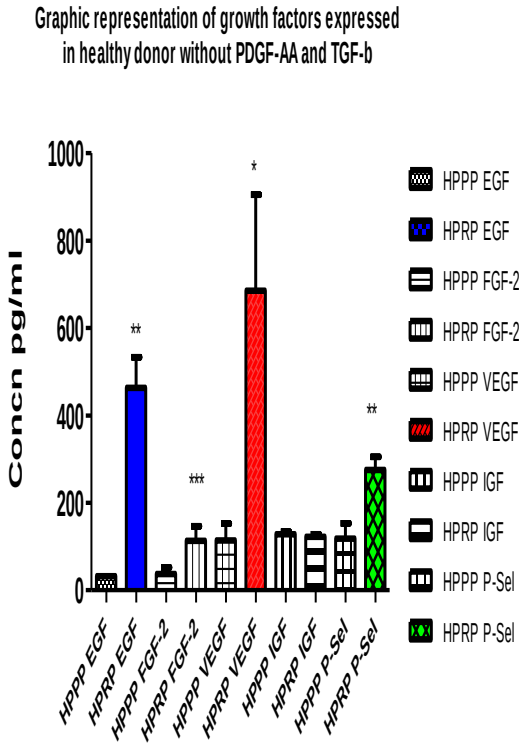
B


Comparing the expression of TGFβ-1between healthy and diabetic patient
Concn pg/mL
40000
30000
20000
10000
0
**
*
HPPP TGFb1
HPRP TGFb1
DPPP TGFb1
DPRP TGFb1
Treatment groups

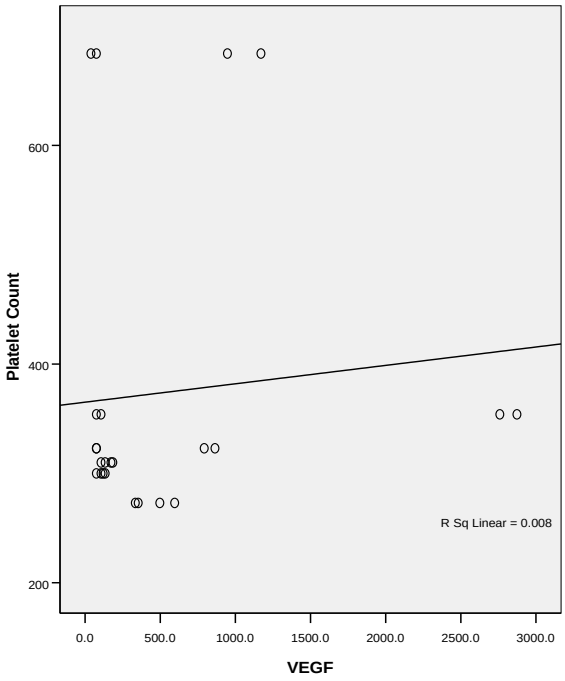
C

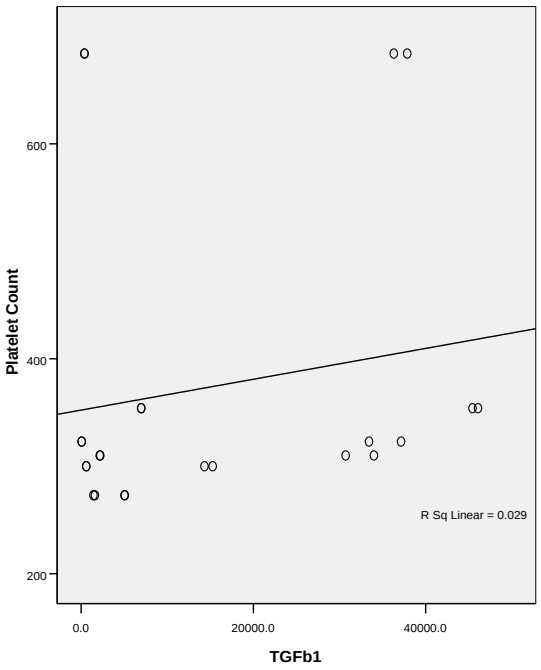


D



E **Plots with Linear Regression Line superimposed**


Platelet Count
600
400
200
R Sq Linear = 0.008
0.0
500.0
1000.0
1500.0
2000.0
2500.0
3000.0
VEGF


Platelet Count
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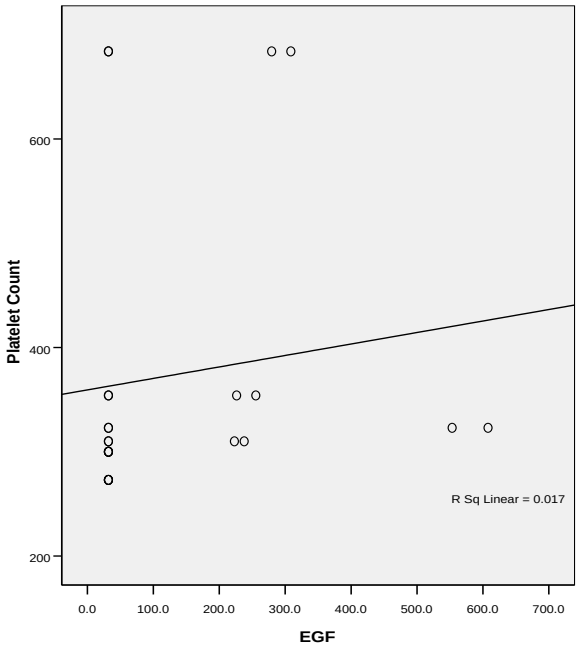
400
200
R Sq Linear = 0.029
0.0
20000.0
40000.0
TGFb1


Platelet Count
600
400
200
R Sq Linear = 0.017
0.0
100.0
200.0
300.0
400.0
500.0
600.0
700.0
EGF

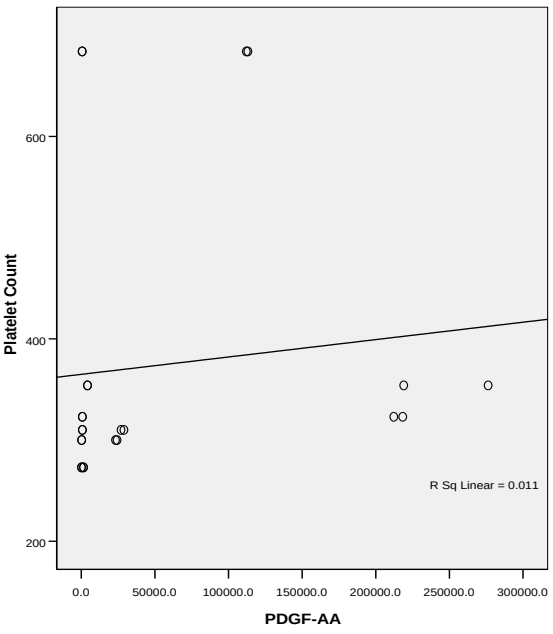
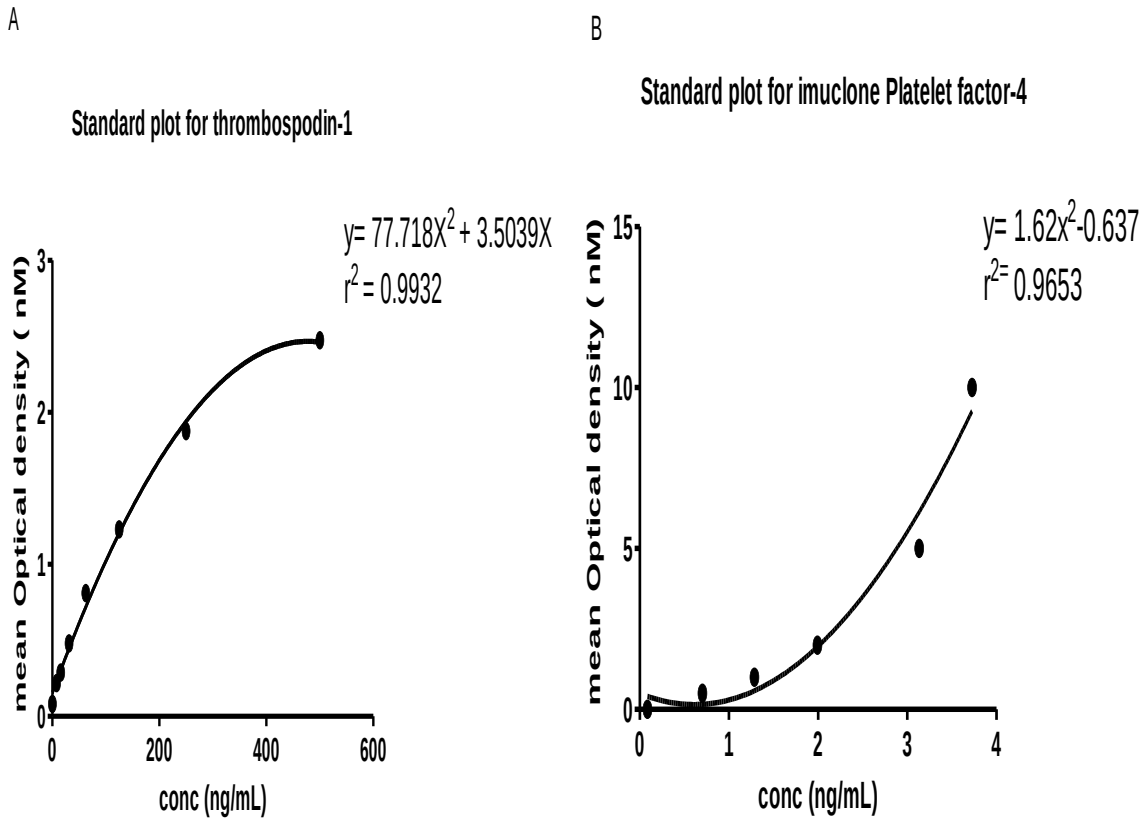

Platelet Count
600
400
200
R Sq Linear = 0.011
0.0
50000.0
100000.0
150000.0
200000.0
250000.0
300000.0
PDGF-AA

Figure 3.1 Graphical illustrations represents the different expression of growth factors expressed from platelete-derived platelet gel from PRP vs. PPP. **A)** Compares the expression of PDGF-AA between PRP and PPP in the healthy, PRP showed a significance of p< 0.0001 over PPP and similarly in the diabetic group, PRP has a significant difference over PPP <0.001. **B)**. The expression of TGF-β showed p value of <0.004 when PRP and PPP were compared in the healthy donor group and <0.002 for the diabetic patient but there was no statistical difference when the PRP from both groups were compared. **C&D)** showed graphical representation of the less expressed growth factors, VEGF and EGF are the 2 most prominent with p values of <0.001 when PRP and PPP were compared in both groups. E) Pearson correlation test revealed that none of the growth factors has a positive linear correlation with the platelet concentration (R^2 for TGF, EGF, VEGF and PDGF-AA are 0.029, 0.008, 0.017 and 0.011 respectively)



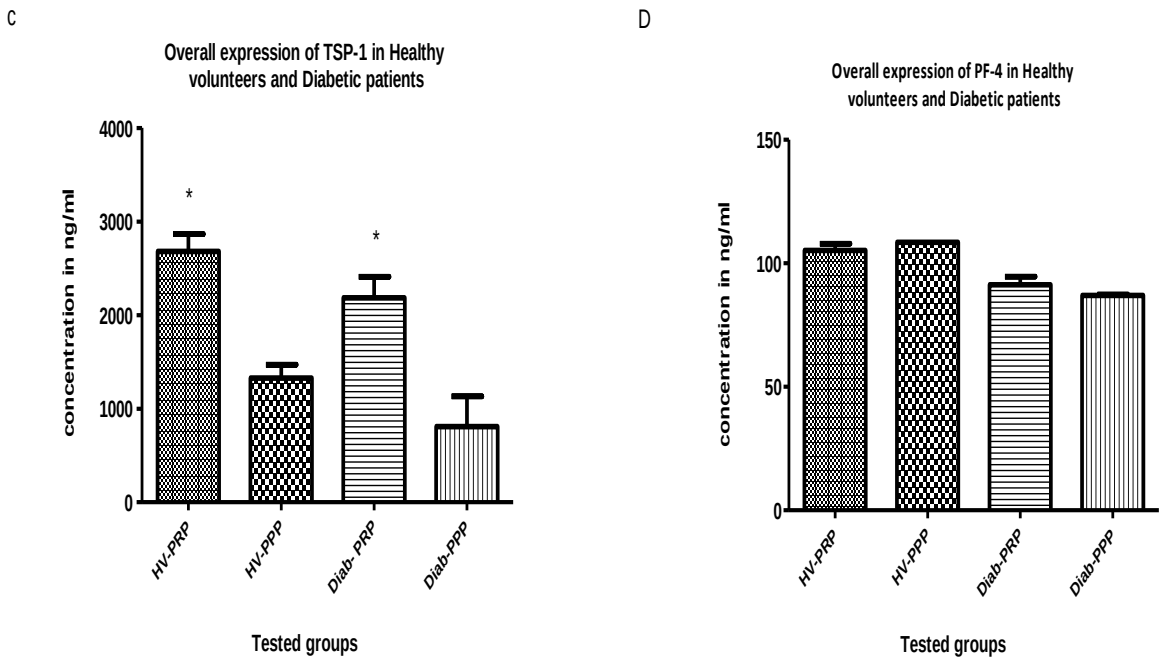


Figure 3.2. Graphic illustration of TSP-1 and PF-4 A & B) showed the standard plot for TSP-1 and PF4 with there equations and the degree of correlation. The final concentrations were derived through the equation obtained. **C)** Compared the expression of TSP-1 on-way analysis of variance, a significance of P value 0.015 was reached. The expression of PRP is significantly higher in the healthy group as compared to PPP with p value of <0.05, a similar pattern was noticed amongst diabetic patient with a p value of <0.05. Although, the expression of TSP-1 in PRP was higher from Healthy volunteers compared to diabetic patient it is not significant statistically **D)**. The expression of PF-4 is was significant on one-way analysis of variance with P value of 0.005. However, no statistical significant was reached when comparing PRP and PPP in either group.

3.7 DISCUSSION

All measurements were performed in duplicate using validated commercially available ELISA and millpore kit, and coefficient of variance was < 5%. For all growth factors analysed, specimen were used after storage in -80 ºC. Studies have shown that deep freezing and thawing is a safe method for releasing intracellular thrombocyte growth factors (Saratzis, 2008). Zimmermann et al., in there study showed that at the end of the 5-day storage period, the mean supernatant levels of PDGF-AB and PDGF-BB were about twice as high as they had been on the day of preparation. The supernatant levels of TGF-β1 remained constant. At the same time, the total growth factor levels had decreased substantially. However, Sekido et al. 1987; reported that PDGF activity of platelet concentrates in a

bioassay was at the same level during preservation for up to 5 days. Other researchers reported an approximate accumulation of three to five times the levels of PDGF and TGF-β1 in the supernatants of platelet components during storage (Ledent, 1995). The platelet counts from the blood sample from the diabetic patients were within acceptance range. The platelet counts in the platelet concentrate were also in the expected range and corresponded to values previous reported in literature (Sekido, 1989).

The correlation between the GFs and the platelet count from the PRP were weak, this might be due to small sample size, or might be due to isolated incidents of inefficient concentration of PRP. From the clinical point of view, it is important that the platelet concentration process is reliable by using an approved and tested platelet separation device. The data obtained from this study further supports other published series, that neither the direct platelet count nor the platelet in PRP could predict the expected expression of GFs (Weibrich, 2002). It is critical to minimize platelet activation that occurs during PRP preparation. In our study P-Selectin was measured after activation of PRP as means of defining the degree of activation, and a 3 fold increase was noticed when the value of P-selectin in PRP was compared with PPP in the healthy donor and the diabetic patient. In a similar study by Eppley et al. P-selectin was measured after centrifugation of PRP before the activation and they reported that there was no significant change in the level as compared with the whole blood (Eppley, 2004). Although our approach was different to that of Eppley et al., the levels of GFs expression in our study compares favourably to other published series after activation (Alsousou, 2009, Weibrich, 2002).

When comparing the GF profile from PRP and PPP. It is interesting to see that PPP has some degree of expression of GF, contrary to the popular opinion that it's merely useful as a sealant and it contains no growth factor. Hence, an added advantage could be derived when PPP is used a sealant. The theoretical levels of platelet- derived growth factors in PRP might be expected to depend on the number of platelet involved, which have previously been reported in some studies (Webrich, 2002). However, from our study were could not demonstrate a linear relationship. Eppley et al., concluded that at the very best correlation was seen with TGF-β1, which was still quite low, he suggested little correlation between platelet number and GF concentration and that content of the platelets varies from patient to patient. These results might be explained by high individual variability in cellular production or storage of cytokines (Marx, 1998). Of all the GFs identified in the wound cascade, the concentration of IGF-1 was not significantly increased. IGF-1 is primarily excreted by the liver into blood plasma; hence the value of IGF is marginally higher in PRP than PPR in both groups. The findings in this study corroborate with previous reports that significant inter-patient variability of

growth factors occurs. Several other parameters influence the relationship between platelet concentration and GF level measured.

There are other methods by which platelets can be activated such as the use of adenosine diphosphate and repeated freeze-thaw, but in this study human thrombin with a combination of calcium chloride was used. The method of PRP preparation in our study achieved a greater yield of growth factors as compared to other published series. But has its inherent practical drawbacks such as; longer time of preparation and problem of fixed volume of whole blood needed to operate the device, which means the processor is unable to handle volume of less than 60ml. However, it's debatable whether this is the most effective method, but it's clinically convenient and user-friendly for the preparation process. In our study, there is a disparity in the age and gender profile of the healthy donors and the diabetic patients. The control did not matched age and gender for the diabetic group. Other studies have also suggested that gender and age are not predictive factors in determining GFs expressions (Kevy, 2001).

There were few *in vitro* researches that discussed the platelet gel composition, strength and degradation characteristics using point-of-care technologies. There must be a quantifiable way of determining the structural integrity of the resulting formed platelet gel thrombus. The thrombelastograph hemostasis analyzer (TEG) and sonoclot measure the elasticity of a clot as it forms and subsequently degrades naturally. Cassidy et al., in there study on the role of TEG concluded that the technology to quantify platelet gels must first be standardized and that the TEG and the sonoclot are not equally capable of analyzing platelet gel clots. The TEG was a valid means for analysis, whereas the sonoclot provided unreliable analysis based on a Chi-squared test (Cassidy et al., 2005). Further study with a control disease group will be more helpful to address the effect of chronic disease on GFs expression. Reliable prediction of GFs levels in PRP samples is necessary to ensure reliable and reproducible use of PRP for clinical treatment, since the regenerative potency of PRP undoubtedly depends on its GFs Level (Kevy, 2004).

3.8 CONCLUSION

Results from this study demonstrated that platelets can be sequestered and concentrated 6-fold from whole blood and that PRP contains significantly higher levels of GFs when with PPP; PDGF-AA, VEGF, TGF-1, with potential benefits on wound healing. PRP also had significantly higher levels of IGF in diabetic PRP (but not healthy control PRP) and FGF-2 in healthy control (but not diabetic) PRP. Furthermore, we demonstrated that activating PPP will aid release of the above GFs as shown in PRP but to a lower degree. Prediction of GFs levels based on platelet count or PRP have not been

possible in this study. Further investigation might be needed to resolve the issues of unpredictability release of growth factor from PRP. Furthermore, a technique whereby GFs could be rapidly assessed in PRP before application may be of therapeutic benefit.

CHAPTER 4

Biological Effect of Platelet–Rich Plasma & Platelet-Poor Plasma on Human Dermal Fibroblast, Human Epithtelia Keratinocyte and Human umbilical Vein Endothelial Cells

4.1 Introduction

Platelet-rich plasma gel is generated by differential centrifugation, in which platelets are concentrated into a small plasma volume. The use of PRP is based on the premise that platelets constitute a reservoir of critical growth factors (GFs), such as platelet–derived growth factors (PDGF), transforming growth factors-β (TGF-β), vascular endothelia growth factor (VEGF), Fibroblast growth factor (FGF) and more which once released from the platelet alpha granules, may positively regulate the wound healing process (conway 2001 et al. and koveker 2000).

There have been successful reports on the use of Platelet –rich plasma (PRP) in various clinical setting such as reconstructive plastic surgery, orthopaedic surgery and in treating cutaneous wounds / ulcers. However, an increasing body of evidence suggests that the clinical benefits related to this technique may be limited (Weibirch 2002, & Weibirch 2004). Steed and the Diabetic Ulcer study group have shown PDGF-BB to be effective in clinical controlled study in the treatment of diabetic ulcers. They studied recombinant human PDGF-BB and reported significant difference in the percentage of patients that reached complete wound healing (48% in treatment group vs. 25% control group at the end of 20 weeks. (Steed et. al., 1995).

Growth factors are a class of natural biological mediators that regulate key cellular events in tissue repair, including cell proliferation, differentiation and extracellular matrix synthesis. Previous studies have demonstrated that local application of GFs alone or mixed with bone allograft is capable of increasing bone growth and accelerate healing of soft tissue (Cochran and Woozney, 1999). Based on in vitro studies, GFs have beneficial effect on wound healing, for example, PDGF stimulates the mitogenesis of mesenchymal cells, TGF-β stimulates matrix synthesis and activates pre-osteoblasts to initiate mitosis and promote their differentiation. The use of autologous source of GFs (PRP) seen to be more beneficial because higher level of GFs concentration may be achieved, which could stimulate the process of tissue repair in a more physiological way than delivery of a single growth factor (Marx et.al 1998, Kim et.al 2001). Although individual purified GFs have been extensively studied in vitro prior to in vivo, PRP has only recently started to be assessed in a similar manner. However, studies investigating the effect of PRP on cell function *in vitro* have utilized a wide variety of cell types (periosteum derives cells, human osteoblast, mesenchymal cell and endothelial), which has shown conflicting results (Liu et.al. Lucarelli et. al 2003, Gruber et al 2004, kilian et. 2004; kanno shownet. al 2005). More basic research are still required in this growing field of regenerative science and its therapeutic applications. It still uncertain the concentration of PRP that is optimal in

regeneration and promoting wound healing. It is assumed that PRP would act in a similar manner to individual growth factors and that the preparations containing maximal concentration of growth factors would be the ideal. However, there is little evidence to support this assumption, which may be flawed on the basis that PRP is a combination of different growth factors, each of which exerts a unique influence on the complex cascade of events that occur during wound healing and tissue regeneration.

Furthermore, in vivo animal studies and clinical studies appeared to have benefit of PRP may be as results of different techniques of harvesting PRP, various animal model and methodologies used in assessing the effect of PRP and possible presence of negative regulators in PRP (Hsu et. al, 2009). It has been observed that in addition to GFs released from platelets and their demonstrated positive effects on wound repair, thrombospodin (TSP) is also abundant in the α-granule of platelet (Switalska 1985). Thrombospodin consist of five members TSP 1-5, TSP is a glycoprotein, with 3 identical di-sulphide–linked polypeptide chains. Thrombospodin -1 inhibits adhesion, proliferation and tube formation of endothelial cells in culture, and it has been found to block neovascularization of chick-chorioallantoic membrane (Iruela- Arispe et.al 1999). However, there is no evidence to suggest that TSP-3, TSP-4 and TSP-5 have such inhibitory functions on wound healing. In another study published by Hus et.al, 2009, abundant secretion of TSP-1 from concentrated PRP may contribute to the antiproliferative effect on oral cells via cell apoptosis. In view of the controversies surrounding the efficacy of PRP in tissue reparation, we have developed an in vitro experimental model through which the basic principal elements involved in wound healing i.e. proliferation, migration and angiogenesis can be investigated. The study focuses on the biological effect of PRP on human endothelial, fibroblast and keratinocyte as it relates to the key principal element of wound healing through biological assay.

4.2 Effect of Growth Factors on Cell Cycle and it Functions

Growth factors play a key role in initiating and sustaining the different phases of tissue repair (Bennet and Schultz, 1993). Growth factors are characterized by their capacity to induce mitosis in different cell types *in vitro*.

- FGF and PDGF-AB (competence factor) act by making quiescent cell in G0 phase of the cell cycle competent to respond to a second group of growth factors called the progression factors (Flyvbjerg, 1990)
- Epidermal growth factor and Insulin like growth factor appears to drive cell through G1 of the cell cycle into the DNA synthesis, these growth factors are referred to as the progression factor.

- Each growth factor uses a distinct tyrosine kinase receptor but the receptors share common signal transduction pathway.
- PDGF-AB is a major serum mitogen and induces fibroblast proliferation, matrix production and maturation of connective tissue.
- FGF-basic has its main stimulatory effect on the growth and differentiated function of fibroblasts and on proliferation of vascular smooth muscle cells and endothelial cells.
- EGF stimulate cell proliferation by binding to it's on own receptor on the different cell types

4.3 MATERIALS AND METHODOLOGY

Quantification of GFs from PRP and PPP have been described and discussed in details in **Chapter 3 section 3.4 and 3.7** respectively. In addition, measurement of TSP-1 and PF-4 was carried out from the same aliquot prepared at the start of the study, where other GFs were measured from. This was as an attempt to explain the decreasing dose phenomenon seen from the initial proliferation assay experiment. Hsu et.al, 2009 showed that TSP-1 might be an inhibitory cytokine expressed in PRP, hence, TSP-1 and PF-4 was measured. The GFs were measured according to the manufacturer's instruction.

Isolation and subculturing of HEK/NEB-1 cell, HD, HUVEC/EA.hys used in the assay have been fully described in **Chapter 2, sections 2.4, 2.5, 2.6, 2.9 and 2.10** respectively. Details of *in vitro* wound assay and transwell migration assay and the modification peculiar to this study have been fully described under **Chapter 2, sections 2.13.1 and 2.14.2**. Furthermore, the seeding density for the all the different cell types and the assay are detailed in **Chapter 2, section 2.11.**

4.4 Morphological Changes Following Subculturing with PRP/PRP

During optimization and cell viability experiments with PRP/PPP, morphological changes were observed with the varying concentrations of PRP/PPP over the 5 day time course **(Figure 4.1 and 4.2).** Observed cellular proliferation correlated with Alamar Blue cell quantification technique. The plate was further incubated for 24 h at 37^{o} C. The number of viable cells correlates with the magnitude of dye reduction and is expressed as percentage of AB reduction (Ahmed et al., 1994; Goegan et al., 1995). The values of % AB reduction were corrected by deducting the background values of negative controls containing medium without cells from the true test. In order to eliminate

differences due to medium colour, serum free keratinocyte (K-SF) was used for keratinocyte proliferation and DMEM without phenol red was Fibroblast whilst EMG-2 was used for HUVEC proliferation assay**.**

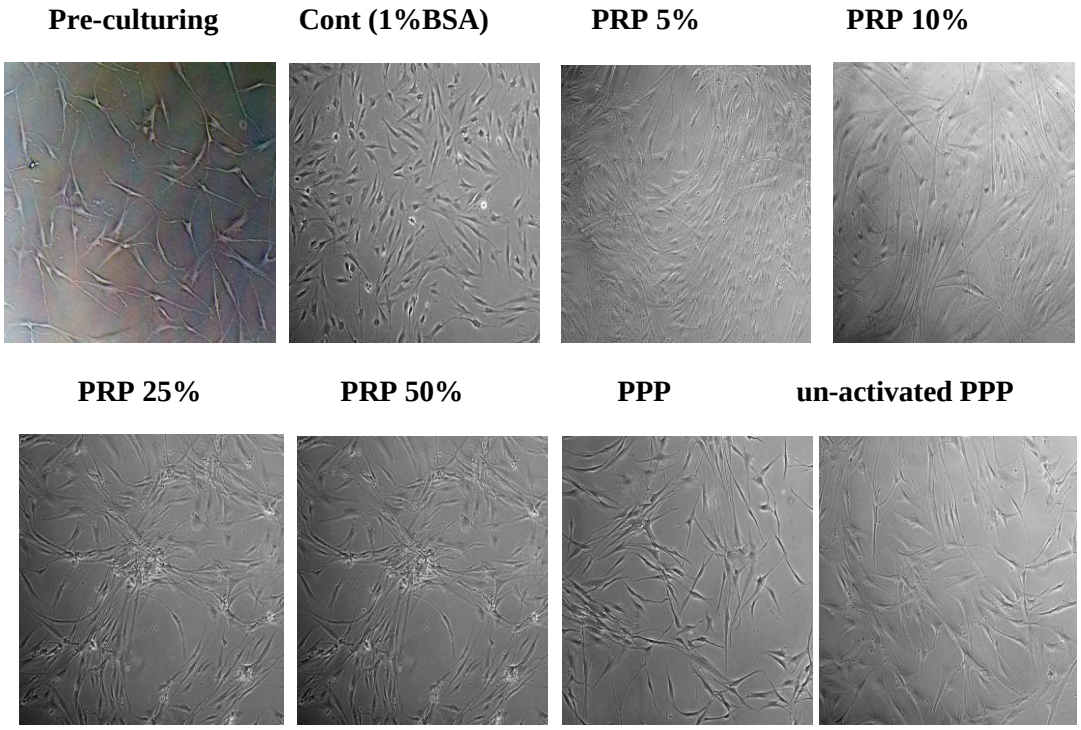


Figure 4.1 Morphological changes after sub-culturing Human dermal fibroblast with v/v of PRP/PPP and 1% BSA over 7 days period.

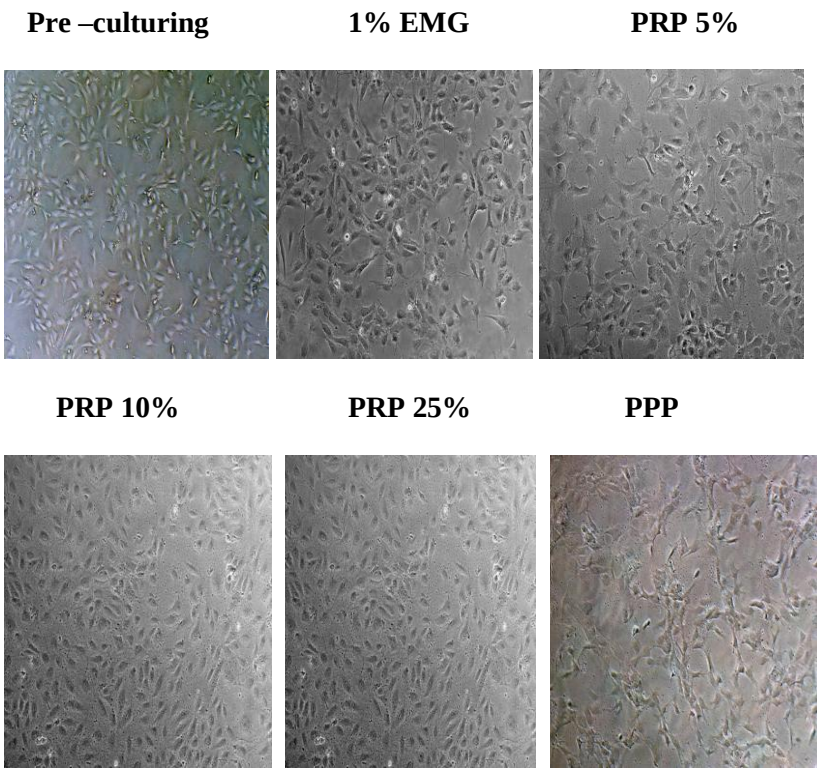


Figure 4.2 Morphological changes after sub-culturing Human umbilical vein endothelia cell with v/v of PRP/PPP with 1% EMG after 5 days.

4.5. Angiogenesis assay on matrix gels.

Growth factor-reduced Matrix gels (Matrigel, Becton Dickinson) were allowed to polymerize in a 24-well plate for 1h at 378C. HUVEC were seeded at $2x10^5$ per well and grown for 36hrs in a humidified 37^0C, 5% CO_2 incubator. In some experiments, cells were cultured in the presence or absence of different kinds of reagents. Tube formation was documented using an inverted fluorescence microscope and pictures were captured with a digital camera and stored in a computer system. Matrigel angiogenesis and collagen based angiogenesis were both optimized; the results from both were illustrated in **Figure 4.3 and Figure 4.4** respectively. Complete angiogenesis process lasted 36hrs with matrigel, whereas it last last 7days with collagen based angiogenesis. Matrigel was preferred in our study, because of quick turnover from the experiment, the methodology was more straightway and the final images were easily quantifiable, hence matrigel was the preferred technique in our study.

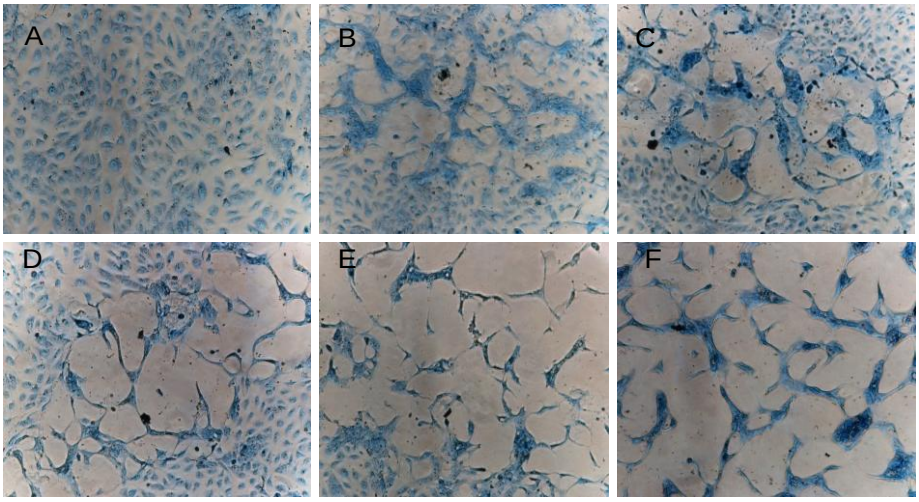


Figure 4.3. Complete process of angiogenesis assay with HUVEC on reduced Matrigel. A) Seeding of HUVEC, B) Process of sprouting and tube formation at 6hrs, C) tube formation and capillary networking at 12hrs D) angiogenesis at 20hrs , E) at 24hrs and F) complete angiogenesis at 36 hrs.

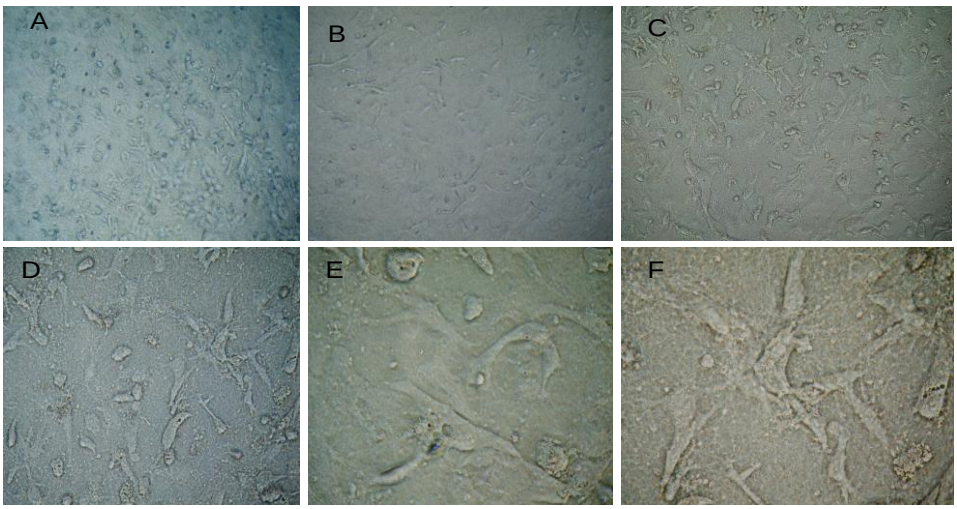


Figure 4.4. Illustrates the Collagen based angiogenesis. A) Seeding of HUVEC on Polymerized collagen gel at 6hrs, **B)** Sprouting of HUVEC at 24hrs, **C)** Development of capillary and tube formation on day 3 day, **D)** Capillary network evolving on day 4 **E)** Capillary network are fully developed but the processes are short on day 6, **F)** Poorly defined but fully formed short and thick capillary network on day 7.

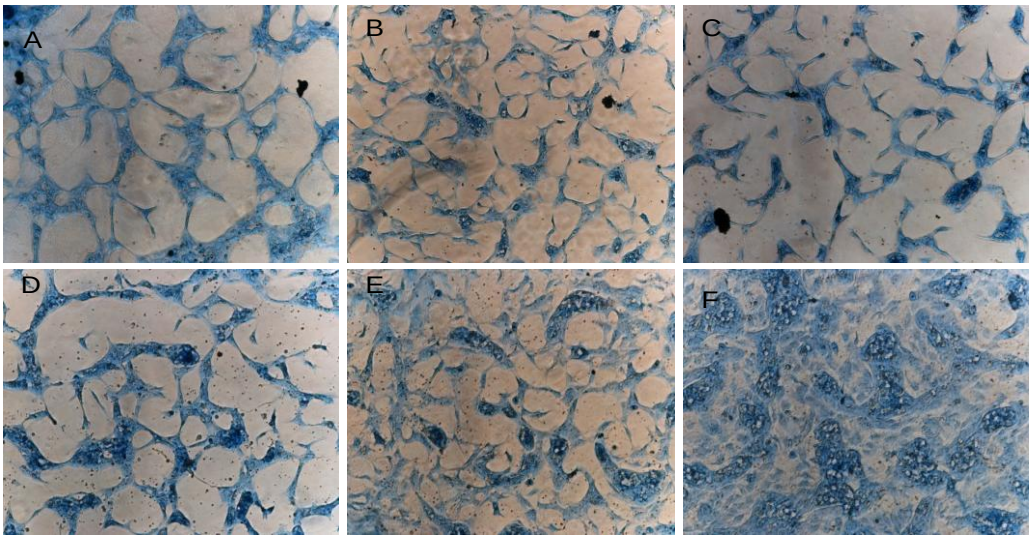


Fig 4.5 Illustrates the final stages of Matrigel angiogenesis assay with varying concentration of PRP/PPP A) Is the control experiment (EMG 1%) with polygonal tube -like network formation; **B)** 5% PRP+ RM , with more polygonal network formation; **C)** matrigel+ 10% PRP with tube-like network but less define mesh work formation; **D)** reduced matrigel +25% with thicker diameter of the tube-like structure; **E)** reduced matrigel +50% PRP with poorly defined network; F) PPP with no particular tube network formation pattern.

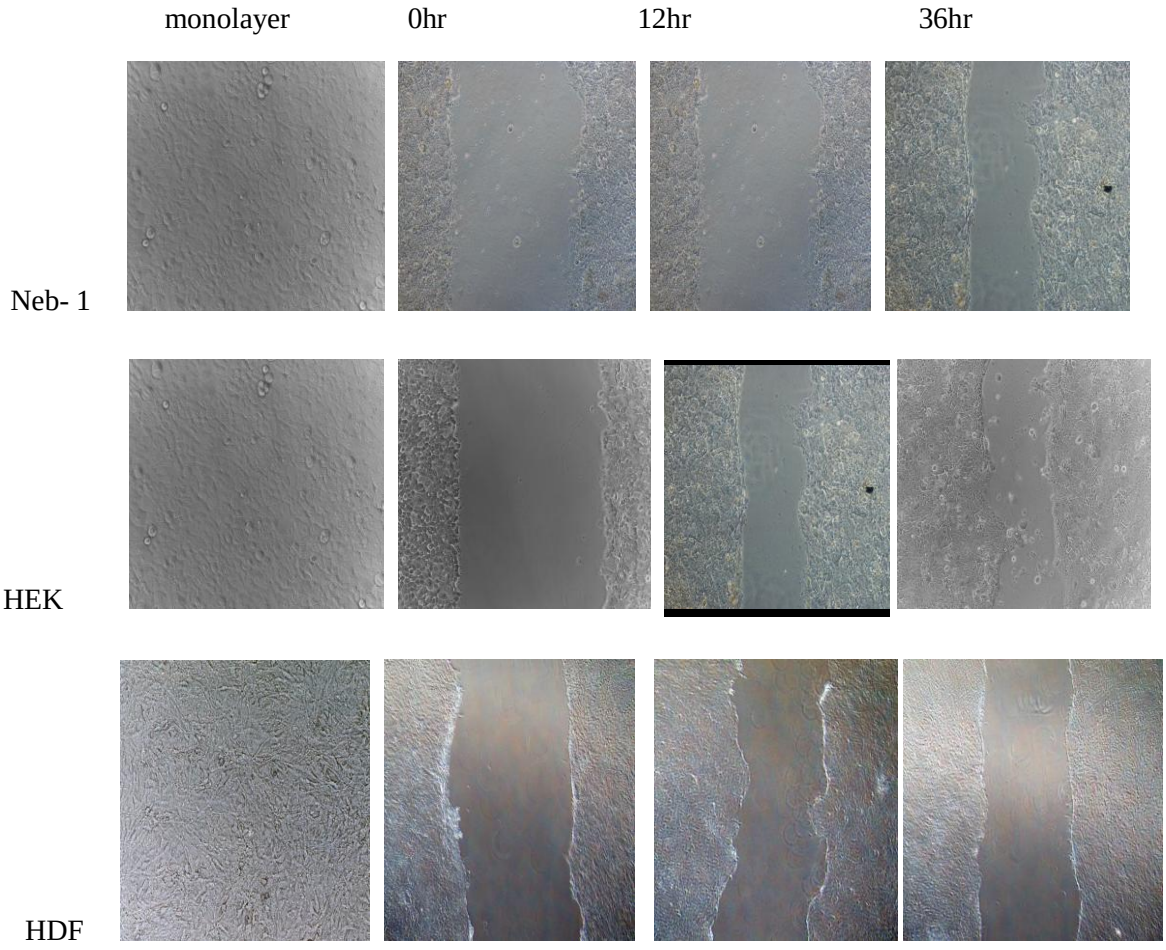
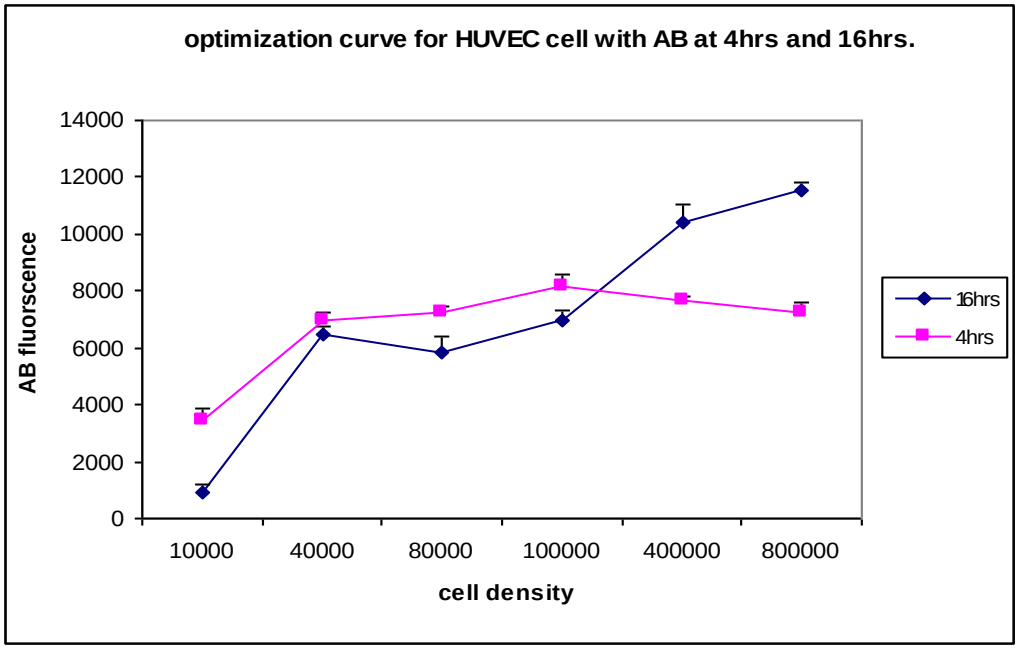


Figure 4.6 Showed photomicrographic images of scratch/ in vitro wound assay with Neb-1 cell, HEK and HDF respectively from the start of the experiment (monolayer), (0hr) a scratch was made with the tip of the pipette at the centre of the monolayer to mark the start of the experiment, changes in the scratch gap was observed over time; 6hr, 12 hrs and 36hrs respectively. The experiment can be terminated at any convenient point and the gap closure over time can then be analyzed either by directly measuring the gap at several points and finding the average or exporting the images to Photoshop and analyzed the area change over time.

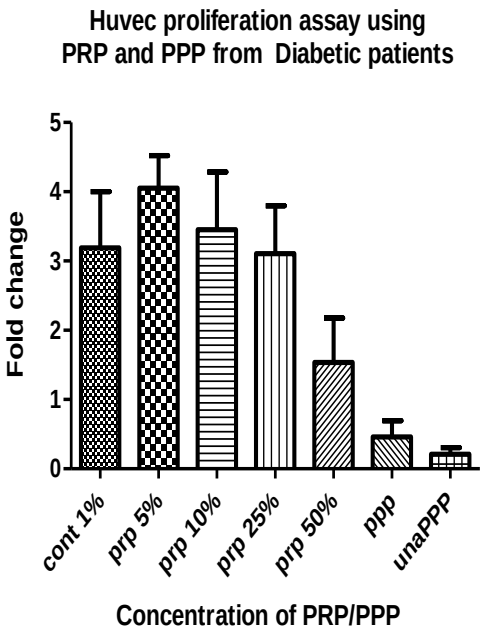
4. 6 Statistical Analysis

A standard curve of % AB reduction was plotted for each of the cell types during the optimization stage. For the proliferation assay; N-fold increase calculated from the Alamar blue fluorescence was used to represent the change in proliferation between day 0 and day 5. Results of cell culture experiments (Proliferation, transwell and scratch assay) were statistically evaluated using one-way analysis of variance, followed by post-hoc Turkey–Kramer multiple comparisons test. Results of the tube-formation assay were statistically evaluated, using one-way analysis of variance, followed by post-hoc Dunnett multiple comparisons test. The significance level for both tests was set at $p<0.05$. Calculations were performed in Graph Pad Instat, Version 5.01 (Graph Pad Software).

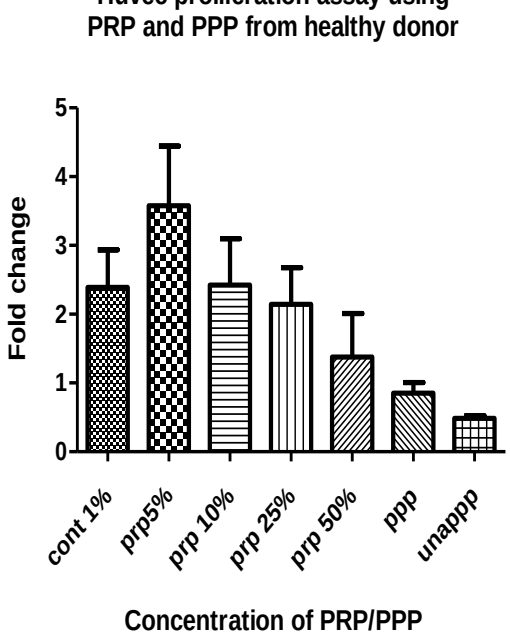
A.



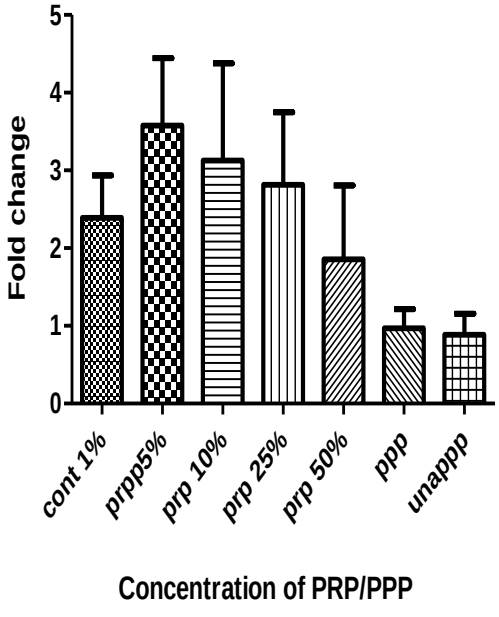
B


Huvec proliferation assay using
PRP and PPP from Diabetic patients
Fold change
0
1
2
3
4
5
cont 1%
prp 5%
prp 10%
prp 25%
prp 50%
ppp
unaPPP
Concentration of PRP/PPP

C


Huvec proliferation assay using
PRP and PPP from healthy donor
Fold change
0
1
2
3
4
5
cont 1%
prp5%
prp 10%
prp 25%
prp 50%
ppp
unappp
Concentration of PRP/PPP

D


Proliferation assay with EA.hy cells
Healthy PRP and PPP
Fold change
0
1
2
3
4
5
cont 1%
prpp5%
prp 10%
prp 25%
prp 50%
ppp
unappp
Concentration of PRP/PPP

E

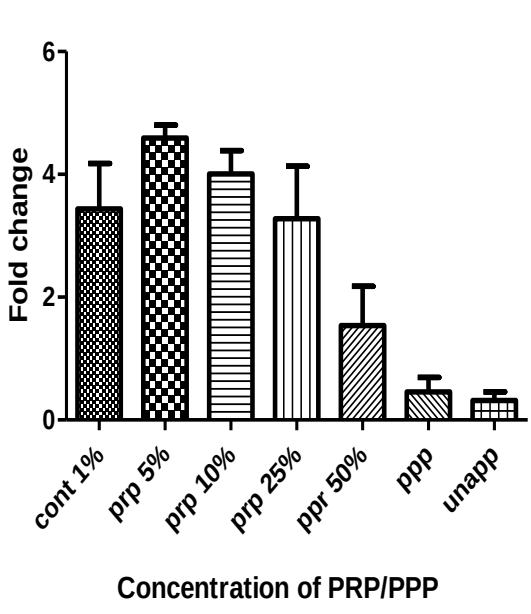
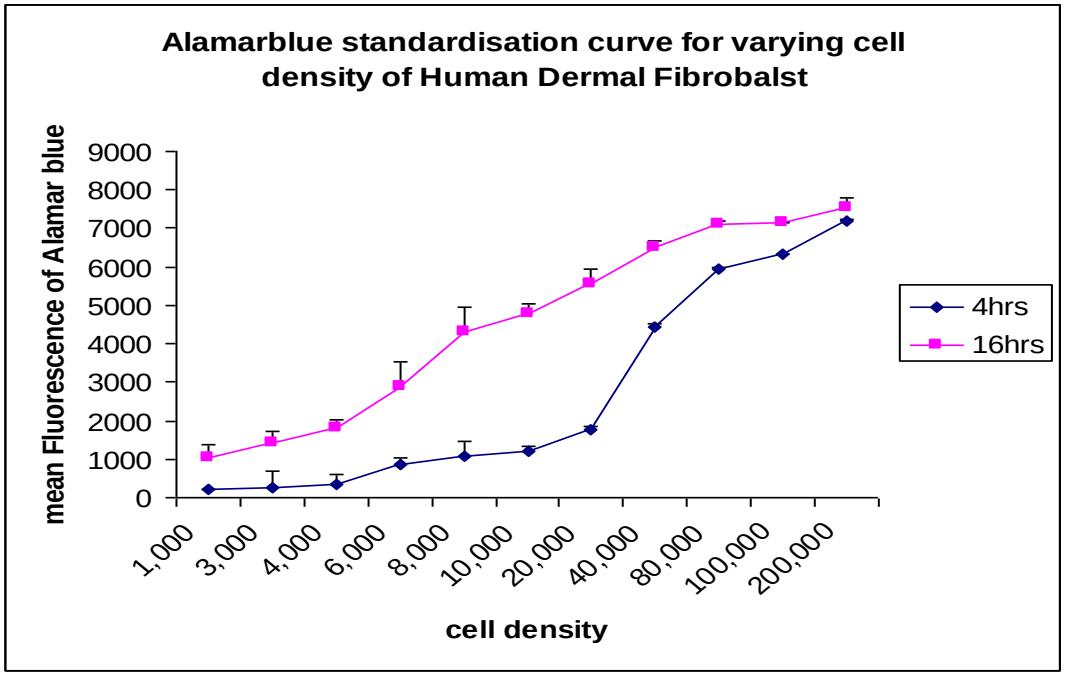

Proliferation assay with EA. hy cell
and PRP/PPP from Diabetic patient
Fold change
0
2
4
6
cont 1%
prp 5%
prp 10%
prp 25%
ppr 50%
ppp
unapp
Concentration of PRP/PPP

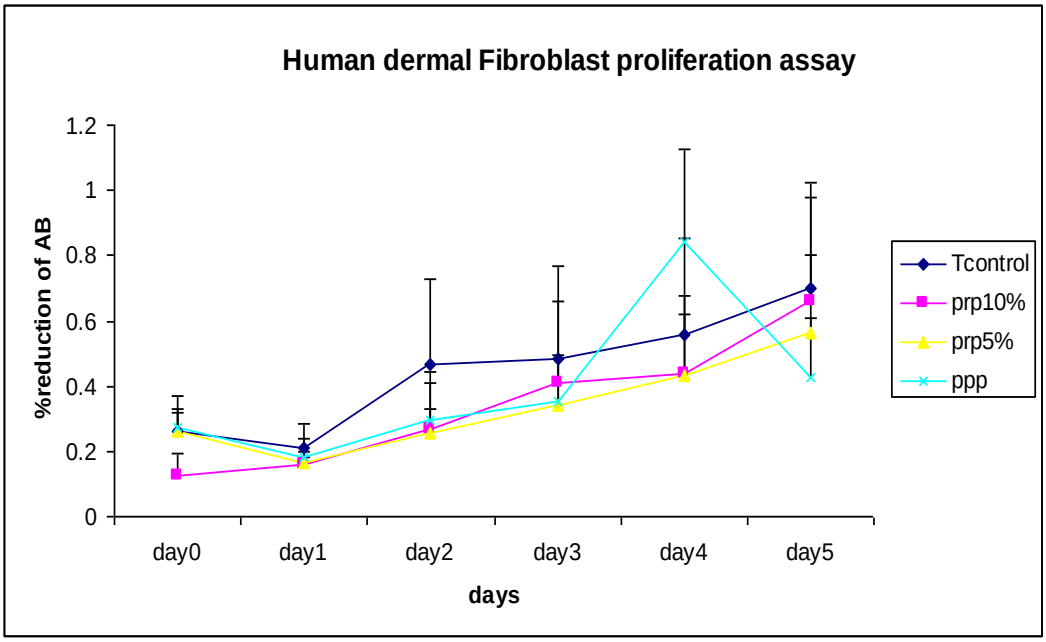
Figure 4. 7 Represents the mean ± SD of the three replicates for the proliferation assay of HUVEC; **A)** the optimization experiment to determine the optimum incubation period for HUVEC with alamar blue. A Plot of N-fold increase against varying concentration of PRP/PPP was subsequently performed. **B&C)** showed proliferation assay of HUVEC with varying concentration of PRP/PPP derived from healthy volunteers and diabetic patients, both showed significance with p value of 0.02 and 0.002 ANOVA analysis of variance respectively. A similar pattern of significance with 5% PRP over PPP and unaPPP was noticed in both groups (p of 0.05). **D&E)** were the results from EA.hy which showed a similar pattern with HUVEC; however no significance was reached either on analysis of variance or on multiple comparison post Hoc testing.

Graphs for all the proliferation assay

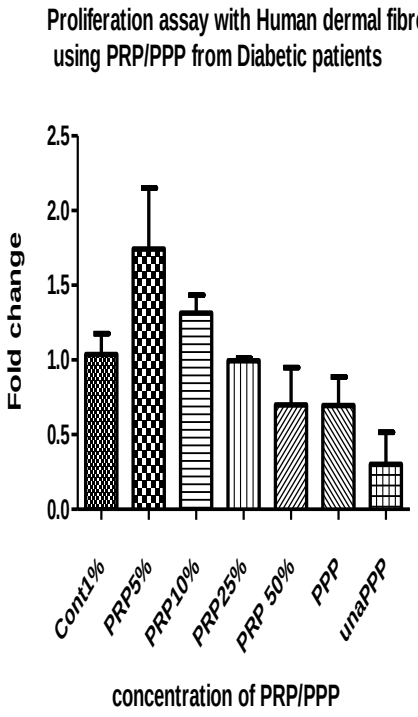
A



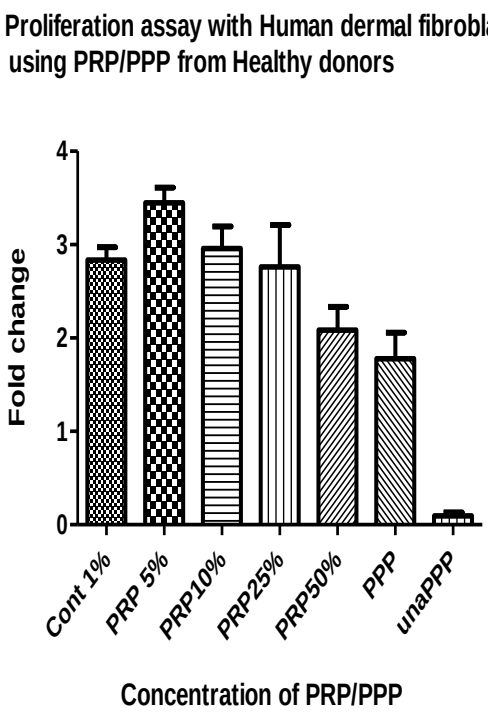
B


Human dermal Fibroblast proliferation assay
%reduction of AB
1.2
1
0.8
0.6
0.4
0.2
0
day0
day1
day2
day3
day4
day5
days
Tcontrol
prp10%
prp5%
ppp

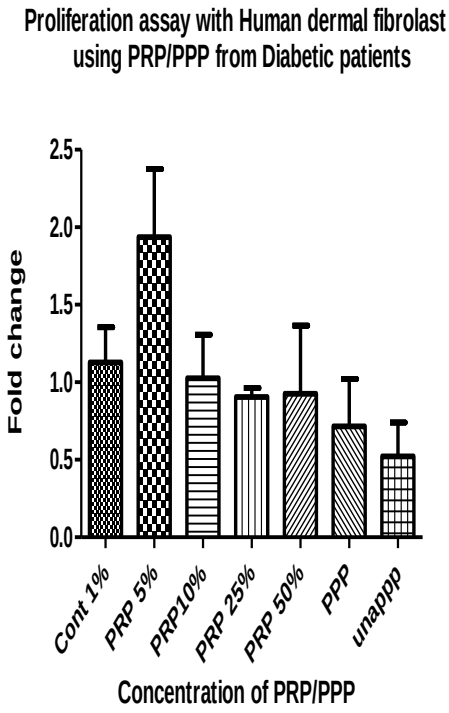
C


Proliferation assay with Human dermal fibroblast using PRP/PPP from Diabetic patients
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
Cont1%
PRP5%
PRP10%
PRP25%
PRP 50%
PPP
unaPPP
concentration of PRP/PPP

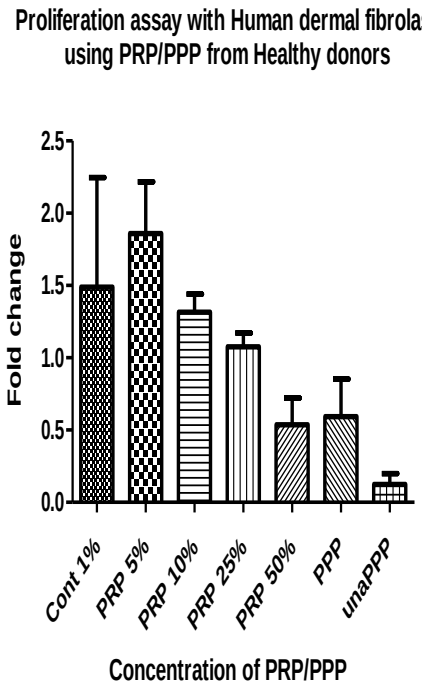
D


Proliferation assay with Human dermal fibroblast using PRP/PPP from Healthy donors
Fold change
4
3
2
1
0
Cont 1%
PRP 5%
PRP10%
PRP25%
PRP50%
PPP
unaPPP
Concentration of PRP/PPP

E


Proliferation assay with Human dermal fibrolast
using PRP/PPP from Diabetic patients
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
Cont 1%
PRP 5%
PRP10%
PRP 25%
PRP 50%
PPP
unappp
Concentration of PRP/PPP

F


Proliferation assay with Human dermal fibrolast
using PRP/PPP from Healthy donors
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
Cont 1%
PRP 5%
PRP 10%
PRP 25%
PRP 50%
PPP
unaPPP
Concentration of PRP/PPP

G

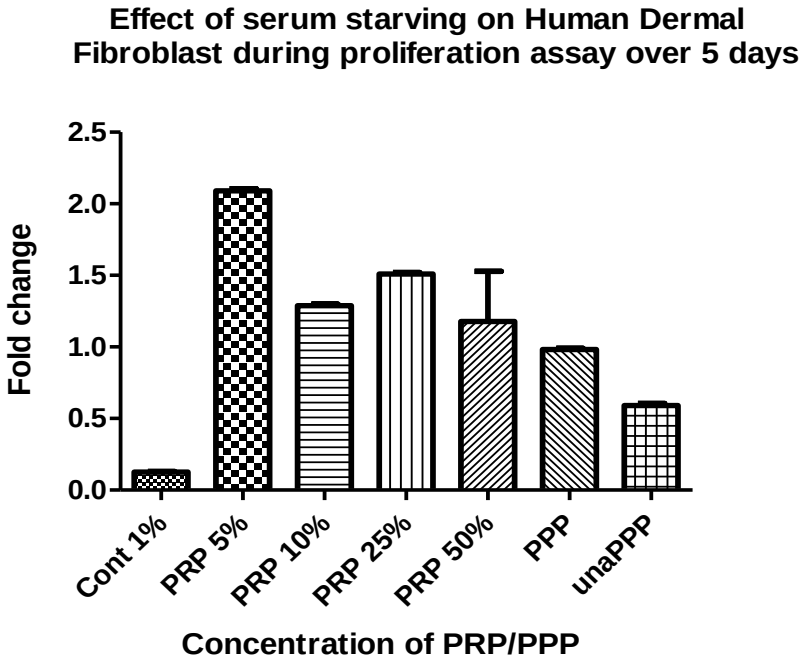
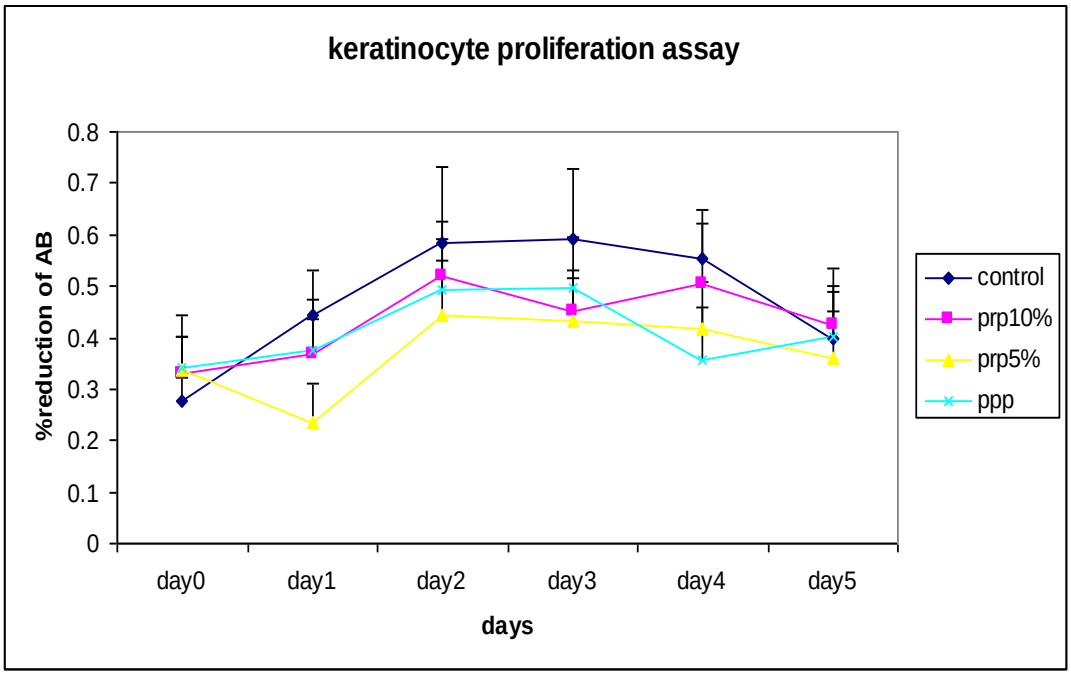
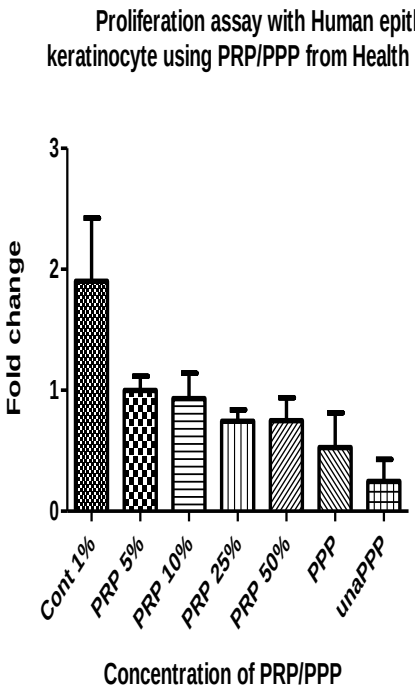


Figure 4.8 Graphic illustration of fibroblast proliferation with PRP/PPP. **A)** Showed the optimization experiment with fibroblast proliferation and optimal incubation period was set at 16hrs. **B).** Showed proliferation over a 5 days with control at FCS 10% producing the maximal effect. There was no statistical significance when a repeated analysis of variance was performed over the 5 day period. **C&D)** Proliferation effect of PRP/ PPP from Diabetic and Healthy donor on fibroblast with maximal effect seen on PRP 5% (v/v) of 1% BSA and an inverse dose dependent relationship was observed. C&D showed significance of 0.01 and 0.0001 on analysis of variance. PRP 5% was also showed statistical significance over PRP 50%, PPP and unaPPP respectively. **E&F)** illustrates proliferation of fibroblast with 1% FCS mixed with (v/v) of PRP/PPP. Analysis of variance for **E** was not significant however **F** reached significance at 0.04, PRP 5% from the healthy donor reached a significance over unaPPP at 0.04 **G)** Serum starvation effect on fibroblast proliferation over 5 days produced the most marked effect at PRP 5% with statistical significance of $p< 0.0001$. In addition PRP 5%, 10%, 25% and 50% all showed significant fold proliferation over the control experiment at <0.0001 for 5% PRP and < 0.001 for the other concentrations.

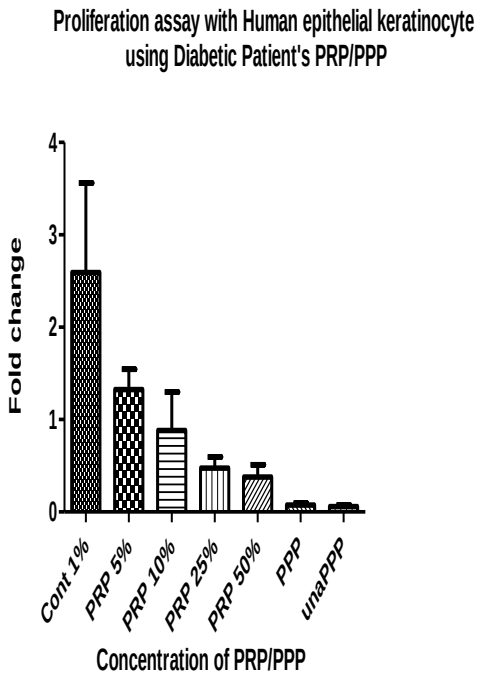
A


keratinocyte proliferation assay
%reduction of AB
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
day0
day1
day2
day3
day4
day5
days
control
prp10%
prp5%
ppp

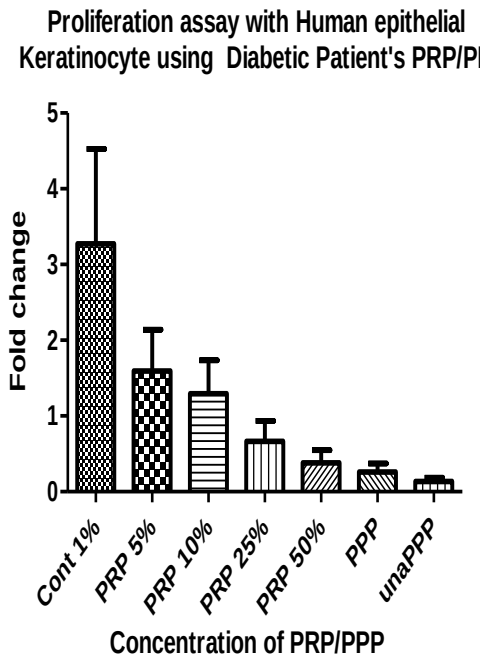
B


Proliferation assay with Human epithelial keratinocyte using PRP/PPP from Health donors
Fold change
3
2
1
0
Cont 1%
PRP 5%
PRP 10%
PRP 25%
PRP 50%
PPP
unaPPP
Concentration of PRP/PPP

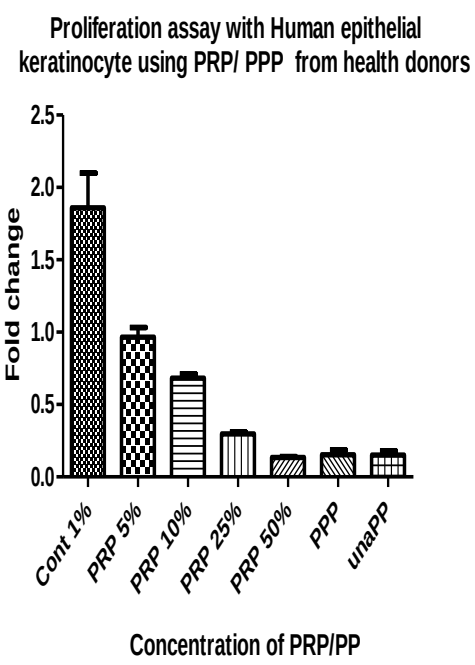
C


Proliferation assay with Human epithelial keratinocyte using Diabetic Patient's PRP/PPP
Fold change
4
3
2
1
0
Cont 1%
PRP 5%
PRP 10%
PRP 25%
PRP 50%
PPP
unaPPP
Concentration of PRP/PPP

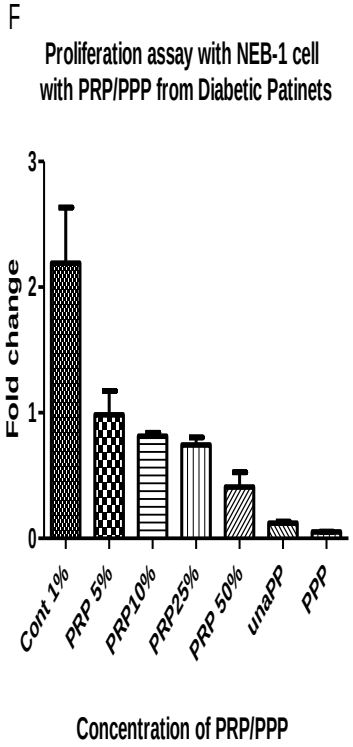
D


Proliferation assay with Human epithelial Keratinocyte using Diabetic Patient's PRP/PPP
Fold change
0
1
2
3
4
5
Cont 1%
PRP 5%
PRP 10%
PRP 25%
PRP 50%
PPP
unaPPP
Concentration of PRP/PPP

E


Proliferation assay with Human epithelial keratinocyte using PRP/ PPP from health donors
Fold change
0.0
0.5
1.0
1.5
2.0
2.5
Cont 1%
PRP 5%
PRP 10%
PRP 25%
PRP 50%
PPP
unaPP
Concentration of PRP/PP

F


Proliferation assay with NEB-1 cell with PRP/PPP from Diabetic Patinets
Fold change
0
1
2
3
Cont 1%
PRP 5%
PRP10%
PRP25%
PRP 50%
unaPP
PPP
Concentration of PRP/PPP

G

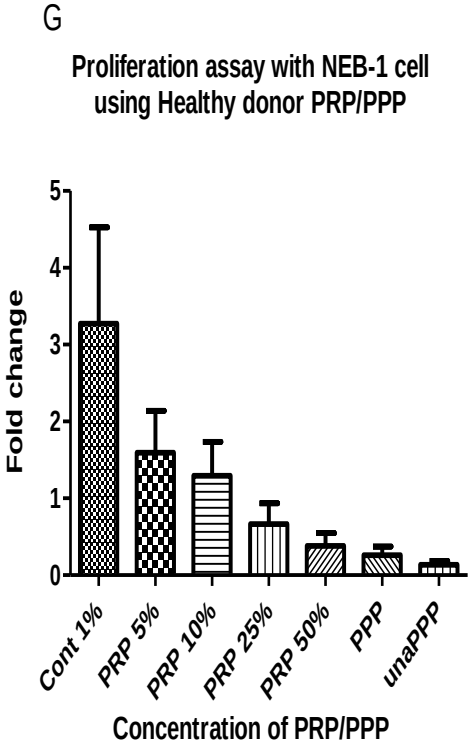
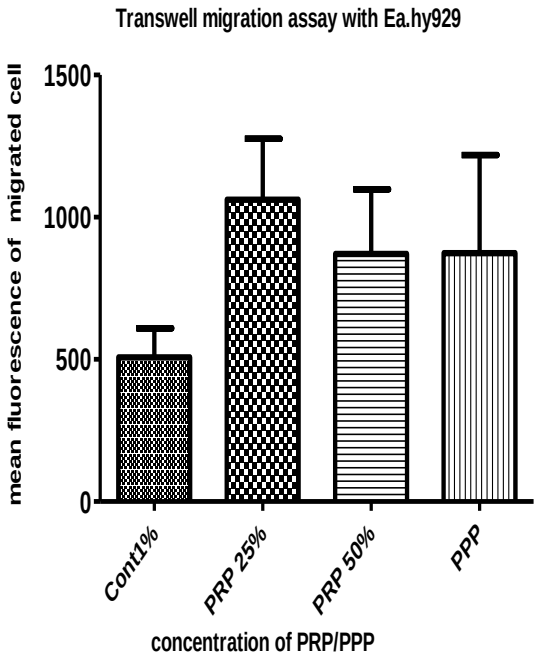

Proliferation assay with NEB-1 cell using Healthy donor PRP/PPP
Fold change
0
1
2
3
4
5
Cont 1%
PRP 5%
PRP 10%
PRP 25%
PRP 50%
PPP
unaPPP
Concentration of PRP/PPP

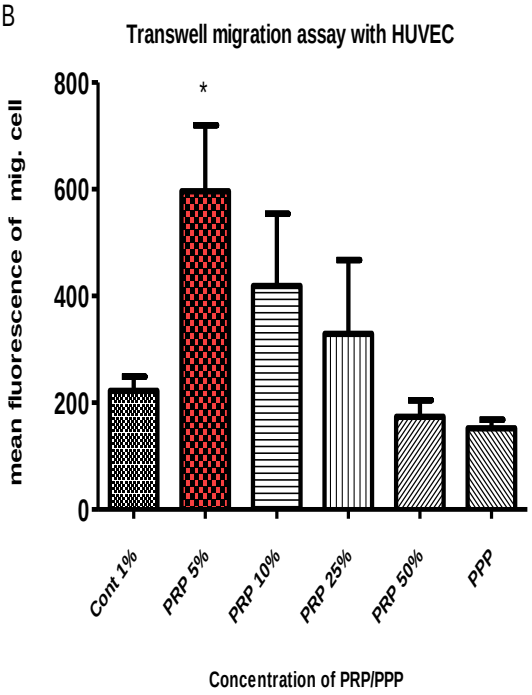
Figure 4.9 Showed proliferation assay with keratinocyte. **A).** Time course plot of keratinocyte proliferation over 5 days. Varying concentration of PRP/PPP with *v/v* of FCS 10% and control at 10% FCS. The proliferative effect was higher in the control experiment but was not statistically significance on repeat analysis of variance with $p > 0.5$. **B&C)** Showed proliferation effect of PRP /PPP from with v/v 1% BSA for both experiment with most marked effect seen on the control, however the decreasing dose pattern was still evident with the varying concentration of PRP. Univariate analysis showed of $p< 0.02$ and 0.008 for **C&D** respectively. **D&E)** Kerationcyte proliferation with 1% FCS showed marked significance on control experiment and a similar pattern with BSA. Univariate analysis was p of 0.02 and <0.0001 respectively. **F&H)** Neb-1 cell proliferation assay behaved in a similar pattern to keratinocyte, with 1% control displaying a statistical proliferative effect over all other treat with p value of 0.02 and 0.0001.

Graphs for transwell migration assay

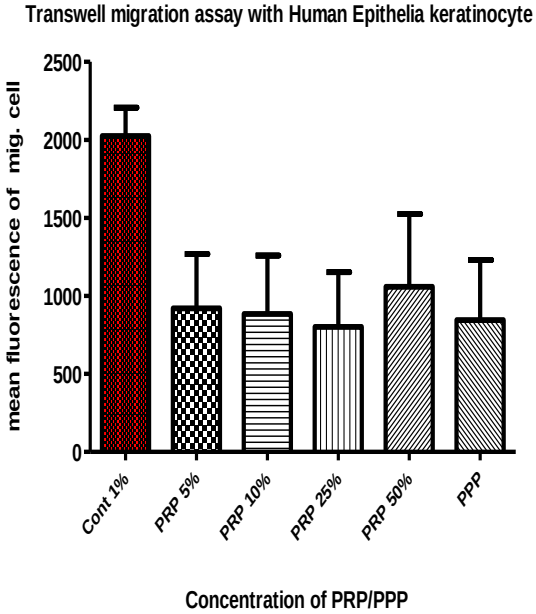
A


Transwell migration assay with Ea.hy929
mean fluorescence of migrated cell
1500
1000
500
0
Cont1%
PRP 25%
PRP 50%
PPP
concentration of PRP/PPP

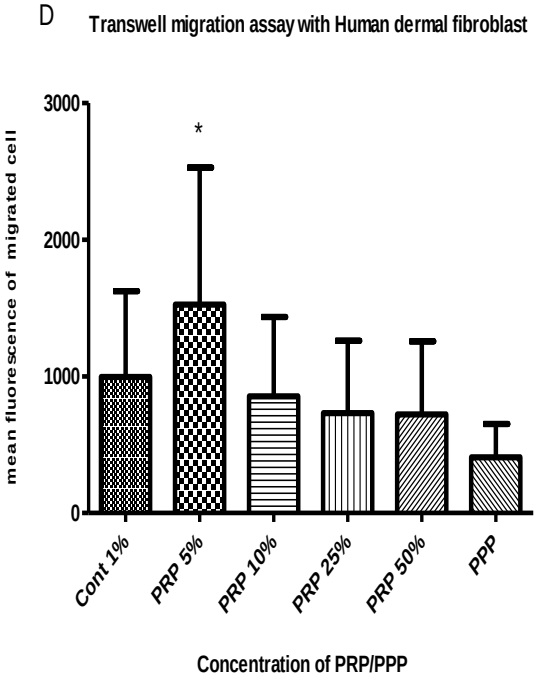
B


Transwell migration assay with HUVEC
mean fluorescence of mig. cell
800
600
400
200
0
*
Cont 1%
PRP 5%
PRP 10%
PRP 25%
PRP 50%
PPP
Concentration of PRP/PPP

C


Transwell migration assay with Human Epithelia keratinocyte
mean fluorescence of mig. cell
2500
2000
1500
1000
500
0
Cont 1%
PRP 5%
PRP 10%
PRP 25%
PRP 50%
PPP
Concentration of PRP/PPP

D


Transwell migration assay with Human dermal fibroblast
mean fluorescence of migrated cell
3000
2000
1000
0
*
Cont 1%
PRP 5%
PRP 10%
PRP 25%
PRP 50%
PPP
Concentration of PRP/PPP

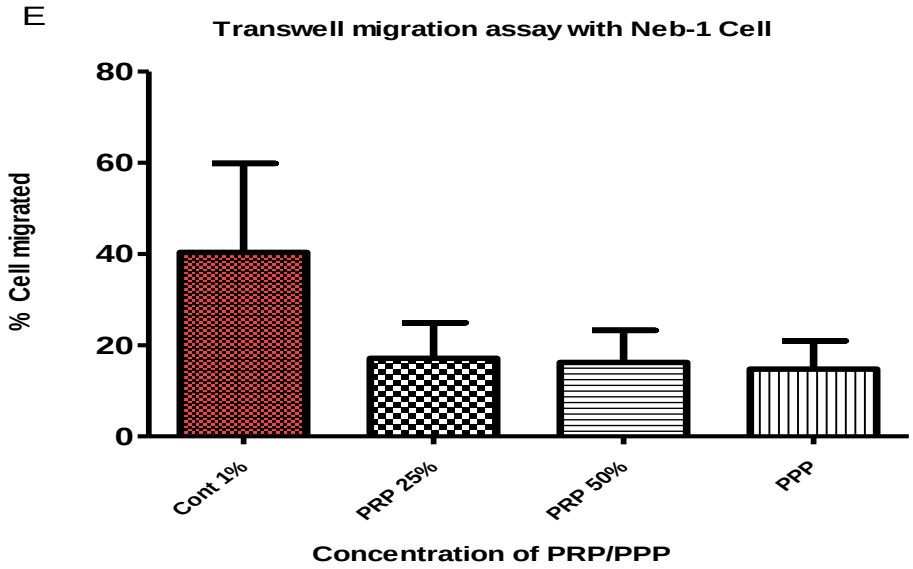
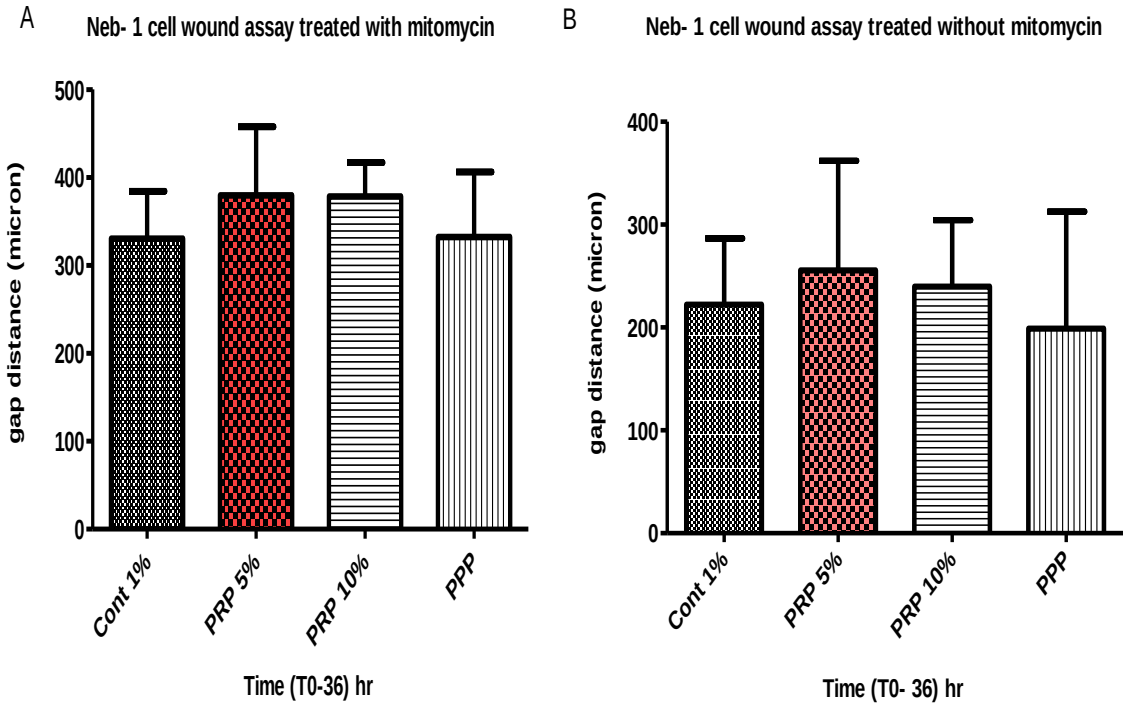
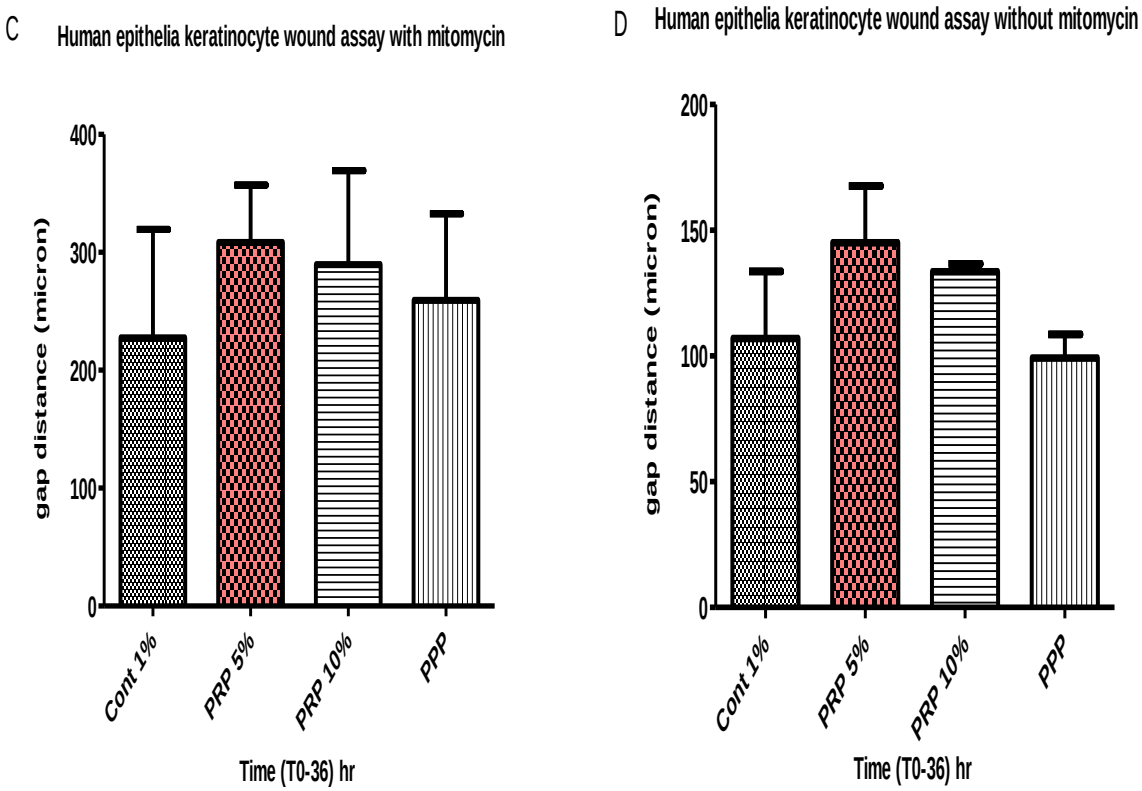


Figure 4.10 Represents the graphic illustration of transwell migration assay for all the different cell types. **A)** Showed transwell with Ea.hys, there was no significance on the analysis of variance or on comparison of the individual treatment groups. **B)** HUVEC showed significance of 0.0031 on ANOVA and Post –Hoc test demonstrated that PRP 5% was statistically significant over cont 1%, PRP 50% and PPP with p value of < 0.005. **C&E)** Keratinocyte and NEB-1 cell behaved in a similarly pattern on transwell migration assay with no significant amongst all the treatment as well on analysis of variance **D)** fibroblast has a p value of 0.012 on analysis of variance and PRP 5% showed a statistical significance over PPP with p of 0.05.

Graphs for *in vitro* wound assay





E

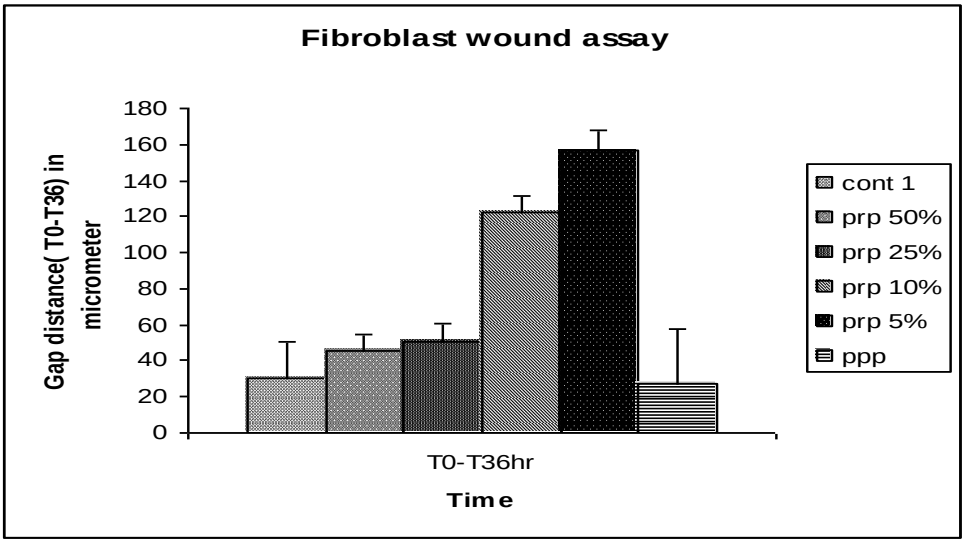
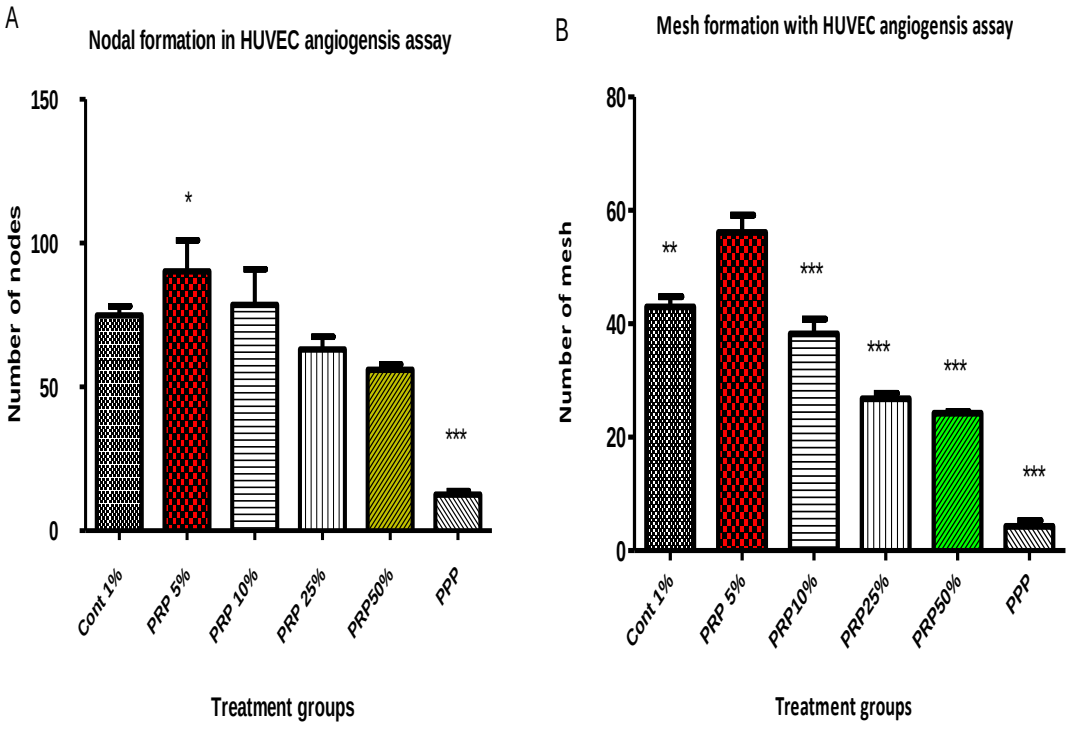


Figure 4.11 Represents scratch / in vitro wound assay with HEK/ NEB -1 cell and HDF. **A&B)** illustrates wound assay with NEB-1 cell with and without mitomycin treatment. Although the treatment concentration was for control, PRP 5%, 10% and PPP. There was no statistical significance

on analysis of variance or comparison of the treatment groups. **C&D)** HEK behaved in a similar pattern to NEB -1 cell. Although the treatment did not reach statistical significance, PRP 5 % has the most positive effect on gap closure during the assay. **E)** HDF showed a different characteristic behaviour with PRP 5% showing statistical significance over the control, PRP 25% &50% and PPP treatment and reaching a statistical significance of <0.05.

Graphs for angiogenesis assay



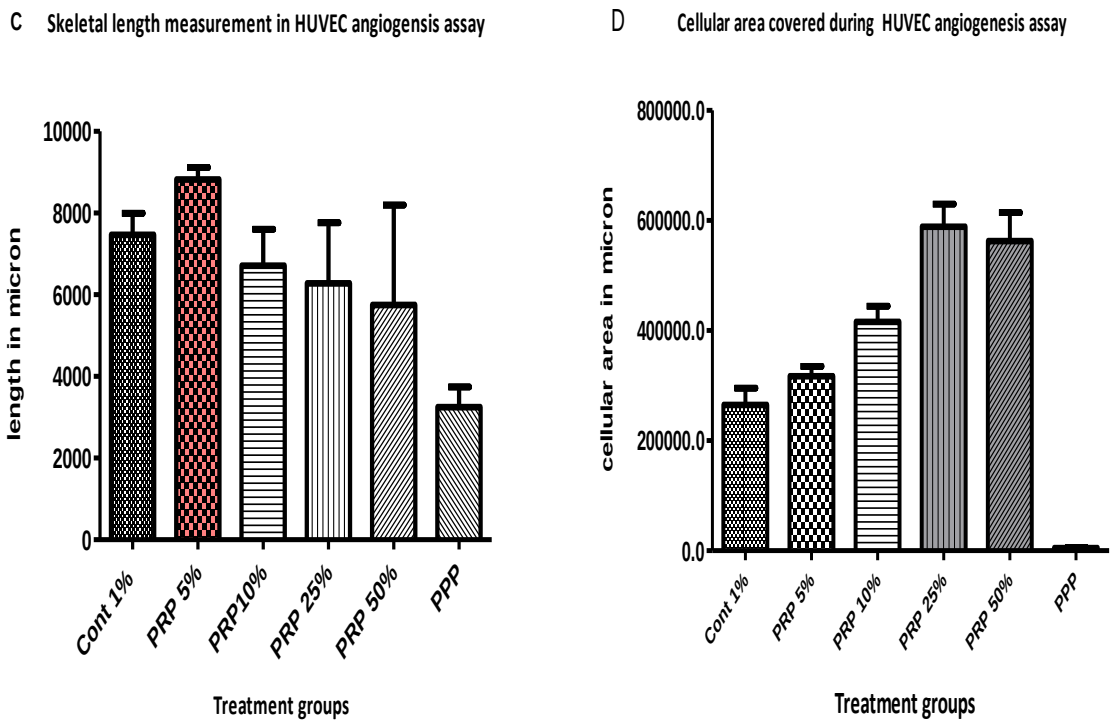


Figure 4.12 Angiogenetic effect of PRP on HUVEC cell. Control 1% is the positive control (full complement of EMG with 1% FCS), PRP derivatives (without any of the EMG complement) ranging from 5% to 50% and PPP was the negative control. All experiments were performed in triplicate, the bars represent mean ±SD. **A)** Considering nodal formation; PRP 5% showed a significance over 50% and PPP at p value of <0.05 and <0.001 respectively and a p value of <0.0001 on one-way analysis of variance for the entire group. **B)** Mesh formation also showed a p value of <0.0001 on one-way analysis of variance. PRP 5% showed a significance of <0.0001 over PRP 10%, PRP 25%, PRP 50% respectively and PPP but showed significance of <0.001 over the positive control. **C)** There were no statistical difference amongst the entire group either on one-way analysis of variance or on the post –hoc testing. **D)** PRP 25% and PRP 50% showed a level of significance over the other modes of treatment when considering the cellular area cover during angiogenesis.

4.7 RESULTS

4.7.1 Evaluation of Cell Proliferation

After the initial experiment were carried out to determine the optimal seeding density and culture period. Cells were seeded and incubated for a 5 days period, proliferation rate were determined using alamar blue (AB) technique. Proliferation measurement was done spectrophotometricaly using absorbance test wells at 540 and 630 nm and flurometeric measurement by exciting samples at 560nm and measuring emission at 590nm. The number of viable cells correlates with the magnitude of dye reduction and is expressed as percentage of AB reduction (colour change in the medium, which is usually from blue to pink). To calculate AB reduction for a given test well plate, a blank reading (negative control medium without cell) was subtracted from the test well plate reading. The readings were taking at the start of the experiment (day 0) and at the end (day 5) after incubation with alamar blue for 16hours. A fold change (increase or decrease) in the proliferation rate was calculated by dividing the day 5 plate reading with day 0.

$$\text{Fold change} = \frac{\text{Plate reading at day 0}_{590nm} - \text{Blank reading at day 0}_{590nm}}{\text{Plate reading at day 5}_{590nm} - \text{Blank reading at day 5}_{590nm}}$$

4. 7.1.1 Human umbilical Vein Endothelia Cell and Ea. Hys Cell

When fluorescence reduction was plotted against the cell concentration, the standard curve was linear between 80,000 and 800,000 cells /mL with a (correlation value) r^2 values of 0.8799 **(Fig 4.7a)**. A significant increase in endothelial cell proliferation was observed on univariate analysis with the addition of PRP supernatants, as compared with minimal medium alone. The HUVEC proliferation with diabetic patient PRP and the healthy donor had p values of 0.002 and 0.02 **(Fig 4.7b &c).** A similar pattern was seen with EA.hy cells **(Fig 7d &e),** which reached significance at 0.04 and 0.01 with healthy and diabetic PRP respectively. The proliferation of HUVEC and EA.hys cell is most marked at PRP 5% with p value of <0.05 over activated and inactivated PPP. Although PRP 5% produced the most marked proliferative effect; there were no significant differences over the other forms of treatment (PRP 10%, 25% & 50%). Furthermore, a decrease dose dependent pattern was observed in both the different sources of PRP (healthy and diabetic donors) and the two cell types.

4.7.1.2 Human Dermal Fibroblast

When fluorescence reduction was plotted against the cell concentration, the standard curve was linear between 8,000 and 80,000 cells /mL with an r^2 values of 0.9715 **(Fig 4.8a).** An initial proliferation experiment was performed with the positive control media at 10% of FCS (v/v) and the PRP were at 5% and 10% and PPP over a 5 day period **(Fig 4.8b).** The positive control showed the most promising proliferative effect over the other form of treatment, however did not reach statistical significance on repeated analysis of variance (p value >0.05). The initial thinking was that by increasing the concentration of PRP to 25% and 50% and reducing the positive control media concentration a more positive proliferative result might be observed but a decreasing dose dependent was observed. Furthermore, a different serum protein at 1% concentration was introduced (Bovine serum albumin, {BSA}), and a comparison was then made with 1% FCS.

A significant statistical difference was observed when 1% BSA was used as the diluting media for PRP/ PPP concentration as well as for the positive control, with a p value of 0.001 and 0.01 on univariate analysis for PRP from healthy donor and diabetic donors. PRP 5% showed a significant fold increase over PRP 50%, PPP and inactivated PPP with p value of 0.01, 0.001 and 0.0001 respectively **(Fig 4.8c &d).** On the other hand with 1% FCS as the diluting media (v/v) PRP/PPP, the supernatant from diabetic donor did not reach significance (p of >0.05), whereas the supernatant from healthy donor reached significance with p value of 0.04 **(Fig 4.8 e& f).** Although, the statistical significant was not reached with supernata from diabetic patients , the two sources of PRP/PPP still maintained a decreasing dose dependent relationship and PRP 5% produced the most positive proliferative effect. Further more, the effect of serum starvation on fibroblast proliferation produced a more remarkable proliferative effect of PRP /PPP over the positive control media **(Fig 4.8 g).** With serum starvation all the varying concentration of PRP/PPP showed a statistical significance difference over the positive control experiment with p value of <0.001.

4. 7.1.3 Human Epithelia Keratinocyte (HEK) and NEB-1 cell

An initial proliferation experiment was performed with the positive control media at 10% of FCS (v/v) and the PRP were at 5% and 10% and PPP over a 5 day period **(Fig 4.9a).** The positive control showed most promising proliferative effect, however did not reach statistical significance on repeated analysis of variance (p value >0.05). Further experimental repeats were carried out with further reduction in FCS to 1% and another serum protein was introduced (BSA). HEK proliferation assay was performed as outlined for fibroblast above. Both the positive control at 1% FBC and 1% BSA had a greater proliferative effect as compared to the varying concentration of PRP/PPP from the

2 different **supernatants (Fig. 4.9b, c, d& e**). Similarly, NEB-1 cell also used a similar pattern of proliferation as HEK **(Fig 4.9 f &g).** Overall, a lesser proliferative effect was observed with treating HEK with PRP/PPP as compared to the positive control. The decreasing dose dependent was still evident in amongst the well plates treated with PRP/PPP for both HEK and NEB-1 cell proliferation assay.

4.7.2 *In Vitro* Cell Migration Assay

4. 7.2.1 Transwell Cell Migration Assay

Transwell migration with EA.hys cell did not reach statistical significance either on one-way ANOVA or on post –hoc testing **(Fig 4.10a).** Transwell migration with HUVEC was significant on one-way ANOVA with a p value of 0.0003 and trans-migration with PRP 5% was significant over the positive control, PRP 25%, 50% and PPP with P values of < 0.005 **(Fig 4.10b).** Interestingly, transwell migration with HEK and NEB-1 cell behaved similarly to wound/ scratch assay; the positive control showed better migration than the varying treatment concentrations of PRP **(Fig 4.10c&e).** Finally, transwell migration with human fibroblast was significant on univariate analysis with a p value of 0.01 and transmigration with PRP 5% has the most positive effect with a significant difference over PRP 50% and PPP with p values of <0.05.

4.7.2.2 Wound / Scratch Assay

There were no demonstrable statistical difference between the mitomycin treated and non-treated wound assay for either HEK or NEB-1 cell **(Fig 4.11 a, b, c & d).** Treatment with PRP 5% used a promising migratory effect over all the other treatment although did not reach statistical significance. On the other hand, fibroblast wound assay **(Fig 4.11e)** with PRP 5% showed a significant cell migratory effect over the other treatment concentration with p value of 0.001. Elimination of cell proliferation bias was not achievable with human fibroblast, the mitomycin had a strong apoptotic effect on fibroblast, and hence, the assays were performed without mitomycin treatment. Furthermore the decreasing dose dependent pattern was clearly demonstrated with fibroblast scratch assay.

4.7. 3 Angiogenesis Assay with HUVEC

Nodal formation with HUVEC showed significance on univariate analysis with p value of <0.0001and PRP 5% has the most positive angiogenic effect and also reaching significance over PRP 50% and PPP with p value of <0.005**(Fig 4.12 a)**. Mesh formation analysis was similar to nodal formation, with significance on one –way ANOVA of variance of < 0.0001 and PRP 5% has the most angiogenic effect. PRP 5% was significant over positive control (p of <0.001) and p value

of <0.0001 for PRP10%, 25%, 50% and PPP **(Fig 4.12b)**. The decreasing dose dependent trend was also observed with nodal and meshes formation. Skeletal length measurement did not show any significance both on univariate analysis and on post –hoc testing **(Fig 4.12c)**. Finally, analysis of cellular area covered showed an inverse proportionality graphical when compared with mesh and nodal formation **(Fig 4.12d).** This could mean that this particular parameter is not a reliable means of assessing angiogenic effect.

4. 7.3.1 Rationale for Combining Morphometric and Topological Parameters

The combination of the morphometeric and topological parameters seems to sufficiently describe morphological changes in angiogenesis. Hence, the observation qualifies these 2 parameters as a significant characteristic of a 2D branching and anatomising structure. Although, the matrigel assay does not simulate the whole process of angiogenesis, it provides valid and objective assessment of the two key angiogenic pathways which are the migration and differentiation of endothelial cells. Until recently, morphometric methods to evaluate the effects on Matrigel of angiogenic inducer or inhibitors were mostly descriptive. The changes in tubule formation present the principal angiogenic response in this assay.

The combination of the topological and morphometric parameters provides an objective and reproducible measure in assessing anti or pro-angiogenic effect of a given experimental condition (Guidolin D et al,. 2004) in their recent study showed that total length of the formed capillary next work per unit field area appears to be more accurate descriptor since its values is less influenced by the presence of cell aggregates among the tubules. It is important to know that morphometric parameters do not fully characterise the architecture of the tubule pattern, in that there other physiological features of angiogenesis that this does not account for, such as branching density and sprouting. Also the topological parameters have the added advantage of allowing detection of changes in the cell arrangement even in the conditions where no meshes are formed, in cases of high inhibition and early stages of angiogenesis. New techniques are available that allow morphometry of three-dimensional structures allowing quantitative measurement of vessel diameter and lengths, intervascular and interbranching distances as well as branching (Brey et al ,2002).

4. 4 DISCUSSION

The wound-healing process is a complex mechanism characterized by four distinct, but overlapping phases: haemostasis, inflammation, proliferation, and remodelling. The proliferative phase includes blood vessel formation by endothelial cells and bone synthesis by osteoblasts. We developed an experimental model to test our hypothesis that PPR has a biological effect in the wound healing process. It has been demonstrated that the release of PDGF-BB, TGF-β1, bFGF, and VEGF is

significantly regulated by the amount of calcium and thrombin added to the PRPs, and that PRP supernatants are more mitogenic for endothelial cells than whole-blood supernatants, reinforcing the scientific evidence for a beneficial role of PRPs in tissue regeneration (Lacoste *et al.*, 2003; Martineau *et al.*, 2004). Other GFs such as epidermal growth factor (EGF), transforming growth factor-alpha (TGF- α), and insulin-like growth factor-1 (IGF-1), angiopoietin-2 (Ang-2), and interleukin-1beta (IL-1 β) are also known to play important roles in the wound-healing process (Werner and Grose, 2003). All these events are coordinated by cell-cell interactions and by soluble GFs released by various cell types. Thrombin represents a strong inducer of platelet activation leading to GF release (Furman *et al.*, 1998). It is also known that particulate grafts, when combined with calcium and thrombin-treated PRPs, possess better handling characteristics and higher GFs content (Froum *et al.*, 2002; Weibrich *et al.*, 2002). Hence, in this study both the PRP and PPP were activated with autologous derived thrombin before quantification of the growth factors of interest. PRPs from 14 healthy donors and 6 diabetic patients were prepared according to the PCCS system, with platelet yields and recoveries similar to already published series.

4.4.1 Proliferation of HDF, HUVEC and HEK

PDGF is a powerful mitogen for fibroblasts and smooth muscle cells and is involved in all the three phases of wound healing (Hosgood, 1993). PDGF is composed of two polypeptide chains (A and B) combined in three disulfide-linked dimeric forms (AA, AB, and BB). Human platelets have all three PDGF dimmers, in quantities of approximately 65 percent AB, 23 percent BB, and 12 percent AA. In this study, PDGF-AA was evaluated because of its easy commercial availability. In contrast, the TGF-β family of proteins has a distinguishing ability in that they reversibly inhibit the growth of a number of cell types, particularly cells derived from the ectoderm such as keratinocytes and leukocytes (Olashaw et.al, 1986). However, it has been reported that TGF-β are weak mitogens for cells derived from the mesoderm, such as fibroblasts. Furthermore, activated PRP has also been shown to be mitogenic for a variety of cell types, such as human mesenchymal progenitor cells, mesenchymal stem cells from adipose tissue, endothelial cells, and human osteoblasts and gingival fibroblasts. (Kilian et.al 2004, Kocaoemer 2007, Graziani et .al, 2006)

Graziani et al. 2006, reported that the maximum effect of activated platelet-rich plasma was achieved in a concentration of 2.5 x platelet-rich plasma, with higher concentrations resulting in a reduction of cell proliferation. Choi et al, 2005, examined the influence of platelet-rich plasma concentrations on the viability and proliferation of alveolar bone cells, and the results showed that the viability and proliferation of alveolar bone cells were suppressed by high platelet-rich plasma concentrations but were stimulated by low platelet rich plasma concentrations (1% to 5%). Another study by Kakudo et. al, 2008, showed that activated PRP also stimulated the proliferation of human adipose–derived

stem cells and human dermal fibroblasts. This result showed that 1% and 5% were suitable concentrations for human adipose–derived stem cells proliferation, and 5% was a suitable concentration for human dermal fibroblasts. Kakudo et. al, postulated that the difference in the degree of cell differentiation (adipose-derived stem cells and mature fibroblast) may have influenced the sensitivity of PRP to cytokines leading to differences in the appropriateness of concentrations.

Endothelial cell proliferation, which represents a key step in the angiogenic process, was chosen as an *in vitro* model, based on the work of Knighton et al., who demonstrated that the use of thrombin-treated platelets significantly increased the healing rate of the rabbit cornea, which directly correlated with the induction of angiogenesis. In this study, we found that supernatants collected after PRP activation with thrombin and calcium significantly enhanced proliferation of HUVECs in vitro. A similar decreasing dose dependent pattern was noticed with 5% producing the optimal proliferative effect. A similar biological effect of PRP on HUVEC has been published by Martineau, 2004 and Bertrand-Duchesne, 2009. Human epidermal keratinocyte behaved slightly different to the HUVEC and HDF cells, in that the control experiment showed the maximal proliferative and statistical significant effect over the various concentration of PRP. Although the decreasing dose trend was still maintained, the positive control cultured well showed a far more proliferative effect in both HEK and NEB-1 cell.

Switalska et. al 1985, demonstrated that in addition to the growth factors released from platelets and their positive effect on wound healing, thrombospondin (TSP) is also abundant in the α- granule of platelets. TSP family consist of five members (TSP 1-5). TSP-1 is believed to inhibit adhesion, proliferation and tube formation of endothelial cells in celluture, and has been found to block neovascularization of chick chorioallantoic membrane (Bornstein, 1994). TSP-1 also inhibits the migration and proliferation of endothelial cells invitro. However, there is no evidence to suggest a similar inhibitory function on wound healing for TSP 3-5 (Armstrong, 2003). Thrombospodin-1 is a well investigated angiogenesis inhibitor released by platelets, but this molecule is seldom mentioned in studies of PRP. Weiberch et al., 2002, Okuda etal., 2003 and Eppley 2004 reported that the range of TGF-β1 concentration is 120-169.4 ng/mL and that of PDGF- AB is 117.-182 ng/mL. In our study we reported quantified PDGF-AA and TGF- β1 from healthy donor as 131- 123.6 ng/ml and 27.24- 32 ng / ml respectively.

The concentration of TSP-1 in this study was reported as 1184- 2866 ng/ml which is obviously about 10 times the greater than PDGF-AA and 90 times greater than TGF- β1. It's currently unknown whether TSP-1 serves as a more potent negative healing factor than any of the specific

growth factors found to be released by platelets in PRP preparation. From the published series, Hsu et.al 2009 showed that TSP-1 has similar antiproliferative effect on human osteoblast, periodontal ligament fibroblast and endothelial cell. Although there were no sufficient prove that TSP-1 was responsible for the antiproliferative effect of high concentrations of PRP on the cell investigated. This result is very similar to our result, in that an antiproliferative effect was observed on Ea.hys cell, HUVEC, HDF, Neb-1 cell and HEK. This phenomenon appears to be in an increasing dose dependent fashion. Furthermore, HDF showed marked reduced cell proliferation when serum-free controls was tested, but cell proliferation markedly increased in the presence of activated platelet-rich plasma on day 5 of cell culture to more than 2-fold increase. It is worth mentioning at this point that platelet poor plasma and inactivated plasma showed some positive biological proliferative effect in all the cell type, howbeit, little effect.

Based on our study and other published data, the proliferative effects of PRP on cells have an optimal range of concentrations instead of a continuous linear relationship. One plausible mechanism for the nonlinear relationship may be the complex composition of proteins released by the platelets. Negative regulators may nullify or reduce the positive effect of growth factors released by platelet. Further research with TSP-1 neutralized antibody or a competitive assay to clarify the mechanism of TSP-1's negative effect on wound healing might be helpful in understanding this phenomenon. In addition to TSP-1, there are other cytokines which may have a negative biological effect from PRP. In our study, we also measured Platelet factor-4 (PF-4) along with TSP-1 from activated PRP, PF-4 have been shown to have potent anti-angiogenic effect but has not been directly related to cell proliferation (Taylor and Folkman, 1982).

4. 4. 2 Migration Assay with HDF, HUVEC and HEK

In this study, we have demonstrated using two different *in vitro* assays of cell migration (modified Boyden chambers and wound /scratch assay) that PRP has a positive migratory effect on HDF. In addition, PRP also produced a positive migratory effect on HUVEC and Ea.hys cell on transwell migration assay, however PRP show some migratory effect on HEK and NEB-1 cell but less pronounced as compared with the positive control experiment, the scope of this study did not allow us to fully explore this seemingly different response of keratinocyte to PRP.

The mechanisms of keratinocyte motility are not fully understood however, Odland and Ross, 1968, demonstrated that lamellipodia formation occurs when human keratinocytes become migratory during reepithelialization. Ezrin, moesin, and radixin are constitutive proteins involved in the formation of lamellipodia (Pestonjamasp, 1995). Another mechanism involved in cellular motility is the expression of metalloproteinases (Pilcher, et.al 1997). It has been shown that dermal fibroblasts,

human keratinocytes synthesize and secrete type I and type IV collagenases (Petersen, 1990). Moreover, these collagenases exhibit increased expression when the keratinocytes are migrating. A matrix of interstitial collagen induces keratinocyte motility and the expression of collagenases (Petersen, 1990). In study coordinated by Decline and Rousselle, 2001, confirmed that keratinocyte migration on plastic, fibronectin or collagen IV substrates requires endogenous laminin-5 depositions, which is predominantly detected under its unprocessed form. In addition, they evidence that the alpha2beta1 integrin-laminin 5 interaction is absolutely required for keratinocyte migration and that the alpha2beta1 integrin is responsible for cell spreading on laminin 5.

O'Toole et. al. 1997, in their study demonstrated motility of human keratinocyte on extracellular matrices under normoxic and hypoxic conditions in two independent migration assays. The colloidal gold assay measures the migration of single keratinocytes apposed to a matrix and used the in vitro scratch assay measures the migration of a confluent sheet of keratinocytes over matrix. The study showed that hypoxia significantly enhanced keratinocyte motility on collagens and fibronectin compared with normoxic conditions. The pro-migratory effect of hypoxia is comparable in potency to soluble factors like EGF, TGF-*a*, and IL-1a (Cha .et.al. 1996). This result is counter to the notion that reduced oxygen tension has a negative effect on wound healing (Conlon et.al .1994). While hypoxia may not be beneficial for overall wound healing, certain elements of the wound healing process such as re-epithelialization may be initiated and promoted by hypoxic conditions.

Celotti et al. 2006 showed from their study that PRP in vitro has a strong, dose-dependent, chemoattractant activity on human (SaOS-2) osteoblasts cell line and the effect of PRP on SaOS-2 cell migration is maximal with protein of higher molecular weight. Among the GFs released in PRP from platelet α-granules upon activation, are PDGF and TGF- β, which have higher molecular weight and more abundant. To understand the role of these two growth factors in PRP-induced chemotaxis, PRP was preincubated with specific neutralizing antibodies directed toward human PDGF and TGF-β. The concentration of the antibodies was selected based on the reported concentrations of these growth factors in PRP and on the neutralizing capacity of the antisera.

The data presented show that the chemotactic activity exerted by PRP on SaOS-2 cells is completely neutralized by the anti-PDGF. These results are in substantial agreement with those of Gruber et al., 2004 and Fiedler et .al, 2004, whom have both shown that PRP of adult volunteers, obtained from thrombin-activated platelets, increases migration of bone marrow-derived mesenchymal progenitor cells and bone marrow derived cells. Furthermore, neutralization study indicates that TGF-b does not produce a significant effect on SaOS-2 cell migration.

4. 4. 3 Angiogenesis Assay

The angiogenic process is regulated both by positive and negative cytokines that modulate the migration, proliferation, proteolytic activity, and differentiation of endothelial cells (Risau, 1997). One of the most specific tests for angiogenesis is the measurement of the ability of the endothelial cells to form three-dimensional structures (tube formation). Endothelial cells of all origins appear to be able to form tubules spontaneously, given time *in vitro* to lay down appropriate extracellular matrix components. Note that tubule formation in vitro hardly simulate the whole process of angiogenesis, it has become a useful *in vitro* assay of at least two key steps in angiogenic pathway, the migration and differentiation of endothelial cells. The discovery of Matrigel has completely changed the *in vitro* assessment angiogenic properties. However, it is crucial to understand that most commercially available Matrigel has high level of growth factors and proteins that have been shown to have angiogenic property. Therefore for the purposes of our study we used growth factor reduced Matrigel.

The angiogenic effect of a compound is the ability to increase or decrease capillary-like structure formation in matrigel when added directly to medium covering endothelia cell. After completing the *in vitro* angiogenesis assay, we can categorically say that the observed angiogenic effect seen after incubating PRP with HUVEC Matrigel is a true reflection of the tubular forming potential of PRP. All the varying concentrations of PRP (5%, 10%, 25% and 50%) in our study showed a positive angiogenic effect, however this effect appears to be in the decreasing dose dependent pattern with PRP 5% showing the most positive effect. PPP did not show quality or well define tubular formation; the HUVEC when treated with PPP appears to form clumps of cells. To justify this observation we decided to analyse the morphometeric and topological parameters after the complex tubular network process.

With the use of matrigel assay, dimensional (percentage area covered by endothelial cells and the length of the cellular network per field), and topological (the number of meshes and the number of branching points per field) parameters can easily be assessed. These two parameters collectively provide a measure of the amount of formed tubules. Changes in tubule formation represent the principal *in vitro* angiogenic response that can be used as a measure of angiogenic activity. Dimensional parameter does not fully characterize the architecture of the tubular pattern. Topological parameters in addition provide physiological information concerning endothelial cell organisation. Branching point appear more useful, because it allows for detection of changes in the cell arrangement even in conditions where no meshes are formed (Guidolin et.al, 2004)

Qualitative and semi-quantitative were the mainstay of evaluating angiogenesis assay in the recent past, this technique has a number of inherent flaws. The nodal and mesh formation analysis which

are forms of morphometric measurement correlates with the pro-angiogenic process **(Figure 4a &4b).** PRP 5% showed the most predominant angiogenic effect and is statistically significant over the positive control and other varying concentration of PRP /PPP. Furthermore, a similar effect was noticed with measuring skeletal length but PRP 5% did not reach any statistical significance over other forms of treatment **(Figure 4c).** This is probably because the software analysed all connections on the acquired image without factoring the morphologic structure of the formed tubule. Analysis of total cellular area showed a reversal of the angiogenic effect observed with PRP 5% **(Figure 4d).** This analysis may appear miss-leading because cellular area takes into consideration the whole field that is involved during the angiogenesis process without consideration for morphologic changes and the quality of the capillary network formation. This proposed method did not take into account the quality of capillary network formation. From our study, we believe that with repeats of experiments that analysis of skeletinised length of the tubular network will be a useful parameter in the evaluation of angiogenesis assay. Although this method of qualitative analysis has not been validated through testing with other endothelial cells from other sources; we proposed that analysing the nodal formation, mesh formation and the skeletinised length of the tubule as has a direct correlation to angiogenic effect.

We concluded that the analysis of the complete process of angiogenesis will give a better quantitative description of angiogenesis assay as compared to analysing the whole process as other authors have argued. Basu et. al, 2008, Movafagh et ,al 2006 and Xu et al. 2008, all of these studies provides measurement of different steps of angiogenesis process. These approaches failed to take into account that a reorganization of endothelial cell occurs once cell-cell contacts have been established and thus this occur over time during angiogenesis. Another draw back of these approaches is that they tend to consider many variables such as the cell sprouting, cell connections, polygonal structure and total number of cells used in the angiogenesis process and the postulation of complex formula before angiogenic activity of a compound could be established.

We have been able to demonstrate a positive angiogenic effect of PRP on HUVEC and a possibility of decreasing dose dependent effect which was also observed during cell proliferation and migration assay with HUVEC. This observed phenomenon raises the suspicion that there might be other anti-angiogenic growth factors present within the PRP. Some authors have suggested that TSP-1 and PF-4 are likely to account for this phenomenon. Jimenez et. al, 2000, demonstrated that TSP-1 inhibits angiogenesis in vivo and in vitro through CD36 binding, triggering a signalling cascade through caspase-3-like protease and stress-ac on such using TSP-1 neutralized antibody or a competitive assay to clarify the mechanism of TSP-1 negative effect on wound healing.

4. 5 CONCLUSION

In conclusion, PRP is a easily available blood derivative containing high concentrations of growth factors and cytokines, when applied has the ability to potentiate tissue repair through acting on cellular recruitment, proliferation, angiogenesis and migration. The role of its individual constituents in promoting tissue repair remains to be fully elucidated. On the basis of the observations made from utilizing human primary cells and cell lines, PRP possess chemotactic effect on HUVEC, Ea.hys and HDF and also on HEK and NEB-1 cell howbeit weak. The PRP also has a great potential to positively exert a mitogenic effect on all the cell types used , although this effect appear minimal on HEK and Neb-1 cell and did not reach a statistical significant level. Perhaps, the net effect of PRP represents the result of agonist and antagonist actions of the different growth factors. Finally, a commonality was observed in all the assay and cells types, which is the decreasing dose-dependent effect, with 5% PRP concentration producing the most optimal condition for proliferation, migration and angiogenesis. PRP has the potential of promoting wound healing and soft-tissue remodelling without the risk of immune rejection or infection, which, when more clearly defined, might allow for better use of this platelet derivative in the future.

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CHAPTER 5

Immuno-histochemistry Changes in Chronic Diabetic Foot Ulcers

5.1 Morphological changes in the Epidermal Structure of Wounds during Healing.

Although an orderly and carefully orchestrated series of events leads to successful repair of acute wounds but the repair of chronic wound is highly compromised. Chronic diabetic cutaneous ulcers

are the most common cause of lower limb amputation (Pecoraro et. al 1990). The inability to heal chronic ulcers has been attributed to impairment of a variety of biological mechanism such as senescent fibroblast, prolonged inflammatory response, cytokines and growth factors deficits, lack of protease inhibitors (Falanga 2004, Tomic-Canic et. al 2004). Failure of re-epithelisation may be the consequence of any combination of the above mentioned factors.

During acute wound injury, basal keratinocytes at the dermal-epidermal junction (DEJ) in uninjured skin undergo differentiation to ultimately create the strong skin barrier to the external environment. The stratified layers of complex epidermis can be distinguished by expression of differentiation-specific pairs of keratin intermediate filaments (Fuchs, 1995). Interfollicular basal keratinocytes express keratins 5, 14, and 15 are attached at the dermis at the DEJ. Expression of keratins 1, 10, and K2 in suprabasal keratinocyte is an indication of keratinocyte differentiation (Usui, et.al, 2008). In acute wound injury, suprabasal keratinocytes adjacent to the site of injury from the wound margin begin to express keratins 6 and 16 after about 24hrs of wounding (Usui et.al 2005). Keratin 6 and 16 are associated with a change in keratin filament arrangement, conferring keratinocyte with a more activated phenotype (Paladini et.al. 1996). After about 48 hrs of wounding, keratinocyte in the migrating tongue of acute excisional wounds begin to down-regulate expression of keratins 1,10,2 (Usui et.al, 2005) while expressing K6 and K16. Normal differentiation resumes after epidermal wound closure is achieved.

In normal acute wounds, a proliferative burst of keratinocytes behind the migratory keratinocyte wound margin occurs between 24 and 72 hrs after injury, with resumption to a constitutive proliferative population of approximately 10% of the basal keratinocytes after wound closure (Usui et.al, 2005). In addition to the changes in the keratinocyte and its keratin expressions, deposition of laminin 5, epiligrin, kalinin and nicein starts from the leading edge of the keratinocyte, these are key basal membrane proteins (Hamil and McLean 2005; Marinkovich et.al 1992). There are also corresponding changes in the expression of trans-membrane receptors (integrins), which laminin ligates. Integrins function as adhesive proteins and play a role in cell signalling (Larjava et.al, 2003). In the wounded keratinocytes, integrin β1 ligates with the precursor form of laminin to mediate keratinocyte migration (Carter et.al, 1991) and also ligates with laminin after undergoing excellular protelytic processing to mediate stable anchorage of keratinocytes to the basement membrane (Mercurio et .al 2001). Integrins plays a role in heterotypic cell-cell interactions, and is said to be the major players in cell matrix interactions and thus also responsible for structural tissue organization (Hynes, 1992). Furthermore, integrins are supposed to function as sensors for the extracellular environment and for mechanical forces activating cellular signal transduction systems which might in turn influence turnover and processing of extracellular matrix (Juliano, 1993). Integrins form obligatory

dimmers of one α- and a genetically distinct β-chain, and in normal adult human epidermis they are represented by α2β1, α3β1 and α6β4, which are all restricted to the cells in the proliferating basal layer

The deposition of collagen VII in healing human surgical wound has been demonstrated from day 7 following wounding and over a similar time course in healing human acute oral wound. Chronic lower limbs ulceration is usually characterized by basement membrane destruction and it is likely that collagen VII synthesis is required for the restoration of functional basement membrane zone (Hopkinson, 1997). The role of Collagen VII in maintaining the stability of the cutaneous basement membrane zone and identification of multiple conserved cell recognition domains may be a pointer to the fact that Collagen VII may be involved in healing of chronic lower-limb ulcers. The behaviour and morphopical changes of keratinocytes in acute wound healing are well understood and the changes are expected to follow a logical sequence that will end in complete re-epithelization. But the complexity and degree of molecular and physio-structural alteration seen in the chronic wound could not allow for a predictable outcome of healing process.

There have been many studies regarding the role of growth factors in the skin, and several growth factors have been reported to have positive effects on skin regeneration through stimulative effects on the proliferation of collagen and fibroblasts, and on the growth of keratinocytes (Ehrlich et.al, 2006; Gold 2007). However, the production, transportation and storage of growth factors require high costs, and unintended complications can develop during the processes. Therefore, if growth factors can be derived from the patients themselves, superfluous costs could be saved and concerns of complications could be set aside to a great extent. Recently, various clinical trials on the role of PRP in the treatments of chronic leg ulcers have been conducted, based on this background knowledge. However, so far, human studies concerning the mechanism of PRP in chronic diabetic wound healing are very limited, perhaps, because of the too many confounding factors and ethical consideration. It is debatable whether the favourable clinical effects reported in literature are the direct result of PRP treatment or other systemic response to healing. Therefore, we designed this current study to evaluate the effect of PRP in the treatment of chronic diabetic foot ulcers. Immnuno-histochemistry was performed on all the biopsy samples taken from all the patients, and redundant normal skin samples obtained from diabetic patients during debridement was used for control experiment.

Connective tissue formations are meant to prevent scarring during tissue repair and optimum deposition of collagen I and III with their proper ratio is important (Wess, 2005). In particular, collagen I is responsible for higher tensile strength, whereas collagen III is predominantly found in early wound healing stages (Rajan and Murray 2008), and they maintain a particular ratio (Junge,

2004) in healthy skin. The geometrical arrangement of fibrillar collagen and their optimum ratio are vital during healing progression to maintain required crosslinked density and mechanical toughness (Lehto, 1985).

5.2.1 Profile of Antibody used in the Study

5.2.1 Cytokeratin1 (K1) / Cytokeratin 10 (K10)

Cytokeratin K1 is a member of the family of intermediate filament proteins that assemble into filaments through forming heterodimers of one type I keratin (keratin 9-23) and one type II (keratin 1-8). Keratin 1 is a differentiation –specific keratin that is predominantly expressed in the suprabasal keratincytes in stratified epithelia (De Berker D et.al 1995). The keratin pair K1 and K10 is a marker of differentiation. Mutations in keratin 1and 10 results in epidermolytic hyperkeratosis. Cytokeratin 1 is a type 1 subclass of keratin; keratin is a suprabasal marker of differentiation in stratified squamous (Porter et al.2000)

5.2.2. Anti-keratin 5 and 14 Antibody profile (K5/K14)

K5/k14 are predominantly expressed in the basal layer of the stratified epithelia; they demonstrate tissue and differentiation specific expression. Keratin 14 is one of two keratins that distinguish stratifying epithelia cell types from simple epithelial cell types. Mutations in keratin 5 and 14 cause the skin blistering disorder of peidermolyis bullosa simplex. K4/K15 may be useful as a basal cell maker and myoepithelial marker in stratified squamous or mixed epithelia (De Berker D et.al 1995)

5.2.3 Anti-keratin 16 Antibody

Keratin 16 demonstrates demonstrate specific tissue differentiation. Keratin 16 is expressed in suprabasal keratinocyte of wounded epidermis, in cell undergoing rapid turnover; it's often referred to as hyperproliferating–related keratin. Hyperproliferation keratins K6 and K16 are not found in normal skin, however they are expressed in oral mucosa, sole and palm skin. Expression of K6 and K16 is seen in wound healing and hyper-proliferative disease such as psoriasis.

5.2.4. Anti keratin 14- antibody

K14 distinguishes stratified epithelial cell types from simple epithelial cell types, it therefore characterises stratified / keratinocyte cell types. Found on all cells of keratinocyte lineage, including tumours and in glandular tissue found on basal layer (Karashima and Watt, 2002)

5.2.5 Ki67 Antibody

They are referred to as proliferative maker; this antibody reacts with a nuclear antigen present in proliferating human cells. It can be used to evaluate the ki-67 labelling index in various tumours. Ki67 antigen is the prototypic cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase). It is absent in resting (G0) cells (McSheehy, 2010). Ki67 antibodies are useful in establishing the cell growing fraction in neoplasm and have also been used in hyper-proliferative wound edges and scars.

5.2.6 Anti –Integrin β1 Antibody

Integrins are heterodimers cell surface receptors composed of alpha and beta subunit, which mediate cell-cell and cell-extracellular matrix attachments. Loss of integrin β1 is associated with the development of some epithelial tumours (Marshall, 1998). Integrin β is expressed in the basement membrane.

5.2.7 CD31

CD31 is also called platelet/endothelial cell adhesion molecule. CD31 is a single chain type transmembrane protein with a molecular mass of approximately 135 kDA. CD31 binds in both a homophilic and heterophilic manner. The heterophilic ligands include heparin sulphate glycosaminoglycans, heparin and integrin αv β3. CD31 is involved in the adhesive interaction between leucocytes and endothelial cell. CD31 is expressed on all continuous endothelial surfaces for example arteries and vein, however, not on discontinuous endothelium surfaces, such as splenic red pulp. It is also expressed diffusely on the surface of platelets and myeloid cells and homeopoetics cells line. The antibody can be a useful tool in determining rate of new vessel formation during malignant and or in wound healing process.

5.2.8 CD44

CD44 is also referred to as homing-associated cell adhesion molecule (H-CAM). CD44 is a type 1 transmembrane adhesion molecule with molecular weight of 85-90 kDa. CD 44 is a glycoprotein with extensive 0-linked glycoslylated and 6 potential N-linked glycosylated sites. CD44 is a cell surface receptor for hyaluronate suggesting a role in the regulation of cell and cell substrate interactions as well as cell migration. CD44 is expressed on wide variety of cells such as on the haematopoietic system, epithelial cells, fibroblast, skeletal muscle and central nervous system white matter.

5.2.9 Collagen I

Collagen has a characteristic uninterrupted Glycine XY triplet repeat that is a necessary part of the triple helical structure. Collagen I is found in bone, cornea, skin and tendon, with a molecular weight

of about 95 kDa. There may be a cross reactivity with other forms of collagen (Ponsioen et. al, 2008). Some class of specific anti-collagens may be specific for three dimensional epitopes which may result in diminished reactivity with denatured collagen or formalin-fixed, paraffin embedded tissues. Mutations of the encoding gene are associated with osteogenesis imperfecta and Ehlers Danlos syndrome.

5.2.10 Collagen III

Collagen III is a fibrillar collagen that is found in extensible connective tissue such as skin and vascular system, frequently associated with type I Collagen. This type specific collagen antibody only recognizes 3D epitopes, has negligible cross reactivity with type I, II, IV, V or VI collagens (Ponsioen et. al 2008). Genetic mutations of this collagen are associated with Ehlers Danlos type IV, and with aortic and arterial aneurysms.

5.2.11 Collagen VII

Collagen VII is a basement membrane component which is the major protein in the anchoring fibrils projecting from the lamina densa into the subject of the connective tissue. Collagen VII is a homotrimer of α1 (VII) pro-collagen chains, with molecular weight of about 350kD, in which the central triple-helical collagenic domain includes 19 non- triple-helical interruption (Christiano, 1994). The structural role of collagen VII may be inferred from the instability of the basement membrane zone observed in epidermolysis bullosa dystrophia, a blistering skin disorder that is often associated with mutations in collagen VII gene (Uttio J, 1992). Collagen VII has been found in the basal lamina of stratified epithelia such in as epidermis, oral oesophageal and cervical epithelium.

5.3 MATERIALS AND METHODOLOGY

After obtaining ethical approval from the east and city of London Committee, a single 6mm punch biopsy was allowed to be taken from patients with chronic diabetic foot ulcers recruited into the study. **(See details in chapter2).** Immunioperoxidase technique was employed for the antibody testing, however some antibody were not detectable on paraffin section, therefore frozen section was employed for K16 and Collage VII. The optimization technique for each antibody has been fully discussed in **chapter 2 under section 2.2.4 & 2.2.5 and tables 2.1 & table 2.2**. The initial design of the study was aimed at comparing 2 groups of patients treated differently. One group was to be treated with PRP (the active group) and the other group treated with PPP (non-active group). However, due to patient choice and time limitation for the study, we could only recruit patients to the active arm. In order to have a comparison group, redundant part of the normal tissue samples from another patient undergoing wound debridement was used for control expierment.

5.3.1 Photomicrography

All slide sections were viewed on a Nikon Microphot-SA microscope using either standard bright field or differential interference contrast. After confirmation of the desired staining intensity, all the slides were scanned using the Nano zoomer digital pathology system (Hamamatsu). This scanner has the ability to capture a complete macro image on the glass slide, and the images are immediately transferred into soft ware programme which allows the acquired images to be viewed at varying magnification. It also allows for the acquired images to be archived, assessed by a separate assessor on stored file without having to carry the slides and microscope about. The software also allowed for annotation and geometric measurement on the scanned images.

5.3.2 Quantification of Haematoxylin and Eosin

After scanning and storing of the H&E slides, with the help of the NanoZoomer Digital pathology system, the epithelial layer of each of the biopsy sample was stratified into the corneum, garnulosum, spinosum and the basal layer and measured as a percentage of the whole epithelial layer **(Figure 5.1).** Furthermore, the presence of inflammatory cells were assessed in the dermal layer and quantified from no inflammatory cell to severe inflammatory cell **(Figure5.2).** The results from all the 7 patients **(Table 5.2)** were plotted and represented on a histogram, which demonstrate the characteristic changes in the different epithelia layer during wound healing process of a chronic wound. The percentage change in the individual layers of the epithelium could possibly to be used to assess normal progression of wound healing towards complete re-epithelisation of the wound in question.

Antibody	Clone	Clonality	Subclass	Species raised in	Tissue Type	Location

Antibody	Clone	Clonality	Subclass	Species raised in	Tissue Type	Location
Cytokeratin K1	LL017	Monoclonal	IgM	Mouse	Paraffin	Suprabasal
Cytokeratin K10	LH2	Monoclonal	IgG1	Mouse	Paraffin	Suprabasal
Cytokeratin K5/k14	LH8	Monoclonal	IgM	Mouse	Frozen section	Basal
Cytokeratin K16	LL025	Monoclonal	IgG1	Mouse	Frozen section	Suprabasal
Cytokeratin 14	LL01	Monoclonal	IgG2ak	Mouse	Paraffin	All keratinocyte layer
Ki67	MM	Monclonal	IgG1	Rabbit	Paraffin	Basal & dermis
integrin β1	4B7	Monoclonal	IgG1	Mouse	Paraffin	Basement membrane
CD31	JC70A	Monoclonal	IgG1	Mouse	paraffin	Endothelia cell
CD44	DF 1485	Monoclonal	IgG1	Mouse	Paraffin	Haemopoietic cells,epithelial and CNS cells
Collagen I		Polyclonal	IgG	Human/ bovine placenta	Paraffin	Dermis
Collagen III		Polyclonal	IgG	Human/ bovine placenta	Paraffin	Dermis
Collagen VII	LH7.2	Monoclonal	IgG1	Mouse	Frozen Section	Basement membrane

Table 5.1 Profile of the different Anticytokeratin, Cell surface maker and collagen Antibody used during immunohistochemistry experiment.

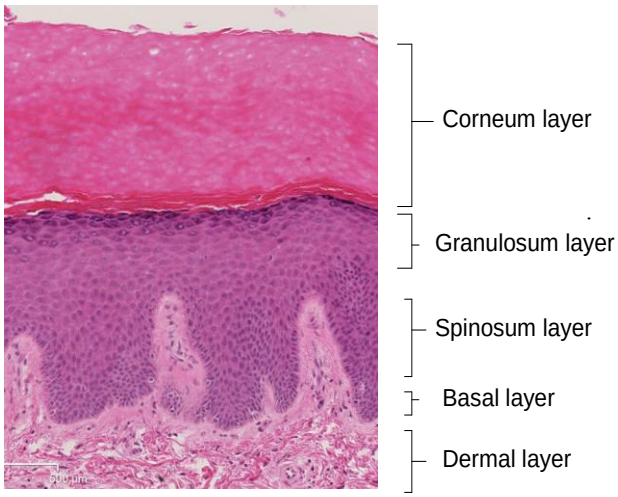


Figure 5.1 Measurement of the stratified epithelial layer from microphotographic H&E staining of biopsy skin. Measurement of individual layer was possible through the Nano Zoomer Digial pathology system.

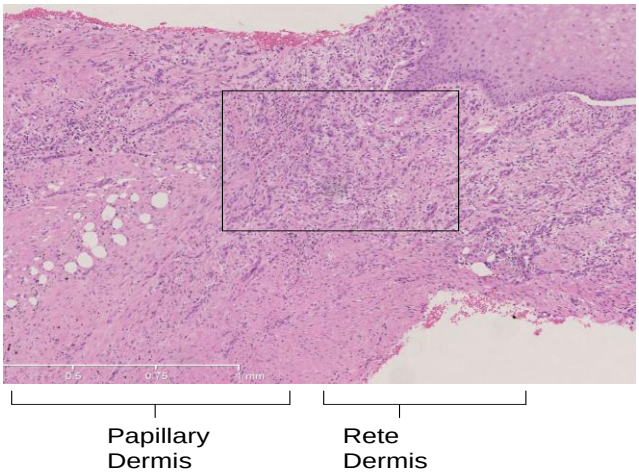
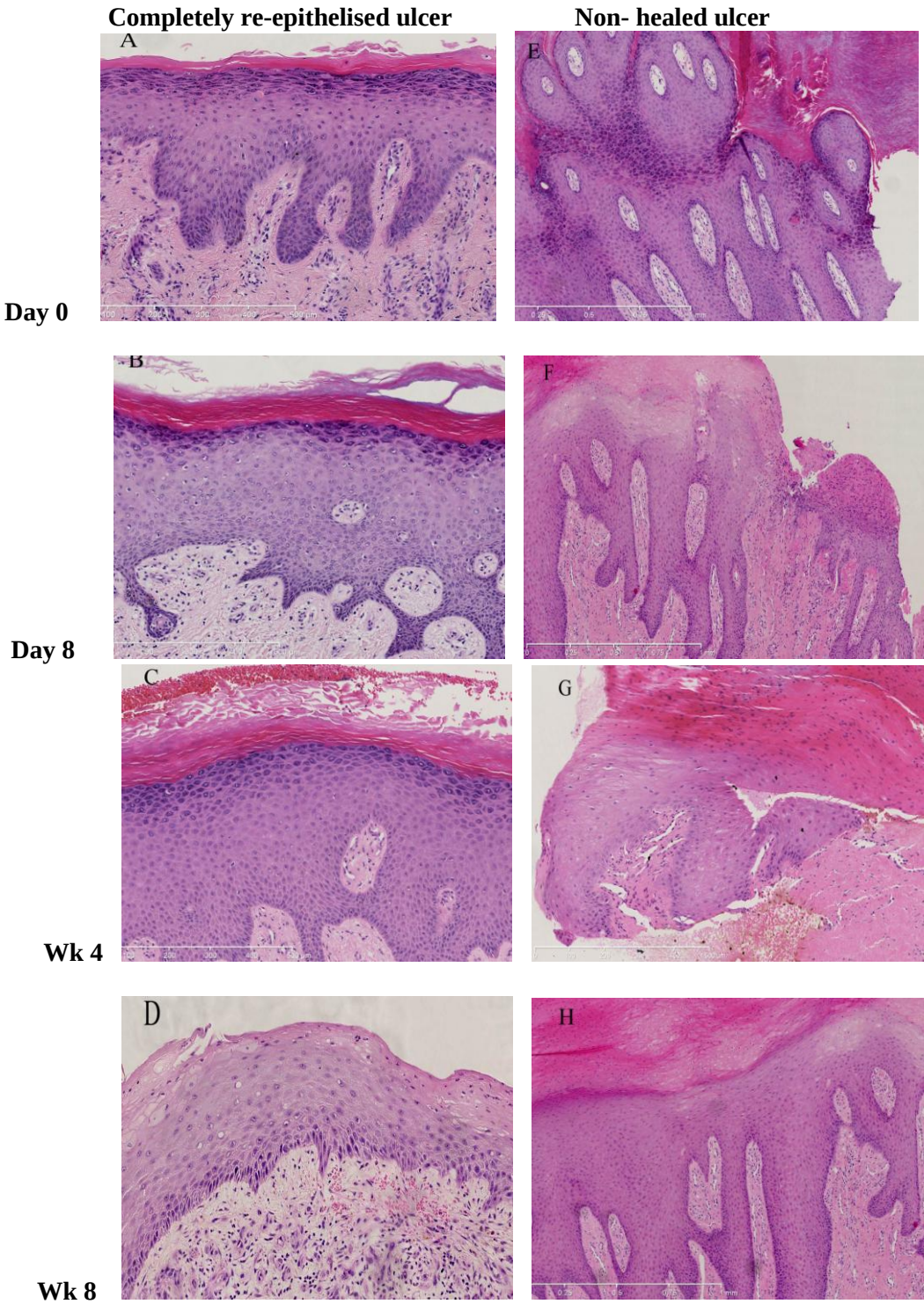


Figure 5.2 Microphotogarhic H&E staining of a wound biopsy with inflammatory cells distributed in the dermis. The rectangular space represents area of aggregation of inflammatory cells, the cell were counted at HPF x20 and graded; as zero for no inflammatory reaction for, + few, ++ mild, +++ for moderate and ++++ for severe inflammatory reaction.


Completely re-epithelised ulcer
Non- healed ulcer
Day 0
Day 8
Wk 4
Wk 8
A
B
C
D
E
F
G
H

Normal

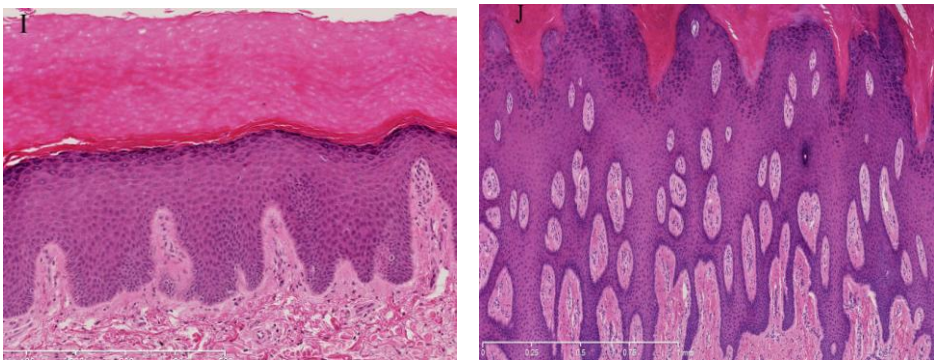
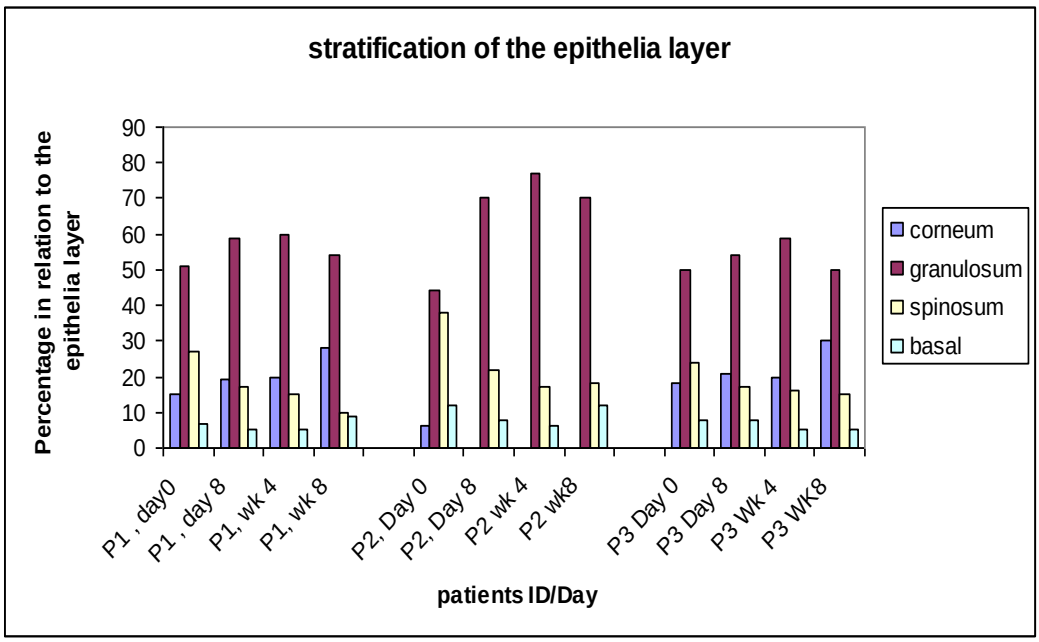


Figure 5.3 Microphotograhic H&E representation of the 2 categorises of chronic diabetic foot ulcer, A-D) represents ulcers that achieved complete re-epithelisation, E-H) represents ulcer that was not healed after 16 weeks of PRP treated and standard wound care. Comparing the histological changes between the 2 groups; the thick and nucleated corneum layer in the non-healed group is characteristic of a recalcitrant ulcer. There is a gross distortion in the structural architecture of the epithelial layer. There is a general decrease in cellular activity in the granulosum and spinosum layer from day 0 to week 16 as evident by decrease in the size of the layer as the wound progresses towards healing. On the contrary, the cellular activity in these layers remains perpetually active in the non healing group. I) represents the normal skin biopsy from a diabetic foot, whereas J) depicts a chronic diabetic foot ulcer with abnormal orientation in the epithelial layer interspared with dermis and thick corneum layer. Some of the characteristic histological features of chronic wound are evident on slides E-H such as koilocytes, hyperkeratosis , hypergranulois and papillary hyperplasia.

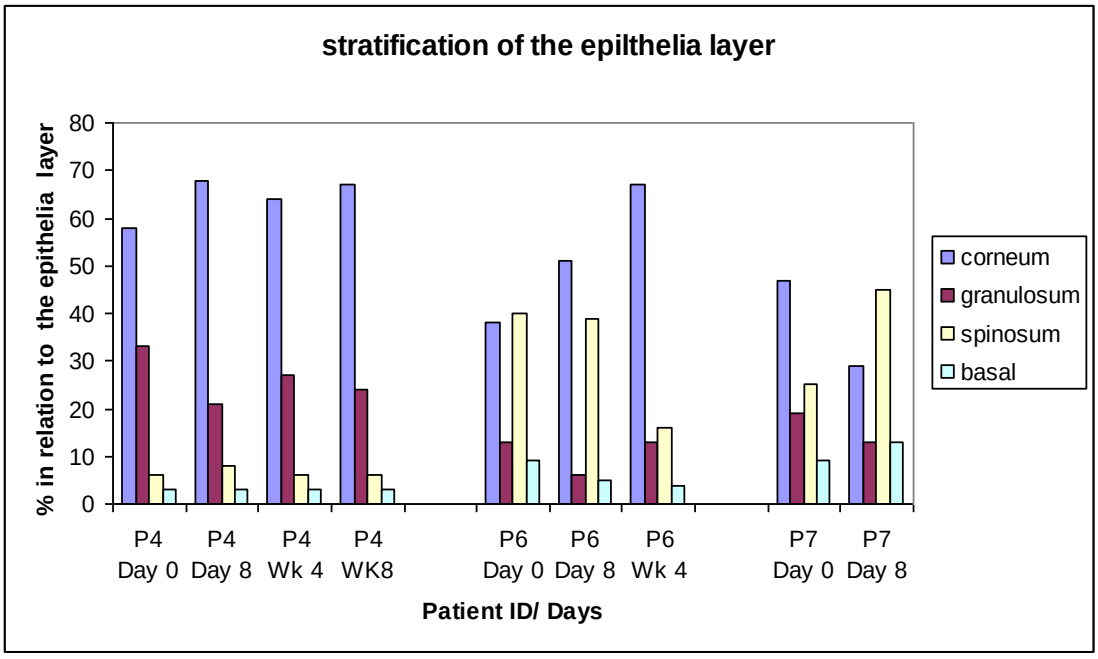
Patient ID	**% Corneum**	**% Granulosum**	**% Spinosum**	**% Basal**	**Papillary dermis inflammatory score**	**Rete dermis inflammatory score**
P1,Day 0	15	51	27	7	++++	++
P1,Day 8	19	59	17	5	+++	+
P1 , Wk4	20	60	15	5	++	+
P2, day 0	6	44	38	12	+++	+++
P2, day8	0	70	22	8	++++	++
P2, wk4	0	77	17	6	++	+
P2, wk8	0	70	18	12	++	0
P3, day0	18	50	24	8	++	+++

Patient ID	% Corneum	% Granulosum	% Spinosum	% Basal	Papillary dermis inflammatory score	Rete dermis inflammatory score
P3, day8	21	54	17	8	++	++++
P3, Wk 4	20	59	16	5	+++	+
P3, wk8	30	50	15	5	++	++
P4, day0	58	33	6	3	++	+
P4,day 8	68	21	8	3	+	0
P4,wk 4	64	27	6	3	+	0
P4, wk8	67	24	6	3	+	+
P5,Day 0	Absent epithelia				++	+
P5, Day 8	Absent epithelia				++++	++
P5, wk8	53	28	16	3	+	+
P6, Day 0	38	13	40	9	+	0
P6, Day 8	51	6	39	5	+	++
P6 wk 4	67	13	16	4	++	++++
P7 , Day 0	47	19	25	9	+	+++
P7, Day 8	29	13	45	13	+	++++
N.diabetic Dorsum skin	56	21	13	10	+	0
N.diabetic sole of foot	71	21	6	2	+	0

Table 5.2 Percentage stratification of biopsied epithelia layer from chronic diabetic foot ulcerson H&E staining. The degree of inflammatory reaction in the dermis was represented by the severity score of 0, +,++,+++ and ++++, which correlates to no inflammation, mild, moderate, severe respectively.



A



B

Figure 5.4 Illustrates the stratification of the epithelia layer obtained from the wound margins into corneum, granlosum, spinosum and the basal layer through H& E staining at Day 0, Day 8, week 4 and 8 respectively during the wound healing process. **Figure A)** represents the group of 3 patients that achieved complete epithelisation; the common feature in this group is the increasing % of the granuosum layer as healing progresses. **Figure B)** on the other hand represents the group that were unable to heal. The common feature is the higher percentage of the corneum layer compared to the granuosum layer which may represent a sign of chronicity.

5.3.3 Quantification of CD 31

Quantitative analysis of angiogenesis is highly suitable for histodiagnosis application because it has been performed primarily by immuno-histochemistry on formaldehyde-fix on paraffin embedded tissue. All the slides were scanned by the NanoZoomer digital pathology system after immuoperioxidase staining. This system allows the area of the fixed histology section to be scanned in the original state; this can then be stored and viewed on the computer system either at the same time or at a later time. Four large squares were drawn in the dermis, in the areas of pre-determined highest micro-vessel density on the tissue section, the drawn area measured 0.361mm^2, which is perpendicular and away from the epithelia layer. The microvessels within the large square under x20 magnification were only counted and the average was used as the vessel density **(See Fig 5.5).** The counting of the microvessel was performed by the researcher and also by a senior laboratory scientist to account for intra-observer variability. According to Vartanian and Weidner, 1995; any vessel that was clearly separate from adjacent vessel and other connective tissue element was considered a countable vessel. The vessel lumen, although usually present was not a criterion used to define a countable microvessel, red blood cells were not counted. Partially identified vessel, which are not contained within the squares were not counted. In each slide, microvessel density was represented by the mean number counted in the 4 larger squares in x20 high per field.

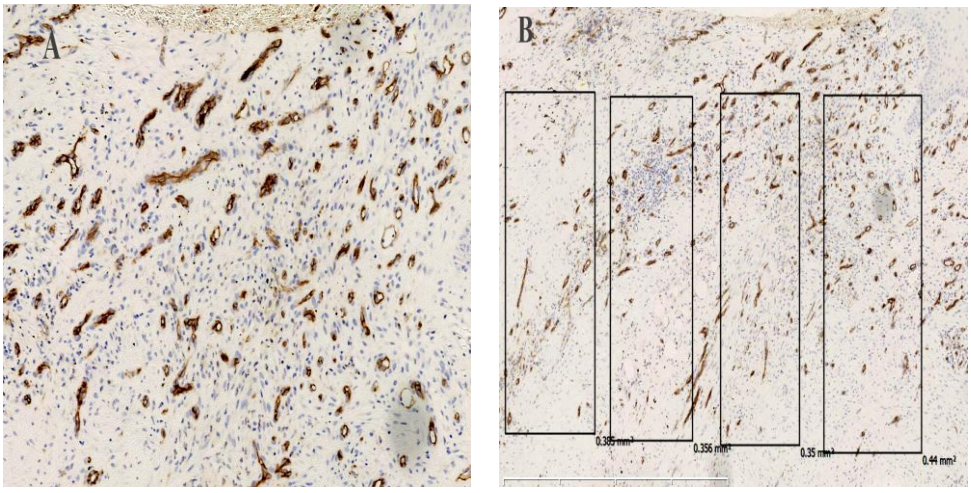
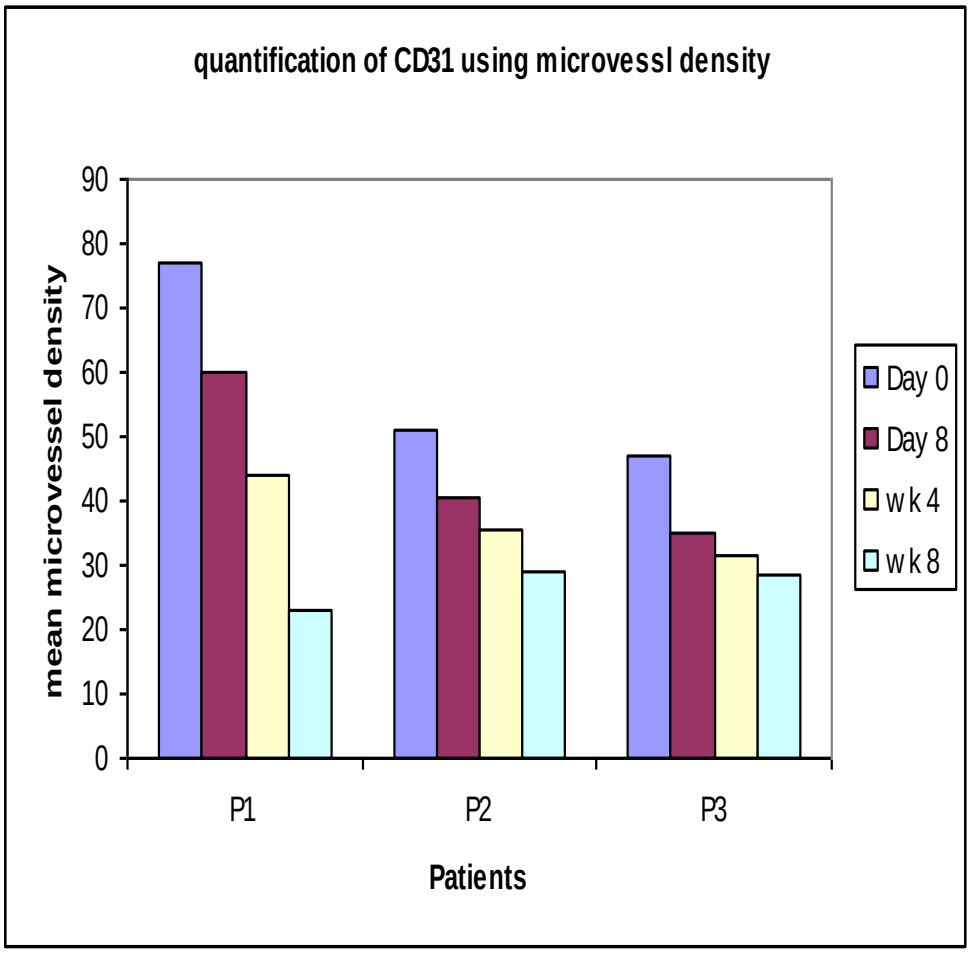


Figure 5.5 Microphotographic representation of immunoperioxidase staining for CD31 antibody. **A)** representation of densely populated microvessel area. **B)** micro-vessels are counted in each of the rectangular spaces and the average represens the total microvessel density of the individual slide section.

5.4 Statistical Analysis

After stratification of the epithelia layer into its respective percentages, the mean of each of layer was calculated for each of the time point of the biopsy and a plot of the mean against the time course was performed for individual patients **Figure 5.4**. Microvessel density was determined by counting the vessels as described above. The mean value of the counted microvessel on CD31 for each patient per time point was plotted as shown in **Figure 5.6**. For the immunoperoxidase staining for K14, k1/k10, k16, CD44, Collagen I, III and VII; the original slides were scanned through the NanoZoomer digital pathology system and achieved, each slides were scored for staining intensity individually and separately by the researcher and a senior laboratory scientist. The degree of agreement between the raters was determined by using the Bland-Altman test on Graph Pad Prism version 5.01



A

B

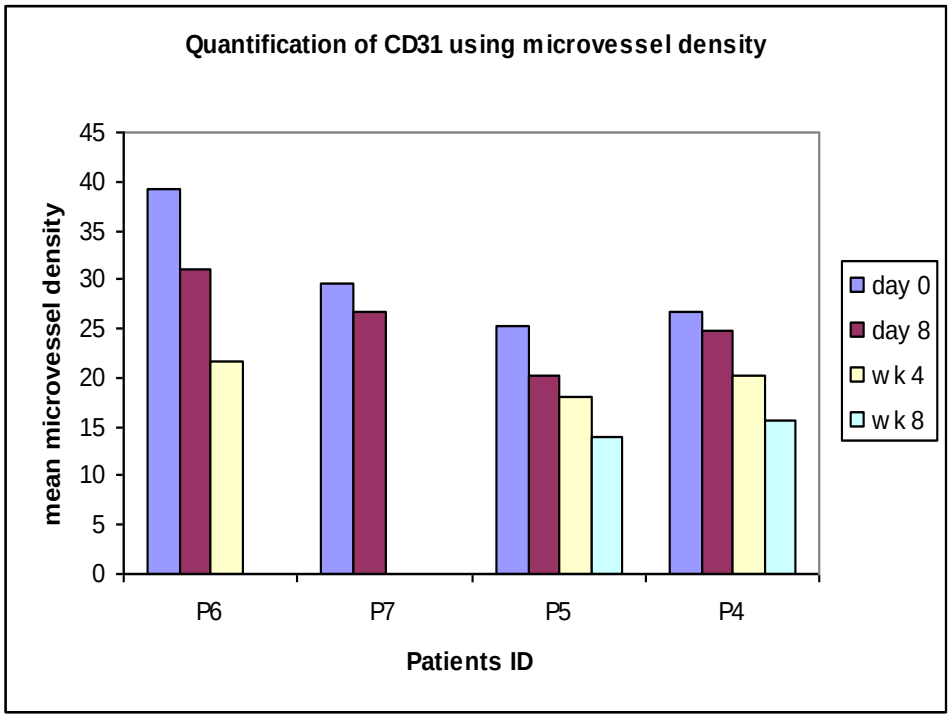


Figure 5.6 Quantification of CD31 through the counting of the micro-vessel density. **A)** represents the expression of CD31 antibody taken at day 0, day 8, wk4 and wk8 for 3 patients that achieved complete wound healing and **B)** represents the patients' ulcer that was not healed. The common feature to both categories of patients is the decreasing trend in the microvessel density from day 0 to week 8. **Figure 5.4B** showed that Patient 6 &7 did not complete the 16 weeks follow up program

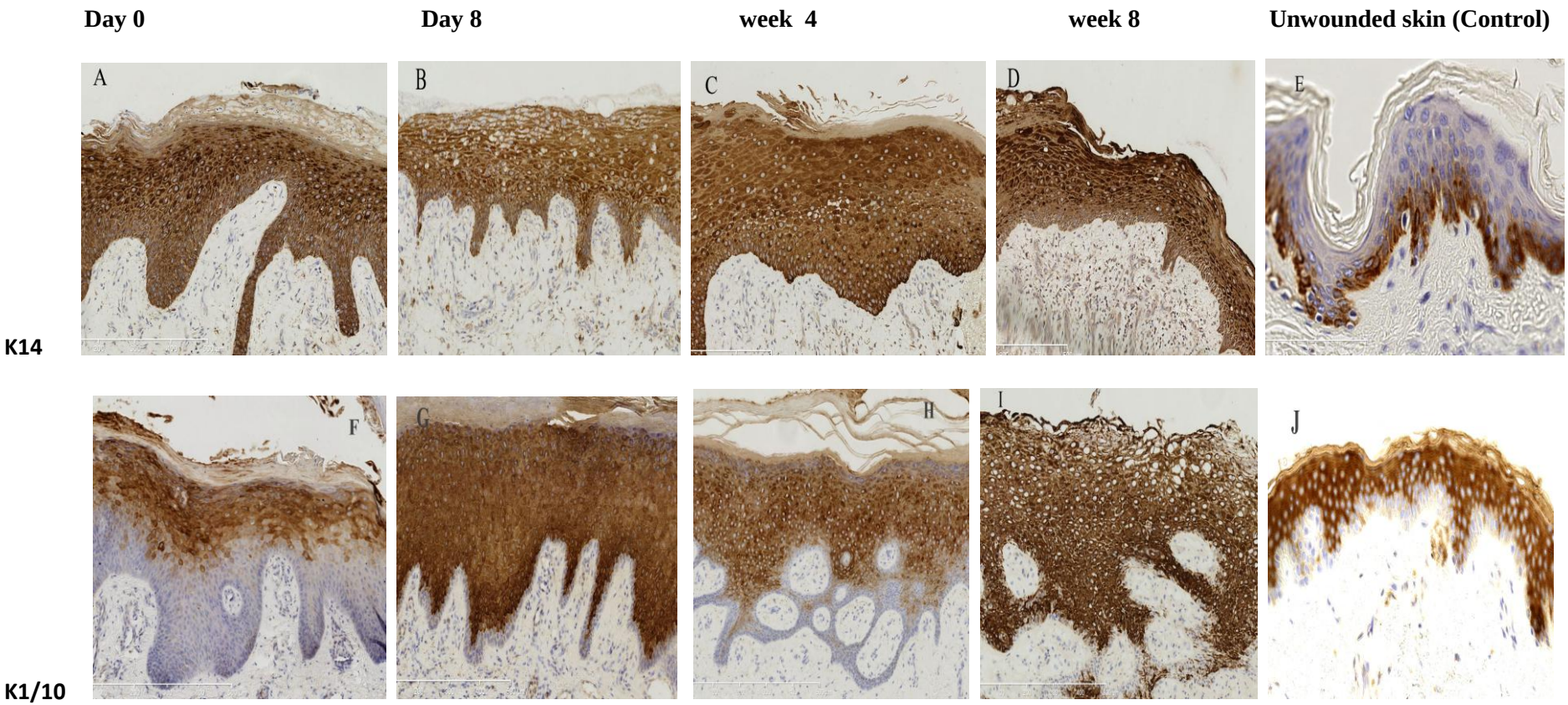


Figure 5.7 Represents photomicrograph from immuoperioxidase staining for cytokeratin antibody K14 (A-E). The cytokeratin stains the whole of the suprabasal layer of the epidermis from day 0 through to week 8, however the positive control in the normal skin was only expressed in the basal layer for a non-wounded normal skin. The expression of cytokeratin K1/K10 antibody usually the two keratin are co-expressed, which was mainly in the suprabasal layer (F-H), the basal layer appeared partly involved wound (I &J) and a similar patter of expression was demonstrated in a unwounded normal skin. However the basal layer appears to be more deeply stained.

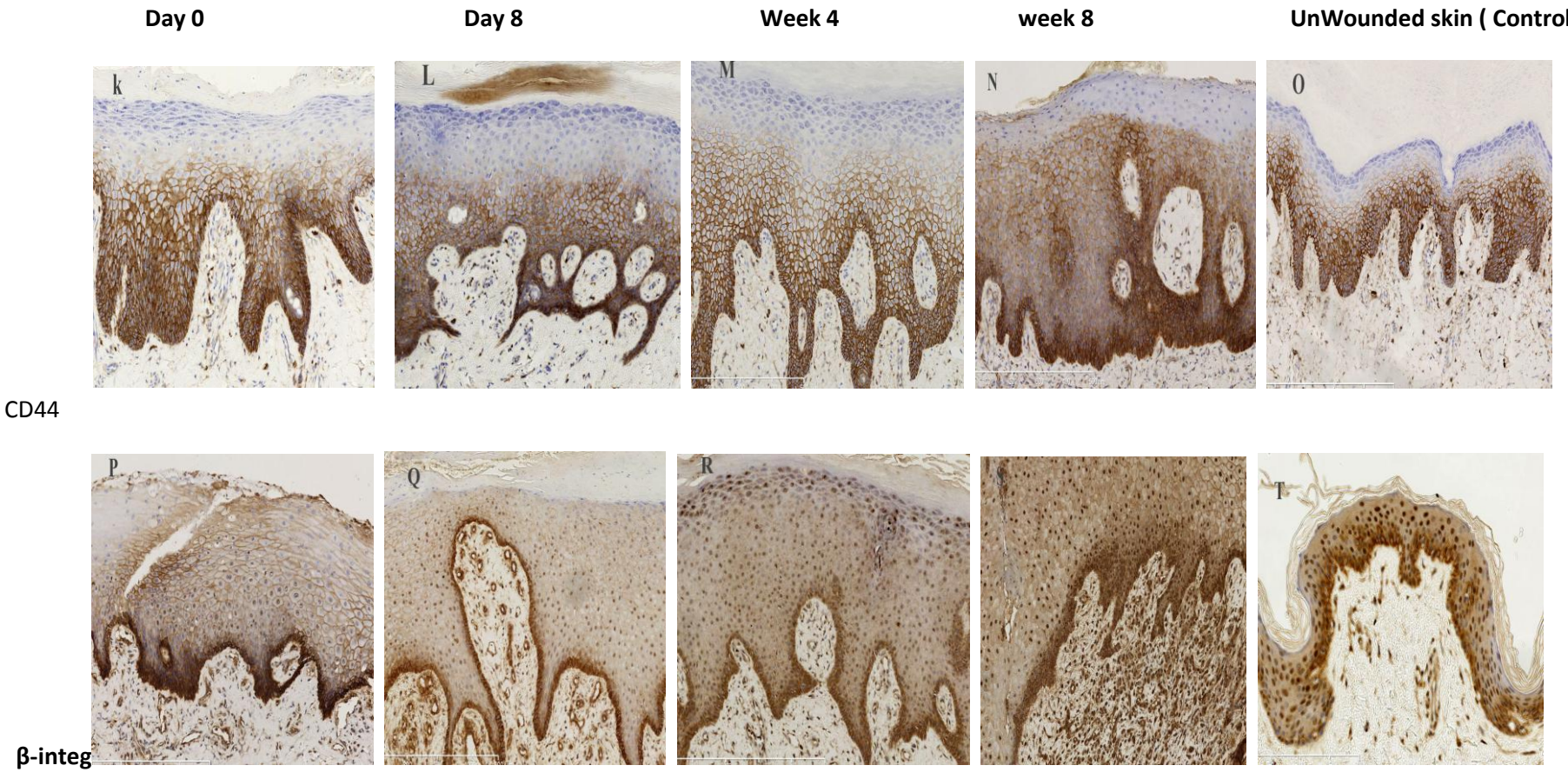


Figure 5.8 Represents the photomicrograph from immunoperoxidase expression of Beta I- Integrin & CD44 from the biopsied wound margin. The expression of beta-I integrin extends from the basal layer (most deeply stained) through to the spinosum, sparing the granulosum layer, a similar pattern of expression was seen from day 0-week 8**(K-N)** and also a similar pattern in the non-wounded skin **(0).** CD44 showed normal distribution along the basal layer only both from the wound edge biopsy and from the unwounded skin.

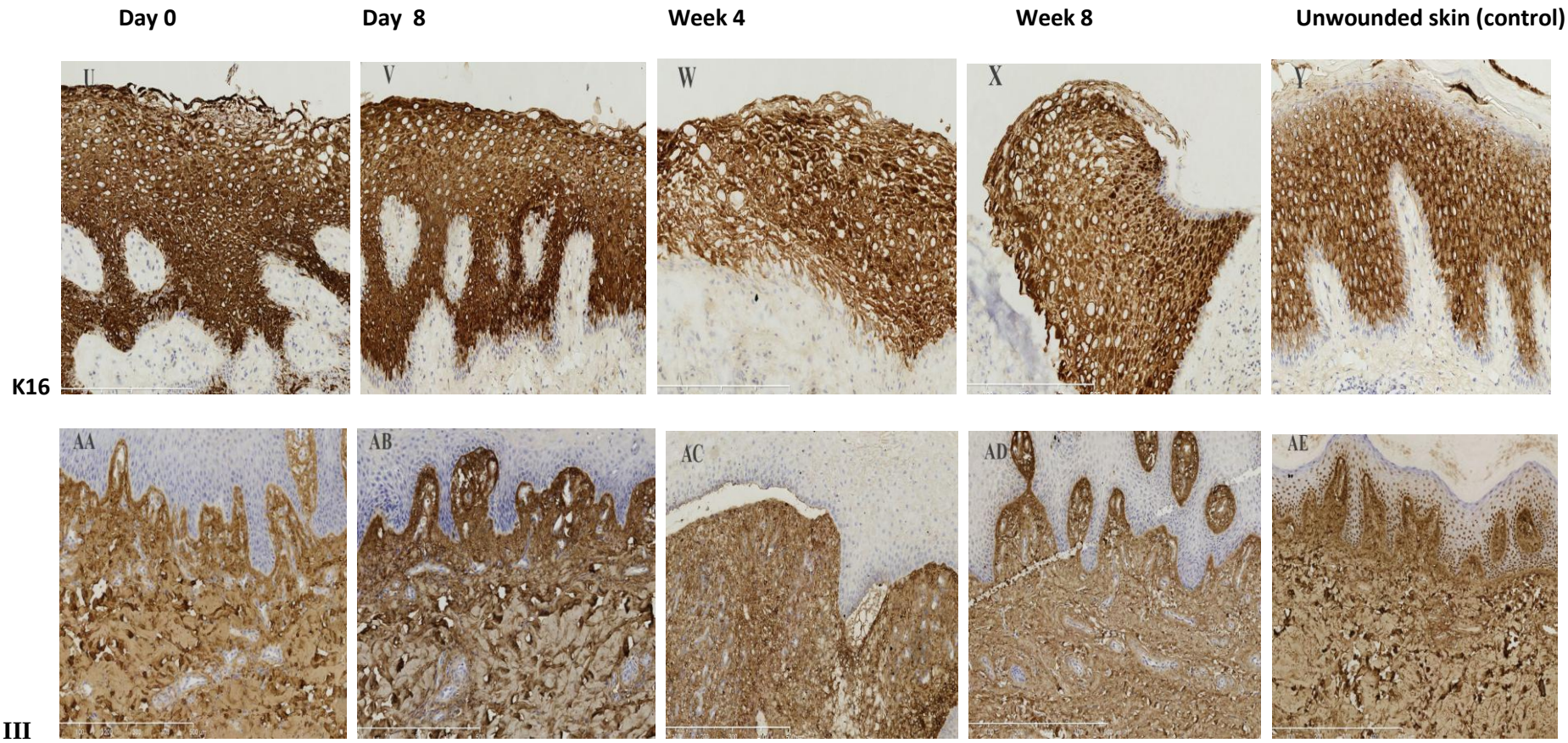


Figure 5.9 Represents the microphotograph from immunoperoxidase expression of cytokeratin K16 antibody and Col III, K16 expression was seen throughout the suprabasal layer; the expression was more pronounced on day 0 and day 8 **(U&V),** the expression in unwounded normal skin **(Y)** appeared similar to week 4&8 **(W,X).** The structure integrity of extracellular matrix protein was maintained throughout the wound healing process **(AA-AD).**

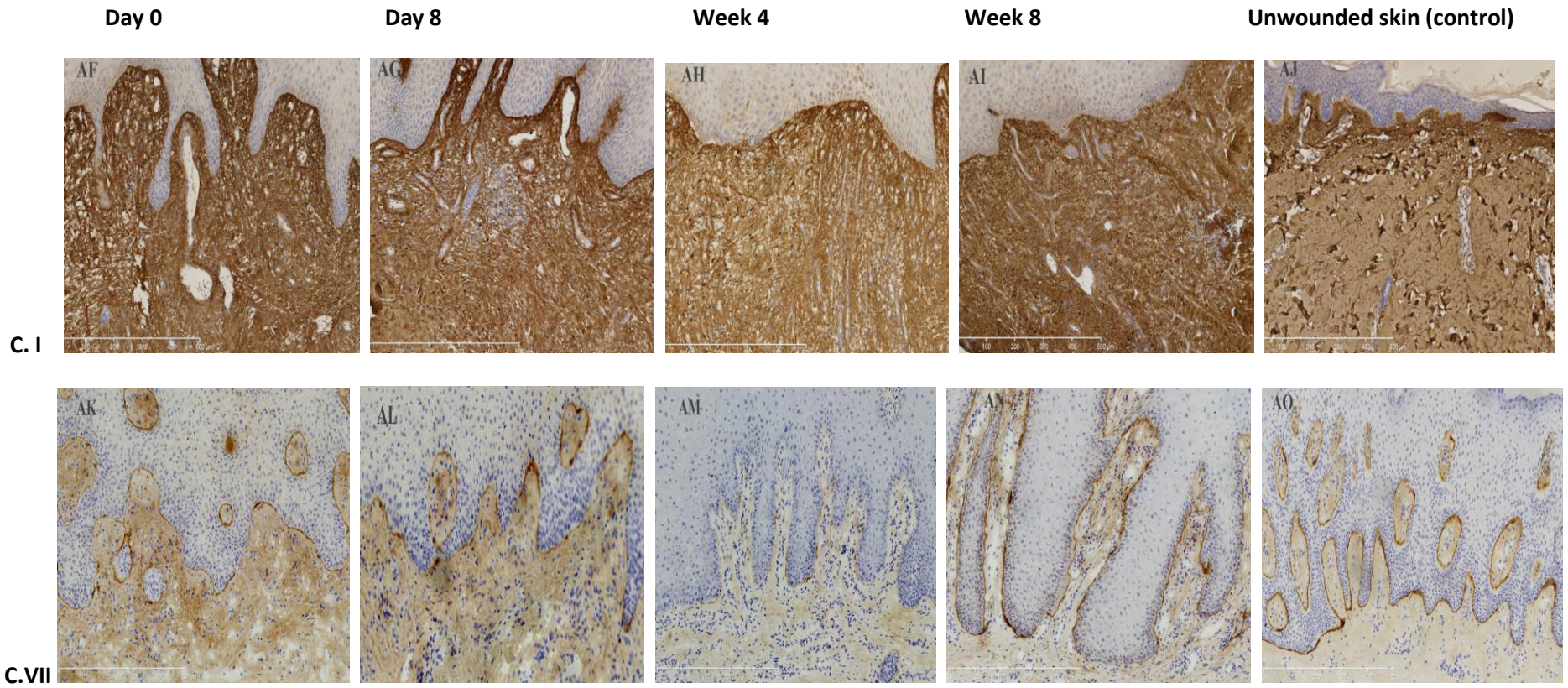


Figure 5.10 Microphotographic representations from immunoperoxidase staining of Collagen I &III. The structure of Collagen I was undistorted throughout the wound healing process (AF-AI). Collagen VII synthesis and incorporation into the anchoring fibrils is a prerequisite for the synthesis of a stable basement membrane, which is a requirement to restore basement integrity as a prelude to re-epithelisation. Restoration of collagen VII was observed from day 0 through week 8 and a similar expression was seen in the unwounded normal skin **(A-O)**. (C I- Collagen I, C VII- Collagen VII)

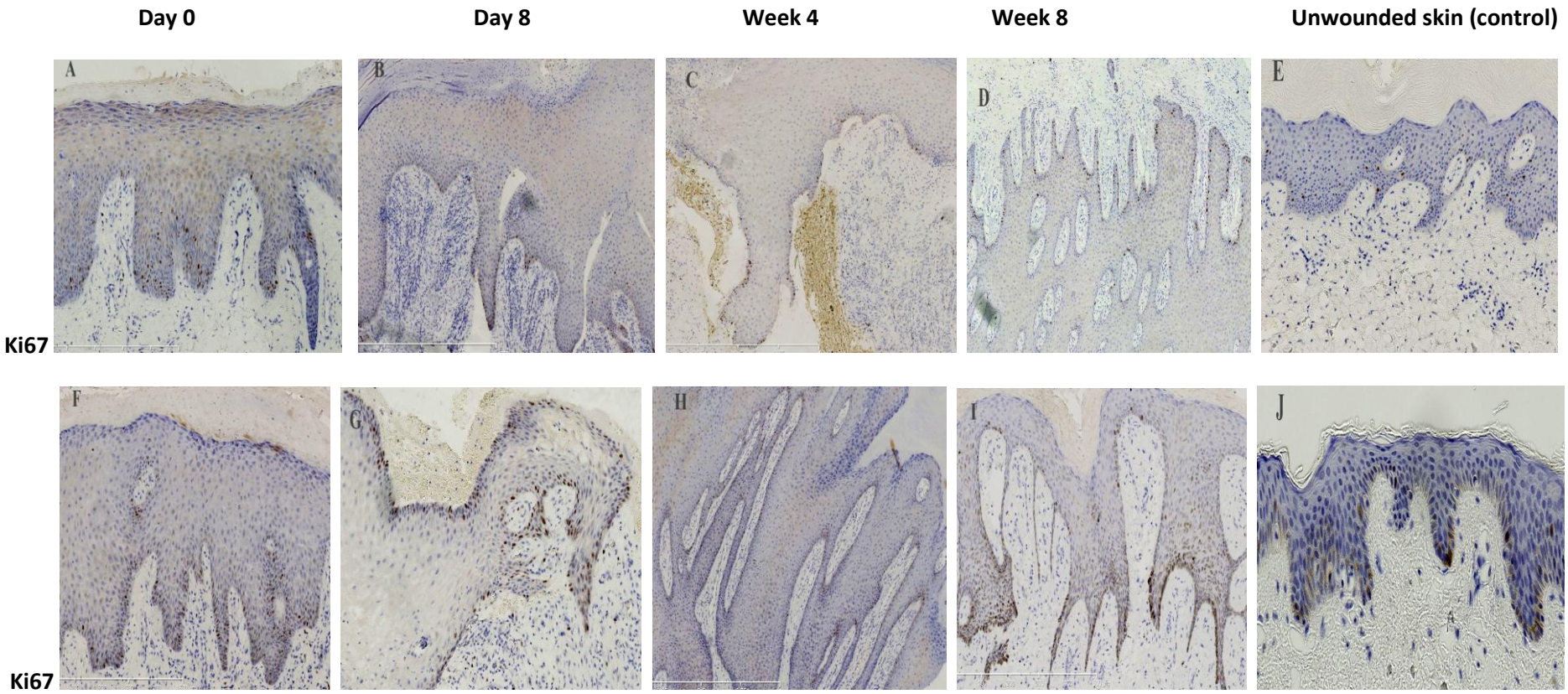


Figure 5.11 Microphotograpic representations from immunoperoxidase staining of Ki67 represents a proliferation marker; approximately 10% of interfollicular basal keratinocytes was seen in unwounded normal skin **(E).** Expression of Ki67 in wound margin of patients that achieved complete wound healing **(A-D**). Biopsy from ulcer margin from patients that did not achieve complete re-epithelisation, maintained a high expression of Ki67, which is a sign of hyper-proliferation, which is a hall mark of chronicity.

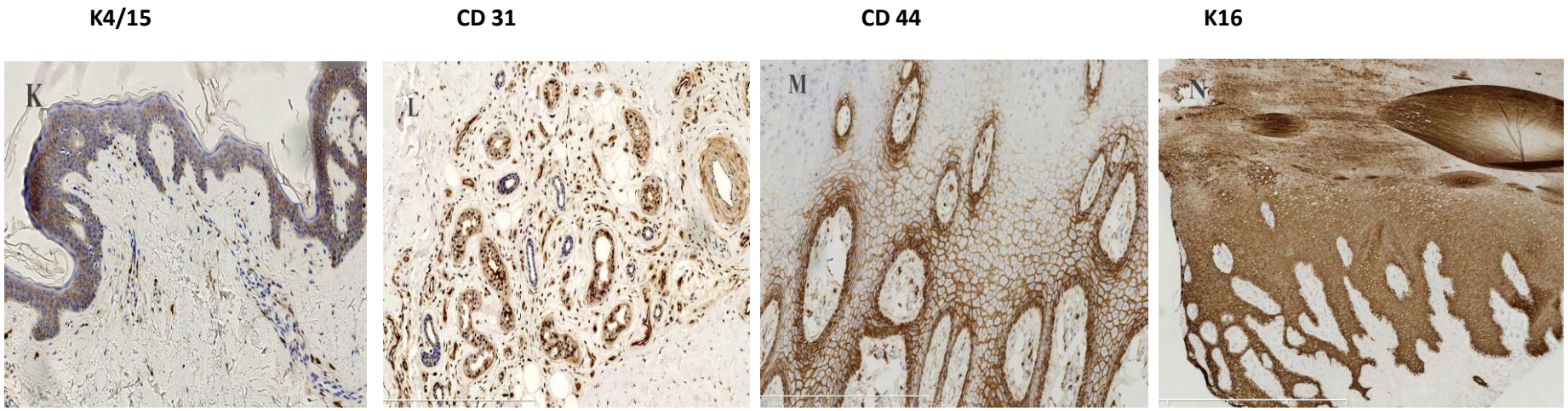


Figure 5.12 Microphotographic representations from immunoperoxidase staining from positive control K) Representation of K4/15; a co-expression of keratins which typify differentiation process of keratinocyte. **I)** Expression of CD 31 in a area of high microvessel density (unwounded skin). **M)** Expression of CD 44 in the normal diabetic skin (Plantar surface of an unwounded skin). **N)** Expression of K16 in the normal diabetic skin (Plantar surface of an unwounded skin)

	Researcher's	**Assessment**		**Operator 2**	**Assessment**	
Patient ID	**K14 Granular**	**K14 Spinosum**	**K14 Basal**	**K14 Granular**	**K14 Spinosum**	**K14 Basal**
P1,Day 0	4+	2+	1+	4+	2+	1+
P1,Day 8	4+	2+	1+	4+	2+	1+
P1 , Wk4	4+	3+	2+	4+	3+	2+
0P1, WK8	3+	2+	2+	3+	2+	2+
P2, day 0	4+	2+	1+	4+	2+	1+
P2, day8	4+	2+	1+	4+	2+	1+
P2, wk4	4+	4+	2+	4+	**3+**	2+
P2, wk8	4+	3+	2+	4+	3+	2+
P3, day0	4+	3+	2+	4+	3+	2+
P3, day8	4+	3+	1+	4+	3+	1+
P3, Wk 4	3+	3+	2+	3+	3+	2+
P3, wk8	3+	3+	3+	3+	3+	3+
P4, day0	4+	4+	3+	4+	**3+**	3+
P4,day 8	3+	3+	3+	3+	3+	3+
P4,wk 4	4+	4+	4+	4+	4+	4+
P4, wk8	4+	4+	4+	4+	4+	4+
P5, day 0	3+	3+	1	3+	3+	1
P5 day8	No Epidermal	No Epidermal	No epidermal	No Epidermal	No Epidermal	No epidermal
P5 wk4	4+	3+	3+	4+	3+	3+
P5 wk 8	3+	3+	3+	3+	3+	**2+**

Patient ID	**K14 Granular**	**K14 Spinosum**	**K14 Basal**	**K14 Granular**	**K14 Spinosum**	**K14 Basal**
P6 day 0	4+	3+	2+	4+	3+	2+
P6 day 8	4+	4+	3+	4+	4+	3+
P6 wk 4	4+	4+	2+	4+	4+	2+
P7 day 0	4+	3+	3+	4+	3+	**2+**
P7 day 8	4+	3+	2+	4+	3+	2+
N.Diabetic skin	1+	2+	4+	1+	2+	4+
Normal skin	0	1+	4+	0	1+	4+

Table 5.3 Immunoperioxidase staining for anticytokeratin K14 antibody examined under X20 High power field. Staining intensity was assessed across the basal, spinosum and granulosum layer and were represented with scores from 0-4; 0- no stain, 1- faintly stained, 2-weak satin, 3-moderately stained and 4- strongly stained . The scanned images from NanoZoomer digital Pathology system were assessed separately by the researcher and a senior laboratory scientist, in order to ascertain the level of correlation between the 2 assessors. Bland–Altman test was used to determine the degree of agreement between the two assessors, the closer the average bias to zero the less the discrepancy in the degree of agreement between the assessors. The average bias for K14 is 0.0512

	Researcher's	**Assessment**		**Operator 2**	**Assessment**	
Patient ID	**K1/K10 Granular**	**K1/K10 Spinosum**	**KI/1K0 Basal**	**K1/K10 Granular**	**K1/K10 Spinosum**	**KI/1K0 Basal**
P1,Day 0	3+	3+	2	3+	3+	2
P1,Day 8	3+	3+	2	3+	3+	2
P1 , Wk4	3+	3+	2	2+	3+	1
0P1, WK8	3+	2+	2	**2+**	2+	1

P2, day 0	3+	2+	1+	3+	1+	1+
P2, day8	3+	2+	1+	3+	2+	0
P2, wk4	3+	3+	1+	3+	2+	1+
P2, wk8	3+	3+	1+	3+	3+	0
P3, day0	3+	1+	0	3	1	0
P3, day8	3+	2+	0	3	2	0
P3, Wk 4	2+	2+	0	2	2	0
P3, wk8	3+	3+	3+	**3+**	3+	2+
P4, day0	3+	3+	0	3+	3+	0
P4,day 8	2	2	0	2	2	0
P4,wk 4	2+	2+	0	2+	2+	0
P4, wk8	3+	3+	0	3+	3+	0
P5, day 0	2+	2+	0	2+	2+	0
P5 day8	3+	3+	1+	3+	2+	1+
P5 wk4	4+	3+	0	3+	3+	0
P5 wk 8	4+	3+	1+	3+	3+	1+
P6 day 0	3+	2+	1+	3+	2+	1+
P6 day 8	3+	2+	0	3+	2+	0
P6 wk 4	4+	3+	1+	3+	3+	1+
P7 day 0	3+	3+	1+	3+	2+	1+
P7 day 8	3+	3+	0	3+	3+	0
N. Diabetic skin	3+	4+	0	3+	3+	0
Normal skin	3+	4+	0	**2+**	**3+**	0

Table 5.4 Immunoperioxidase staining for anticytokeratin K1/K10 antibody examined under X20 High power field. Staining intensity was assessed across the basal, spinosum and granulosum layer and were represented with scores from 0-4; 0- no stain, 1- faintly stained, 2-weak satin, 3-moderately stained and 4- strongly stained. The scanned images from NanoZoomer digital Pathology system were assessed separately by the researcher and a senior laboratory scientist. The average bias on Bland- Altman test was 0.0204

	Researcher's	**Assessment**			**Operator 2**	**Assessment**
Patient ID	**CD44 Granular**	**CD44 Spinosum**	**CD44 Basal**	**CD44 Granular**	**CD44 Spinosum**	**CD44 Basal**
P1,Day 0	1+	2+	4+	1+	2+	4+
P1,Day 8	1+	2+	4+	1+	3+	3+
P1 , Wk4	1+	3+	3+	2+	3+	3+
P1, WK8	0	0	1	0	0	1
P2, day 0	0	3+	4+	1+	3+	4+
P2, day8	0	3+	4+	1+	3+	4+
P2, wk4	0	2+	3+	1+	2+	3+
P2, wk8	0	3+	2+	1+	3+	2+
P3, day0	1+	3+	4+	1+	3+	4+
P3, day8	1+	2+	3+	1+	2+	3+
P3, Wk 4	1+	3+	4+	1+	3+	4+
P3, wk8	1+	2+	2+	1+	2+	2+
P4, day0	0	3+	3+	1+	3+	3+
P4,day 8	0	2+	4+	1+	2+	4+
P4,wk 4	0	2+	4+	1+	2+	4+
P5 day8	0	3+	4+	0	3+	4+
P5 wk4	0	2+	4+	0	2+	4+

	Researcher's	Assessment			Operator 2	Assessment
Patient ID	**CD44 Granular**	**CD44 Spinosum**	**CD44 Basal**	**CD44 Granular**	**CD44 Spinosum**	**CD44 Basal**
P5 wk 8	0	3+	4+	0	3+	4+
P6 day 0	0	3+	3+	0	3+	3+
P6 day 8	1+	3+	3+	0	3+	3+
P6 wk 4	1+	3+	2+	1+	3+	2+
P7 day 0	0	3+	4+	0	3+	4+
P7 day 8	1+	2+	3+	1+	2+	3+
Normal Diabetic skin	1+	2+	4+	1+	2+	4+
Normal skin	1+	3+	4+	1+	3+	4+

Table 5.5 Immunoperioxidase staining CD44 antibody antibody examined under X20 High power field. Staining intensity was assessed across the basal, spinosum and granulosum layer and were represented with scores from 0-4; 0- no stain, 1- faintly stained, 2-weak satin, 3- moderately stained and 4- strongly stained. The scanned images from NanoZoomer digital Pathology system were assessed separately by the researcher and a senior laboratory scientist. The average bias on Bland-Altman test was -0.0307.

	Researcher's	Assessment	Operator 2	Assessment	Researcher	Operator 2
Patient ID	**Colg I P. Dermis**	**Colg I R. Dermis**	**Colg I P. Dermis**	**Colg I R. Dermis**	**Beta-1 int**	**Beta- 1 int**
P1,Day 0	4+	4+	4+	4+	3+	3+
P1,Day 8	4+	4+	4+	4+	3+	3+
P1 , Wk4	4+	4+	4+	4+	4+	4+
P1, WK8	4+	4+	4+	4+	4+	4+
P2, day 0	3+	4+	3+	4+	4+	4+
P2, day8	4+	4+	4+	4+	3+	3+
P2, wk4	4+	4+	4+	4+	3+	3+

	Researcher's	Assessment	Operator 2	Assessment	Researcher	Operator 2
Patient ID	**Colg I P. Dermis**	**Colg I R. Dermis**	**Colg I P. Dermis**	**Colg I R. Dermis**	**Beta-1 int**	**Beta- 1 int**
P2, wk8	4+	4+	4+	4+	3+	3+
P3, day0	4+	4+	4+	4+	3+	3+
P3, day8	4+	4+	4+	4+	3+	**2+**
P3, Wk 4	4+	4+	4+	4+	3+	3+
P3, wk8	4+	4+	4+	4+	3+	3+
P4, day0	3+	3+	3+	3+	3+	3+
P4,day 8	4+	4+	4+	4+	2+	2+
P4,wk 4	4+	4+	4+	4+	2+	2+
P4, wk8	4+	4+	4+	4+	4+	**3+**
P5, day 0	4+	4+	4+	4+	3+	3+
P5 day8	3+	4+	3+	4+	4+	4+
P5 wk4	4+	4+	4+	4+	4+	4+
P5 wk 8	4+	4+	4+	4+	4+	**3+**
P6 day 0	4+	4+	4+	4+	3+	2+
P6 day 8	4+	4+	4+	4+	3+	3+
P6 wk 4	4+	4+	4+	4+	3+	2+
P7 day 0	4+	4+	4+	4+	3+	3+
P7 day 8	4+	4+	4+	4+	3+	3+
Normal Diabetic skin	4+	4+	4+	4+	4+	4+
Normal skin	4+	4+	4+	4+	3+	3+

Table 5.8 Immunoperioxidase staining for Collagen I and beta-1 integrin antibody examined under X20 High power field. Staining intensity was assess in the papillary and rete dermis and were represented with scores from 0-4; 0- no stain, 1- faintly stained, 2-weak satin, 3- moderately stained and 4-strongly stained. The scanned images from NanoZoomer digital Pathology system were assessed separately by the researcher and a senior laboratory scientist. The average bias on Bland-Altman test was 0 for collagen 1 and 0.0054 for beta-1 integrin, which means that there was 100% agreement between the 2 assessors for collagen 1.

	Researcher' s	**Assessment**	**Operator 2**	**Assessment**	
Patient ID	**Colg III P.Dermis**	**Colg III R. Dermis**	**Colg III P.Dermis**	**Colg III Rete Dermis**	**Colg VII BM**
P1,Day 0	4+	3+	4+	3+	0
P1,Day 8	4+	4+	4+	4+	0
P1 , Wk4	4+	4+	4+	4+	1+
P1, WK8	4+	4+	4+	4+	2+
P2, day 0	4+	3+	4+	3+	**3+**
P2, day8	4+	4+	4+	4+	2+
P2, wk4	4+	4+	4+	4+	1+
P2, wk8	3+	4+	3+	4+	2+
P3, day0	4+	3+	4+	3+	3+
P3, day8	4+	3+	4+	3+	1+
P3, Wk 4	4+	4+	4+	4+	2+
P3, wk8	3+	3+	3+	3+	**3+**
P4, day0	3+	3+	3+	3+	4+
P4,day 8	3+	3+	3+	3+	2+
P4,wk 4	3+	3+	3+	3+	3+
P4, wk8	4+	4+	3+	2+	2+

	Researcher' s	Assessment	Operator 2	Assessment	
Patient ID	**Colg III P.Dermis**	**Colg III R. Dermis**	**Colg III P.Dermis**	**Colg III Rete Dermis**	**Colg VII BM**
P5, day 0	4+	4+	4+	4+	4+
P5 day8	4+	3+	4+	3+	2+
P5 wk4	3+	3+	3+	3+	3+
P5 wk 8	3+	3+	3+	3+	2+
P6 day 0	4+	4+	4+	4+	0
P6 day 8	4+	4+	4+	4+	0
P6 wk 4	4+	4+	4+	4+	0
P7 day 0	4+	3+	4+	3+	0
P7 day 8	4+	3+	4+	3+	1+
Unwounded Diabetic skin	4+	4+	4+	4+	3+
Normal skin	4+	4+	4+	4+	4+

Table 5.**7** Immunoperioxidase staining for Collagen III antibody examined under X20 High power field. Staining was assessed in the papillary and rete dermis. Collagen VII staining was assessed across the basement membrane. The intensity were represented with scores from 0-4; 0- no stain, 1-faintly stained, 2-weak satin, 3- moderately stained and 4- strongly stained. The scanned images from NanoZoomer digital Pathology system were assessed separately by the researcher and a senior laboratory scientist. Average bias on Bland –Altman test was 0 for collagen III (100% agreement two the 2 assessors) and 0.066 for collagen VII.

	Researcher's	Assessment	Operator 2	Assessment
Patient ID	**K16 Granular**	**K16 Spinosum**	**K16 Granular**	**K16 Spinosum**
P1,Day 0	2+	2+	1+	2+
P1,Day 8	3+	3+	2+	2+
P1 , Wk4	3+	3+	2+	3+
P1, WK8	3+	3+	3+	3+
P2, day 0	4+	4+	4+	4+
P2, day8	4+	4+	4+	4+
P2, wk4	4+	4+	4+	4+
P2, wk8	4+	4+	4+	4+
P3, day0	3+	4+	3+	4+
P3, day8	4+	4+	4+	4+
P3, Wk 4	3+	4+	3+	4+
P3, wk8	3+	3+	3+	3+
P4, day0	4+	4+	4+	4+
P4,day 8	4+	4+	4+	4+
P4,wk 4	4+	4+	4+	4+
P4, wk8	3+	3+	3+	3+
P5, day 0	4+	4+	4+	4+
P5 day8	4+	4+	4+	4+
P5 wk4	4+	4+	4+	4+
P5 wk 8	4+	4+	4+	4+

	Researcher's	Assessment	Operator 2	Assessment
Patient ID	**K16 Granular**	**K16 Spinosum**	**K16 Granular**	**K16 Spinosum**
P6 day 0	3+	3+	3+	3+
P6 day 8	3+	3+	3+	4+
P6 wk 4	3+	3+	3+	2+
P7 day 0	3+	3+	3+	3+
P7 day 8	3+	3+	3+	3+
Unwounded Diabetic skin	4+	3+	4+	3+
Unwounded normal skin	4+	3+	4+	3+

Table 5.8 Immunoperioxidase staining for K16 antibody examined under X20 High power field. Staining was assessed in the papillary and rete dermis. Collagen VII staining was assessed across the basement membrane. The intensity were represented with scores from 0-4; 0- no stain, 1- faintly stained, 2-weak satin, 3- moderately satined and 4- strongly stained. The scanned images from NanoZoomer digital Pathology system were assessed separately by the researcher and a senior laboratory scientist. Finally, the Bland-Altman test showed that the average bias between the 2 raters was 0.0062.

5.5 RESULTS

There was vast heterogeneity among ulcers from the patients with diabetes. Some ulcers showed no sign of active infection requiring only sharp debridement in the clinic (plantar ulcers), whereas others were severally infected and ulcer with clinical gangrenous required surgical debridement and or digital amputation. The thickness of ulcer edge epidermis ranged from normal (6-10 cell layers) to very hyperplastic (20-40 layer) **see figure 5.3**. There were some structural changes noticed on some of the biopsy samples which resulted in some form of tissue loose either during wound biopsy or tissue processing. The documentation of dates and frequency of treatments, such as debridement, were recorded and discussed in **chapter 6**. The heterogenous nature of the ulcers treated may account for some of the variability of results. All 7 patients recruited were tested with all the antibodies at different time points. A summary of the antibody used and qualitative evaluation of immmunohistochemical data are showed in **tables 5.1, 5.3-5.8**.

5.5.1 Suprabasal Keratins (K16,K1/K10)/ Basal keratin

K16 staining was entirely suprabasal, the pattern of expression was similar in the healed and non healed groups **(Fig 5.11),** although not all the patients in the healed group expressed the similar staining intensity **(Table 5.8),** and the average bias between the 2 raters was 0.0062 on Bland-Altman agreement test which is very close to zero.

K10 mirrored K1 in all samples, the expressions were mainly suprabasal, however some biopsy in the non-healed ulcer group showed an altered pattern with involvement of basal layer which are deeply stained on the immunoperoxidase **(Table 5.4).** A similar pattern was seen in the unwounded normal skin. The average bias between the 2 rater was 0.0204 on Bland-Altman test. Although the main expression of K14 should be mainly basal, we observed that the entire epidermal layer were stained in both groups with the predominate staining in the basal layer, however in the unwounded normal skin and diabetic skin **(Fig 5.7),** the expression of K14 was limited to the basal layer. The average bias was 0.0512 on Bland-Altman agreement test.

The distribution of ki67 is mostly in the basal layer although, Ki67 were occasionally seen in the suprabasal layer. Ki67 is a marker of hyperproliferation which is a hall mark of chronic ulcer. The distribution of Ki67 in the group of patients that achieved complete wound healing decreases as normal pattern of healing is restored. In the unwounded skin, Ki67 stains approximately 10% of the basal interfolicular keratinocyte **(Figure 5.11).** The distribution and amount of Ki67 in the week 8 biopsy when normal wound healing is almost guaranteed wass comparable to the unwounded skin. However, the non-healed group the expression of Ki67 remained throughout the 8 weeks of up.

5.5.2 Basement Membrane Zone (Beta-1 integrin, Collagen VII)

Beta -1 integrin was restricted to the basal keratinocytes in the unwounded skin **(Figure 5.8),** however in the wounded biopsy wound margin, although its mainly found in the basal layer, some extension towards the spinosum was observed. There appear to be increasing pattern of β1- integrin expressing in the basal keratinocyte and some variable expression in the suprabasal layer **(Table 5.6).** The average bias between the two raters was 0.0054 for beta-1 integrin on Bland-Altman agreement test.

Collagen VII is a basement membrane protein, which is evenly distributed and stained in the unwounded skin, however the degree of staining improves as normal re-epithelisation is restored during wound healing process **(Figure 5.10).** The average bias between the two raters was 0.0667 on Bland-Altman agreement test **(Table 5.7)**.

Cell surface marker CD44 were found in the basal and spinous cell layers, but not present in the stratum granulosum and stratum corneum. The expression of CD 44 was more intensed in the post debridement biopsy sample (day 0), the intensity decrease as the wound process towards full re-epitheliasation **(Figure 5.8)**. The average bias was 0.037 on Bland-Altman agreement test **(Table 5.5)**

5.5.3 Extracellular Matrix Marker (Collagen I and III)

Collagen I and III were assessed both in the rete and papillary dermis for staining in intensity, there are to be no difference in the staining intensity. The distribution and integrity of the fibrils appeared undistorted during the process of wound in healing **(Figure 5.9& Figure 5.9).** The average bias was zero for both Collagen on Bland-Altman agreement test, which translate to 100% agreement between the 2 raters **(Table 5.6 &Table 5.7).**

5.4.4 Microvesel/ Endothelial Cell Marker (CD31)

Changes in micro-vessel density from the wound margins were assessed by manually counting the number of microvessel in 4 rectangular boxes from a predetermined area of high vascularity. The average count represents the microvessel density for that slide **(Figure 5.5).** The common trend noticed from both the healed and the non-healed ulcer group was that, the microvessel density of the wound decreases as the wound approaches week 8, which is presumably a sign of wound maturity **(Figure 5.6).**

5. 5 DISCUSSION

Wound healing occurs as a cellular response to injury and involves activation of keratinocytes, fibroblasts, endothelial cells, macrophages and platelets. Many growth factors and cytokines released by these cell types are needed to coordinate and maintain healing. A number of physiological factors have been identified to be contributory to poor wound healing. These include decreased or impaired growth factor production and angiogenic response, macrophage function, collagen accumulation, epidermal barrier function, quantity of granulation tissue, kerationcyte and fibroblast migration and proliferation, balance between the accumulation of ECM component and their remodelling by MMPs (Falanga, 2005 and Lobmann et.al 2002)

Molecular analyses of biopsies from the epidermis of patients have identified a pathogenic marker that correlates with delayed wound healing. These include inhibition of keratinocyte migration, over

expression of c-myc and nuclear localization of β-catenin (Stojadinoviv et.al, 2005 and Brem et. al 2007). In diabetic foot ulcers, the nonhealing wound edges shows absence of keratinocyte migration, hyperproliferation and incomplete differentiation. Fibroblast from similar ulcers demonstrates a phenotypic change as well as decreased migration and proliferation. Lack of healing is also demarcated by a thicker epidermis, thicker cornified layer and presence of nuclei in the cornified layer Brem and Tomic-Canic, 2007. From our result it is also evident that wound categorised into the non-healing group exhibited thicker epidermal layer and distorted architectural pattern, with thicker and nucleated corneum layer **(Figure 5.3).** In agreement with studies conducted on venous ulcers by Andriseen et. al 1995, our results showed that the epidermis of ulcers margins from patients with diabetes are highly proliferative, no matter the thickness of the ulcer edges. The epidermis adjacent to the ulcer, with no gross indications of being affected by the ulcer, is also highly proliferative. Freedberg et al., 2001, reported that signals initiating the keratinocyte activation cyle (Ki67) seem to result in sustained proliferation in the epidermis even at great distance from the ulcer margin. Natarajan et. al .2006, demonstrated that p16 is activated in migrating keratinocytes, and co expresses with uncleaved precursor of 3-alpha chain of lamini 5. This has also been associated with growth arrest. In the absence of p16, transcription for genes encoding proteins necessary to initiate chromosome replicates takes place. Keratinocyte that express p16 are migratory and not mitotic. This study excluded diabetic ulcer; thus we can not draw conclusion about expression of p16 in epidermal margins of diabetic ulcers.

There existed a striking difference between the pattern of immunostaining expression of keratins K14, K1/K10, Ki67 when chronic ulcer margins are compared with the unwounded normal skin. All keratinocyte layers of the chronic wound stained with K14 similarly between days 0 to week 8. Usui et al., 2005 showed that K14 is restricted to the basal keratinoocyte layer after 28 days of wounding in an acute wound. K1/K10 expression is suprabasal and the pattern of expression is very similar in the chronic diabetic ulcer and the unwound skin. Usui et.al 2005 also showed the same result in the migrating wound tongue of acute normal wounds. In chronic ulcer margins and in apparently normal tissue adjacent to the ulcer K16 is dramatically stained throughout the epidermis. Despite the presence of K16, an activated, migratory keratinocyte phenotype, keratinocyte at the ulcer margin are not activated to migrate. Our immunohistochemical studies indicated that all of the samples tested with K16 antibody showed intense staining which is similar to result published in migrating tongue of acute wound studied by Usui et.al, 2008, However there appear to be more intense in the non healing group **(Figure 5.9)**

It has been observed that cutaneous wounds in which the basement membrane remains intact heals rapidly and with little scarring, this may partly be due to the relatively small wound size and or tissue lose but also because the intact basement membrane (BM) provides essential signals required to direct re-epithelisation. Ulcerated lesion in which the BM is histologically absent are usually slow to heal and some form of BM is required before re-epithelisation. We observed a similar pattern with collagen VII synthesis when comparing immunostaining patterns from the completely re-epithelised group and the recalcitrant group. There was impairment of collagen VII expression in the BM of the 4 patients with the non- healed ulcer, the expression and synthesis of collagen VII were inconsistent over the 8 weeks period **(Table 5.7).** On the other hand adequate and consistent synthesis of Collagen VII was an evident feature amongst the 3 patients that achieved complete wound healing. **(Figure 5.10).** Collagen VII synthesis and incorporation into anchor fibrils is probably a prerequisite for the synthesis of BM which is essential for adequate wound healing (Hopkinson et.al, 1997). In a study conducted by Hopkinson et.al, they showed active collagen VII transcription from 13 out of 15 chronic leg ulcers, indicating Collagen VII biosynthesis. Of the two patients that Collagen VII transcription was not detected from, one of the patients was known to have insulin independent diabetic mellitus and the other patient as a non-specific lower limb chronic ulceration.

Under regular culture conditions all integrins present in human epidermis were found in keratinocytes, consistent with previous reports (Carter, 1990). Virtually no integrin staining was observed in cells harbouring differentiation markers, such as keratins K1 and K10, which were mainly in a suprabasal position. In a study conducted by Breitkreutz et al., 1997, on integrin and basement membrane normalization in mouse grafts of human keratinocytes, they demonstrated that the staining for β1 integrins (α2β1, α3β1) was diffuse and extended to suprabasal cell layers, besides a variably intense reaction at the matrix interface. After 1 week, the β1 distribution became more restricted and showed the highest concentration in the basal or the lowermost two layers. The final tissue consolidation with a well-defined BM was underlined by strictly pericellular β1-location in the basal layer.

It has been suggested that β1 could well be an indicator of the actual proliferative potential and elevated β1-levels have been proposed as one criterion for an epidermal subpopulation harbouring putative stem cells (Jones, 1995). In our result a similar pattern of β1 intergrin was noticed as the wound progressed towards week 8 (Figure 5.8), with the expression of β1 more restricted to the basal layer. Furthermore, if the assumption is correct that the β1-distribution spatially defines the compartment of proliferation-competent cells in concert with attachment via α6β4 (Tennenbaum,

1996). The down-regulation of these integrin functions should be involved in the process of regaining tissue normalization, which includes the timely onset of differentiation.

The findings on collagen I and III (Figure 5.9 &5.10) in post-intervention biopsies revealed gradual restoration of collagens towards normalcy in terms of their density distribution in the group of patient that achieved healing. The staining intensity appears the same in both groups of patient treated. However, there appeared to be some distortion in the architectural array of collagen fibrillar in the non healed group.

CD44 is a ubiquitous transmembrane glycoprotein is expressed on the plasma membrane of both epidermal keratinocytes and leucocytes (Wang, 1992 &Tuhkanen, 1999). Previous studies demonstrated that CD44 plays an important role in cutaneous function. For example, CD44 knockout mice was shown to exhibit the following features (Bourguignon et al., 2006); (i) a defect in epidermal permeability barrier homeostasis; (ii) epidermal thinning; and (iii) decreased keratinocyte differentiation. Likewise, in cultured keratinocytes, hyaluronan–CD44 interaction stimulates keratinocyte differentiation and lamellar body formation (Bourguignon, 2006). Accordingly, hyaluronan induces keratinocyte proliferation both in primary cell cultures and epidermal hyperplasia in wild-type, but not in CD44-deficient mice (Bourguignon et al., 2006; Kaya et al., 1997). It remains controversial whether CD44 is pro-inflammatory or anti-inflammatory in skin, however, CD44 regulates lymphocyte proliferation, maturation, activation, homing, extravasations and infiltration (DeGrengele, 1997, Zoller, 2007). From our study there are appears to be down-regulation of CD44 as wound progresses towards epithelial restoration.

5.6 CONCLUSION

In summary, chronic diabetic foot ulcer may be converted to acute ulcers following adequate debridement and display cellular pattern similar to acute wound. Keratinocyte at the margins of ulcers are highly proliferative, they show an activated phenotype as indicated by presence of K16. In addition, the keratinocyte demonstrates evidence of differentiation as exemplified by the expression of K1/K10. Reduction in the expression of Ki67 was seen as proper wound healing process ensures. The expression of CD44 tends to decrease as healing heads in the normal direction. The expression of beta -1 integrin remained unchanged and when compared to the unwounded skin. The restoration of collagen VII in the basement membrane could be a predictable sign of re-epithelisation. The quality and the architecture of collagen I & III remained unchanged during the course of wound

healing. To further understand the effect of PRP on the molecular mechanism involved in keratinocyte migration, basement membrane restoration and changes occurring in extraceullar matrix during healing, a control group (treated with PPP) or a group receiving standard wound care for diabetic ulcer should be equally tested. A better understanding of molecular mechanism involved in the keratinocyte and ECM behaviour may lead to possible molecular targets for therapeutic benefits of chronic diabetic foot ulcers.

CHAPTER 6

Autologous Derived Platelet-rich Plasma Gel may have Therapeutic Implications in the Treatment of Chronic non-Ischaemic Diabetic Foot Ulcers

6.1 Introduction

Chronic wounds pose a major management problem in the health sector, often requiring a multi-disciplinary approach. The majority (90%) of chronic wounds result as a progression from diabetic wounds, venous ulcers (chronic venous insufficiency ulcers), and pressure sores. Diabetic foot ulcers (DFU) seem to account the most for the global burden of the disease, especially with the attending complications of recurrence, chronicity, and the resultant lower limb amputation (Boulton, 2005). There is a global increase in the prevalence of diabetes mellitus, particularly type II diabetes mellitus, which is directly linked with obesity and a sedentary life style. It is believed that 20% or more of the United Kingdom adult population over the age of 65 years suffer with type II diabetes mellitus (Ragnarson, 2004). Two studies from North European countries reported the annual incidence of foot ulcers in the general population to be just more than 2% (Abbott et. al, 2002). The annual incidence rates in diabetic neuropathic individuals vary from 5–7% (Abbott, 1998). The lifetime risk for a diabetic patient to develop a foot ulcer could be as high as 25% (Singh, 2005). Of this population, 15% will either suffer foot or limb amputation (Singh, 2005).

It is likely that the cumulative lifetime incidence of foot ulcers may be as high as 25% (Singh, 2005). Shearer et al. confirmed that diabetic patients with neuropathic risk factors incur five times more direct medical costs for ulcers and amputations, and live for 2 months less than individuals without neuropathy (Shearer, 2003). Lower limb amputation is performed 15 times more frequently among diabetics, as compared with non-diabetic patients (Most, 1983). The American Diabetic Association did a cost analysis of medical expenditure for the United States population with and without diabetes in 2002; the study was based on national health care survey data. The direct and indirect expenditure attributable to diabetes was estimated at 132 billion U.S. dollars. The direct medical expenditure alone totalled 91.8 billion U.S. dollars (23.2 billion U.S. dollars for diabetic care, 24.5 billion for chronic complications, and 44.1 billion U.S. dollars for excess prevalence of general medical conditions). The attributable indirect expenditure resulting from lost workdays, restricted activity days, mortality, and permanent disability due to diabetes totalled 39.8 billion U.S. dollars (Hogan, 2003 and Saar, 2005). Fundamentally, the development of diabetic foot ulcers and the resultant chronicity could be said to have resulted from peripheral vasculopathy, peripheral neuropathy, and immunopathy. There are other contributory factors which stems from these three major risk factors. These include repetitive trauma to areas of the foot exposed to moderate to high pressure, reduced resistance to infection, immunologic disturbances, and microcirculation alterations.

Different authors and researchers have attempted to classify diabetic wounds from the view point of aetio-pathogeneses and failure to demonstrate significant improvement following good clinical care as a guide to determine poor outcome. Presently, there are no early predictive factors to guide clinicians to differentiate patients who will heal readily from those who will have a protracted course of treatment. The American Diabetic Association has attempted to offer a lasting solution by clarifying the definition of a chronic wound, which they defined as; "failure to show continuous progress towards healing. These are wounds that remained unhealed after 4 weeks, were a source of concern and were associated with worse outcomes including amputation" (American Diabetes Association, 1999). In addition, the decision to change to advanced therapeutic intervention should not be based solely on timing, but also in conjunction with careful monitoring of the percentage of the wound closure. A reduction in wound area of 10–15% per week represents a normal healing process and should not necessitate a change in wound management strategy or re-evaluation (Sheehan, 2003). Other authors have demonstrated that chronic diabetic wounds lack significant growth factors which are stimulatory agents for wound healing. These growth factors play important roles in the tissue-remodelling phase through mesenchymal cell recruitment and extra matrix synthesis. This understanding has led to the development of recombinant platelet derived growth factor-BB (becaplermin) that has been approved for use and has shown a degree of success in treating DFU (Robson, 1992).

6.2 Rationale for the Use of Autologous Platelet Gel

In the late 1970s the importance of growth factors within the wound-healing cascade was first identified. This began with platelet-derived growth factors and the subsequent identification of many other growth factors now known to be important within the different stages of the wound healing cascade. These growth factors aid the three phases of wound healing which are the inflammatory, proliferative, and remodelling phases. These phases involve complex paracrine mediated growth factors which influence mitogenic and cellular differentiation activities. Chronic wounds are thought to have increased proteases, increased pro-inflammatory cytokines, decreased protease inhibitors, and decreased growth factor activities. Cooper et al. showed that a number of growth factors were markedly reduced in wound fluid from chronic wounds as compared with acute wounds (Cooper, 1994). Also, FGF and TGF-β concentrations are down regulated in chronic wounds and are significantly lower when compared with acute wounds (Cooper, 1994). It has also been shown that the use of a synthesized single growth factor in treating chronic diabetic wounds has only produced a limited degree of success. However, with the recent understanding of bio-tissue engineering,

autologous derived platelet rich plasma gel can effectively and safely deliver all the needed growth factors to stimulate tissue reparation.

The rationale for employing this technique is to mimic the normal physiological wound healing and reparative tissue process. Autologous derived platelet gel (APG) has a wide and safe application within the post operative surgical field both as a wound sealant and as a tissue repair agent. Its application has extended to patients that are prone to higher surgical complications, and this is also true of diabetic patients. The modification of cell saver technology has made it possible to synthesize AGP with structural and functional properties, as close as possible, to those of natural soft tissue and epidermis. Besides, the ability of AGP to deliver multiple growth factors with a synergistic effect to wound sites, the clot formed from AGP serves as a scaffold and a protein reservoir, thereby locally concentrating and magnifying their effect. The platelet plug acts as a barrier to micro-organism invasion of wounds, achieved with the help of highly concentrated leukocyte buffer, present within APG.

The availability of concentrated leukocyte with about 5-fold the baseline value makes the graft matrix infection free. It also promotes mitogenesis of mesenchymal stem cells at the wound site. As a three dimensional volumetric soft connective tissue replacement, it provides a matrix medium for cell migration, granulation tissue formation, and epithelia wound contracture in addition to serving as a non disturbed wound patch. The overall efficacy of AGP in treating wounds is likely to be a function of many variables such as the platelets concentration, the volume of AGP delivered to the wound, the extent and the type of injury and perhaps, the overall medical condition of the patient AGP contains autologous-derived living cells, which are able to deliver a program of healing to the wound that may not be achieved with a single growth factor. Another added advantage of autologous preparation technique is the inherent safety and therefore freedom from concerns of transmissible disease, such as, human immunodeficiency virus, hepatitis, and Creutzfeldt-Jakob disease. There is anecdotal evidence to suggest APG is effective in the treatment of chronic diabetic ulcer. To our knowledge, there are few studies that have collaborated on the effectiveness of APG with histological changes from treated ulcers. Therefore we analyzed the therapeutic effect of APG and its local biological effect on chronic non-ischemic diabetic foot ulcers. The primary purpose of the pilot study was to determine the number of patients that achieved complete re-epithelisation at the end of 16 weeks treatment.

6. 3 Anti-bacterial Effect of Autologous Derived Platelet rich Plasma

Existing evidence suggests that platelets have many functions in the antimicrobial host defence systems (Krijgsveld et.al, 2000**).** These include expression of immunoglobulin-G Fc receptors,

C3a/C5a complement fragments, and the capacity to generate antimicrobial oxygen metabolites including superoxide, hydrogen peroxide, and hydroxyl free radicals (Yeaman, 1997). Tang in 2002, reported the isolation and tentative identification of seven antimicrobial peptides from human platelets after stimulation by thrombin as follows: fibrinopeptide A, fibrinopeptide B, thymosin β-4, platelet basic protein, connective-tissue-activating peptide 3, RANTES and platelet factor 4. These antimicrobials were found to be more potent against bacteria than fungi, and their activities were dose-dependent. This findings support direct antimicrobial effect of PRP. Platelet-rich plasma also has a high concentration of leucocytes. Furthermore, PRP has been found to possess markedly increased leukocyte counts (more than sevenfold) compared with baseline levels (Bielecki, 2006). In a study by Bielecki, et.al 2007, a strong effect of platelet-rich gel against, *E. coli*, methicillin-resistant *staphylococcus aureus*, and methicillin-sensitive *staphylococcus aureus* was demonstrated. No PRP activity was found against *K. pneumoniae*, *E. faecalis*, or *Pseudomonas aeruginosa*. In contrast PRP was observed to induce the growth of Pseudomonas aeruginosa, suggesting that it may induce a flare-up of infection.

6.4 Evidence to Support the Clinical Application of Platelet Rich Plasma Gel

Different authors have proposed that platelet-rich plasma technology has relevant application in promoting hard and soft tissue wound healing; and potentially decreasing postoperative wound infections, blood loss, and pain. Platelet rich plasma has found clinical application in most fields of surgical practice. There are scientific literatures to support its efficacy in periodontal and oral surgery, maxillofacial surgery, aesthetic plastic surgery, heart bypass surgery, orthopaedic and spinal fusion surgery, and in the treatment of chronic skin and soft tissue ulcers (Eppley, 2004, Marx, 2001 and Marx, 2004). Although the various published data appears promising on its efficacy in wound healing, most are case studies or series; hence, most of the evidence is anecdotal. There are very few prospective randomized controlled clinical trials to support the acclaimed potentials. Knighton et al. reported that 17 of 21 patients with chronic lower limb ulcers achieved complete re-epithelization over an average period of 8.6 weeks, with a course of twice-daily wound treatment with platelet release suspended on a collagen base, compared to two of 13 similar wounds treated with placebo (Knighton,1986). After crossover of the placebo group, all the remaining 11 chronic ulcers were treated with the same bio-active agent (platelet release suspended on a collagen base). All patients in the placebo group eventually achieved 100% re-epithelization in an average of 7.1 weeks after crossover.

6.5 Multidisciplinary Approach to Wound Management and its Future

The complexity and the poor outcome associated with healing chronic diabetic foot ulcers necessitate the need for a multi-disciplinary approach to treating these groups of patients. Most physicians involved with the care of these patients are increasingly advocating for an integrated wound care centre. This centre will foster an environment for effective communication and collaboration for all care givers involved with this patient at each stage of their treatment. Some of the specialists include podiatrists, Plastic, Vascular and Orthopaedic surgeons, diabetologists, nutritionists, radiologists and neurologists. In addition, this centre has the potential of galvanizing already available resources, streamlining protocol implementation, reducing cost, and ultimately accelerating wound healing. This approach has demonstrated significant improvements in outcomes, including reduction in the incidence of major amputation (Dargis, 1999).

6.5.1 Other Adjuvant Treatment

The treatment of diabetic foot ulcers requires management of a number of systemic and local factors, including: `Precise diabetic control which is vital in achieving resolution of the current wound, but also in minimizing the risk of wound recurrence. Close monitoring of Hb1 AC and glycaemia level are vital, as well as management of contributing systemic factors, such as hypertension, hyperlipidemia, atherosclerotic heart disease, obesity, or and renal insufficiency (Margolis , 2001)

The management of diabetic foot ulcers requires offloading pressure from the wound by using appropriate footwear, daily dressings to provide a moist wound environment, debridement when necessary and the use of antibiotic therapy when superficial infection supervene (Hilton,2004). Non-healing deep cavity wounds may require application of negative pressure under vacuum-assisted closure (Evan, 2001) and other adjunct such as cultured human cells (Veves, 2001).to achieve wound coverage and complete healing

6.5.2 The Role of Vascular Surgeon in Managing Chronic Diabetic Foot Ulcers

All patients harbouring diabetic foot ulcers should be evaluated by a qualified vascular surgeon who will consider debridement, revisional surgery, vascular reconstruction, and options for soft tissue coverage. As part of the wound assessment process, the vascular surgeon evaluates blood flow to the wound area by means of handheld Doppler. A triphasic Doppler signal indicates normal blood flow, a biphasic signal indicates adequate blood flow and a monophasic signal warrants further investigation. A questionable blood flow or signal may necessitate a formal non-invasive arterial Doppler evaluation or a conventional angiogram combined with a computerised tomographic scan. If an inadequate flow is established, the wound debridement may be initially delayed in the setting of

dry gangrene until the blood flow is restored. Restoration of blood flow may be by means of endovascular intervention by way of angioplasty and stenting or conventional open bypass surgery. Premature debridement of a devascularised wound may cause future loss of potentially salvageable tissue. However, immediate debridement is mandatory in the presence of wet gangrene, ascending cellulitis from a necrotic wound, or necrotizing fascitis are present. Revascularization should follow as soon as the infection is under control (Attinger, et. al 2001)

6.5.3 Debridement

Proper debridement is defined as the removal of hyperkeratotic, infected, and nonviable tissue from a wound, which is essential to accelerate the process of wound healing (Steed, 1996). Surgical management is indicated for debridement of nonviable and infected tissue from the ulceration, removal of excess callous, curettage of underlying osteomyelitic bone, skin grafting, and revascularization. The potential benefits derived from frequent debridement include removal of inhibitors of wound healing (metalloproteases, collagenases matrix metalloproteinase 1 and 8 and elastases) and more effective growth factor function (Trentgrove, 1999). Furthermore, aggressive removal of all necrotic, infected, and nonviable tissue prevents the establishment of protease rich proteins in the wound base. The wound usually requires an initial surgical debridement and probing to determine the depth and involvement of bone or joint structures. Visible or palpable bone implies an 85% chance of osteomyelitis. Surgical wound closure: Delayed primary closure of a chronic wound requires well-vascularized clean tissues and tension-free apposition; it usually requires undermining and mobilization of adjacent tissue planes by creation of skin flaps or myocutaneous flaps. In general, the indications for vascular surgery in the presence of a reconstructible arterial lesion include intractable pain at rest or at night, intractable foot ulcers, and impending or existing gangrene (Faries, 2004).

6.5.4 Soft Tissue Coverage Options

There are few options available for soft tissue coverage of the clean but non-healing wound: Once a wound has reached a steady clean state, a decision has to be made about allowing healing by natural processes or expediting healing by a surgical procedure. Clinical experience and observation of the healing progress in each case dictates the appropriate management. Surgical options include skin grafting, application of bioengineered skin substitutes, and flap closures (Ehrenreich, 2006).

1. Autologous skin graft is the gold standard for viable coverage of partial thickness wounds. Meshing the graft allows wider coverage and promotes drainage of serum and blood.

2. A cadaveric skin allograft is a useful covering for relatively deep wounds following surgical excision when the wound bed does not appear appropriate for application of an autologous skin graft. The allograft serves as a bridge whilst a more definitive closure method is being planned for.

3. Surgical wound closure: Delayed primary closure of a chronic wound requires well-vascularized clean tissues and tension-free apposition; it usually requires undermining and mobilization of adjacent tissue planes by creation of skin flaps or myocutaneous flaps.

4. Tissue-cultured skin substitutes: Bioengineered skin (Apligraf) and human dermis (Dermagraft) are new types of biologically active implants for ulcers that are derived from fibroblasts of neonatal foreskins (Veves, 2001). These bioengineered products enhance healing by acting as delivery systems for growth factors and extracellular matrix components through the activity of live human fibroblasts contained in their dermal elements.

a. Dermagraft (Smith & Nephew) is a cryopreserved human fibroblast–derived dermal substitute produced by seeding neonatal foreskin fibroblasts onto a bio-absorbable polyglactin mesh scaffold. Dermagraft is useful for managing full-thickness chronic diabetic foot ulcers. It is not appropriate for infected ulcers, those that involve bone or tendon, or those that have sinus tracts. Allergic reactions to its bovine protein component have been reported

b. Apligraf (Organogenesis) is a living, bilayered human skin substitute. It is not appropriate for infected ulcers, those that involve tendon or bone, or those that have sinus tracts. Allergic reactions to the agarose shipping medium or its bovine collagen component have been reported (Streit, 2000). The use of bioengineered skin substitutes has been questioned because the mechanism of action is not clear, the efficacy is questionable, and the cost is high.

c. Xenograft is a xenogeneic acellular collagen matrix derived from porcine small intestinal submucosa in a way that allows an extracellular matrix and natural growth factors to remain intact. This provides a scaffold for inducing wound healing. Xenograft is avoided in patients with allergies to porcine materials.

6.5.5 Biological/ Cellular Therapy

Tissue engineering is a biological science that specializes in the development of biological substitutes to restore, maintain, or improve the function of the skin. It seeks to create a readily available tissue replacement with the biologic and pharmacologic properties of the human skin (Fohn, 2007). The principle of implementing this method in treating chronic wounds is called biological or cellular therapy. Implementation of cellular therapy is recommended when wound size cannot be decreased by more than 10% within 3 weeks. There are a number of commercially available dermal matrices that have been approved by the United States Food and Drug Administration. This includes Alloderm TM (Life Cell Corp., The Woodlands, TX), altered allograft; Integra TM (Integran Life Science Corp., Plainsboro, NJ), dermal regeneration template; Dermagraft TM (Smith and Nephew, London, UK), synthetic dermal replacement/dermal regeneration template; Apligraf TM (Organogenesis, Inc., Canton, MA), composite skin replacement, and Hyalograft-3D TM (Fidia Advanced Biopolymers, Abano T., Italy). Biological therapy may be an ideal treatment for diabetic foot ulcers because it adds cells that release growth factors to a growth factor dependent environment, increases cytokines and matrix proteins, and promotes angiogenesis (Trent, 1998). It is also believed that they accelerate healing time and decrease the risk of wound infection. Of all the available cellular therapy techniques, Apligraf TM (organogenesis, and Hyalograft-3D TM (esterified hyaluronic acid beneath silicone) have shown efficacy in the treatment of chronic diabetic foot ulcers (Stark, 2004).

6.6 Rationale for Gene therapy in wound healing

The combination of bioengineered skin substitutes and the standard of care in treating diabetic foot ulcer, have only achieved a wound healing rate of about 50%, which highlights the critical need for a more practical, safe and effective therapy for non-healing diabetic foot ulcers (Steed et.al., 2006). Delivery of the gene encoding growth factor will resolve the problem of transient expression of GFs at wound site. This could potentially overcome the limited maintenance of the protein at the ulcer site and the need for daily protein administration. In chronic diabetic peripheral neuropathic ulcers, the healing process is impaired in part by the deficiency of growth factors (Cooper, 1994).

GAM501 utilizes a replication-defective adenoviral vector containing the human platelet-derived growth factor-B gene (Ad5- PDGF-B) incorporated into a biocompatible matrix (2.6% bovine collagen) and is applied topically to the ulcer. Preclinical studies have shown GAM501 causes enhanced migration into and proliferation within the ulcer and gene-containing matrix of inflammatory cells, endothelial cells, fibroblasts, and other connective tissue cell types from

surrounding viable tissues (Gu et.al, 2004). The collagen component of GAM501 has two key beneficial functions: (i) it holds the Ad5-PDGF-B vector within the ulcer site until infiltrating cells arrive; and (ii) it promotes the ingrowths of migratory cells responsible for generation of granulation tissue. Once in the matrix, fibroblasts and other cell types take up the Ad5-PDGF-B vector, transcribe, express, and secrete the gene product acting as local in vivo bioreactors that produce PDGF-BB protein. Although the protein is synthesized only as long as the gene persists in the cell (the adenoviral vector transgene does not integrate into the chromosomes of the transfected cells and is inactivated over time), its availability is substantially prolonged relative to topical protein therapy.

In a preliminary study of GAM501 Phase 1 & 2 clinical trials conducted by Mulder et al., 2009, fifteen patients enrolled into the study with chronic, non-healing ulcers received either a single administration of GAM501 at one of three dose levels, or up to four administrations of GAM501 at 1-week intervals. All the patients received standard of care treatment including debridement and were required to wear an off-loading shoe. GAM501 was found to be safe and well tolerated with no evidence of systemic or local toxicity at all doses, no maximum-tolerated dose was reached. Serum antibody titres to platelet-derived growth factor-B homodimer and collagen were negative and adenoviral DNA was not detected in the blood. In the 12 patients that completed the study, ulcer closure was observed in 3 months in 10 patients, seven of whom received a single application of GAM501. In conclusion, GAM501 did not appear to have any toxicity at doses that showed biological activity. GAM501 holds promise as a potentially effective treatment for non-healing diabetic foot ulcers.

6.7 MATERIALS AND METHODS

The protocol employed in conducting the pilot study, the study design, the recruitment process and ethical consideration issues have been discussed in **Chapter 2 section 2.18**. Also details of preparation of PRP can be found in **section 2.19**

6. 7. 1 Characterization and Preparation of PRP

Before undertaking the application of PRP in chronic ulcers, a full characterization of the growth factors present in the patients PRP was carried out. In addition to quantifying the amount of growth factors present in patients' own PRP, we conducted a study to determine the variability in the expression of growth factors amongst the diabetic patients and the healthy non-diabetic subjects **(Details in chapter 3)**. Briefly, platelet-rich fibrin matrix was formed by adding the human derived

thrombin to the platelet rich concentrate. The PRP was divided into two halves: a part was used for wound treatment and the other half was allowed to retract over night for supernatant extraction. The released supernatants was assayed for the expressions of PDGFAA, TGF-b1, IGF-I, VEGF-A, FGF, TSF-1, PF-4 and EGF through the commercially available enzyme-linked immunosorbent assay kits (Quantikine colorimetric ELISA kits, R&D, and Minneapolis, MN). Full details of the processing and quantification of the growth factors have been fully discussed in **chapter 3 section 3.4**

6.7.2 Application of PRP and Study Design

All the patients' recruited into the pilot study received platelet gel therapy in addition to standard wound care (details of standard wound care: **Appendix 5**). The study protocol included an initial period of wound debridement in operating room; application of gel was either carried out in the operating room or a sterile mini- theatre in the surgical Out Patients' Department. Wounds were dried to prevent further diluting of the platelet gel before application. The platelet gel was applied directly over the debrided wound surface; the patients' foot was left for 5- 10 minutes to allow the platelet gel to set. This was followed by gentle application of damp mepitel on the wound to prevent disruption of the germinal layer of the wound. Two layered moist sterile saline gauze were applied on the mepitel layer, to prevent absorption of the gel into the dressing material, followed by a four layer of sterile moist gauze to further protect the wound. An opsite (adhesive dressing material) was applied on the dry gauze, followed by another layer of velband and crepe bandage. Patients with large wound cavity and post digital amputation were admitted for short hospital stay. However, patients who needed a second application of platelet gel were followed up as day cases and given an orthotic foot wear (medisure) to off-load pressure and to allow mobilisation as tolerated.

The first wound inspection was carried out by the research doctor, and post debridement wound biopsies were also taken. Pictures of the ulcers were obtained with a digital camera. Visitrak computerised planimetry was used to determine the surface area of the wound. The percentage of surface area healed was estimated as "initial ulcer surface -final ulcer surface/initial ulcer surface x100". Patients were followed up until complete wound healing (full epithelization) was achieved or 16 weeks of treatment period. The standard treatment for all patients was to receive a single application of platelet gel therapy. A repeat application when necessary was decided by the research team and the doctor. If a second application was recommended, it must not be repeated within 14 days of initial first treatment.

Wounds with superficial slough were debrided in the sterile mini-theatre. The presence of purulent discharge or offensive smell from the wound necessitated taking of wound swab and placing the

patient on empirical antibiotics, pending the outcome of microbiology sensitive pattern. Wounds that showed signs of deep seated infection and sloughiness underwent further surgical debridement and a repeat application of platelet gel therapy. Patients that continued to show signs of delayed wound healing and poor healing progress were reviewed by the principal investigator. Patients in this category were withdrawn from the study and treated appropriately. The primary endpoint of the study was to determine the number of patients that achieved complete re-epithelisation at the end of the study period.

6.8 RESULTS

Patient ID	**Age (yrs)**	**Aetiology of ulcer**	**Pre-treatment Duration of ulcer**	**Duration of Diabetes**	**ABPI**	**Location of ulcer**
P1	42	Neuroischemic	8wks	3yrs	1.4	Post amp big toe
P2	58	Neuroischemic	6 wks	10yrs	1.25	Post amp $4^{th}/5^{th}$ toe
P3	49	Neuroischemic	8wks	15yrs	1.18	Post amp 5^{th} toe
P4	64	Neuropathic	4yrs	18yrs	1.2	Plantar ulcer big toe
P5	59	Neuropathic	10mth	10yrs	1.2	Plantar ulcer Big toe
P6	56	Neuropathic	12 wks	30yrs	1.2	Post amp 2^{nd} toe
P7	50	Neuroischemic	8 wks	6yrs	1.07	Post amp big toe

Table 6.1 Shows the general demographic data, aetiology, location of ulcers, ankle brachial pressure index (ABPI) and Patient's individual identity were represented as P1-P7 (Amp-Amputation).

Patient ID	**Size of Ulcer cm^2**	**Debridement Type**	**Hb1 AC %**	**Platelet Count (x10000)**	**No of treatment**	**Duration of follow up**	**Complete Treatment Duration**
P1	4.2	surgical	12.9	309	single	10weeks	Yes
P2	32.1	surgical	10.8	470	single	16weeks	No
P3	14.3	surgical	15.7	354	twice	16 weeks	Yes
P4	3.3	Non-sur	6.9	280	single	16 weeks	Yes
P5	1.8	Non-sur	6.8	319	twice	16weeks	Yes
P6	4.1	surgical	7.2	293	single	8 weeks	No
P7	16.2	surgical	11	230	single	3weeks	No

Table 6.2 Provides information about ulcer size, pre-treatment glycosylated Haemoglobin (Hb1 Ac) pre-treatment platelet count, duration of follow up and number of platelet therapy application.

Patient ID	DM Type	Previous amputation	Renal failure	Mobility	Wagner Grading	Hospital stay	Complete Re- epithelisation
P1	NIDDM	Nil	Nil	Yes	4	No	Yes
P2	IDDM	Nil	mild	Yes	5	Yes	Yes
P3	IDDM	Nil	mild	Yes	4	Yes	Yes
P4	NIDDM	Nil	mild	Yes	3	No	No
P5	NIDDM	Yes	nil	Yes	3	No	No
P6	NIDDM	Yes	End-stage	Yes	4	Yes	No
P7	NIDDM	Yes	nil	Yes	4	Yes	No

Table 6.3 Provides the information about the patients' pre-morbid state as it relates to the type of Diabetes, previous amputation history, and presence of renal impairment, patient mobility and severity of the ulcer as represented by Wagner classification.

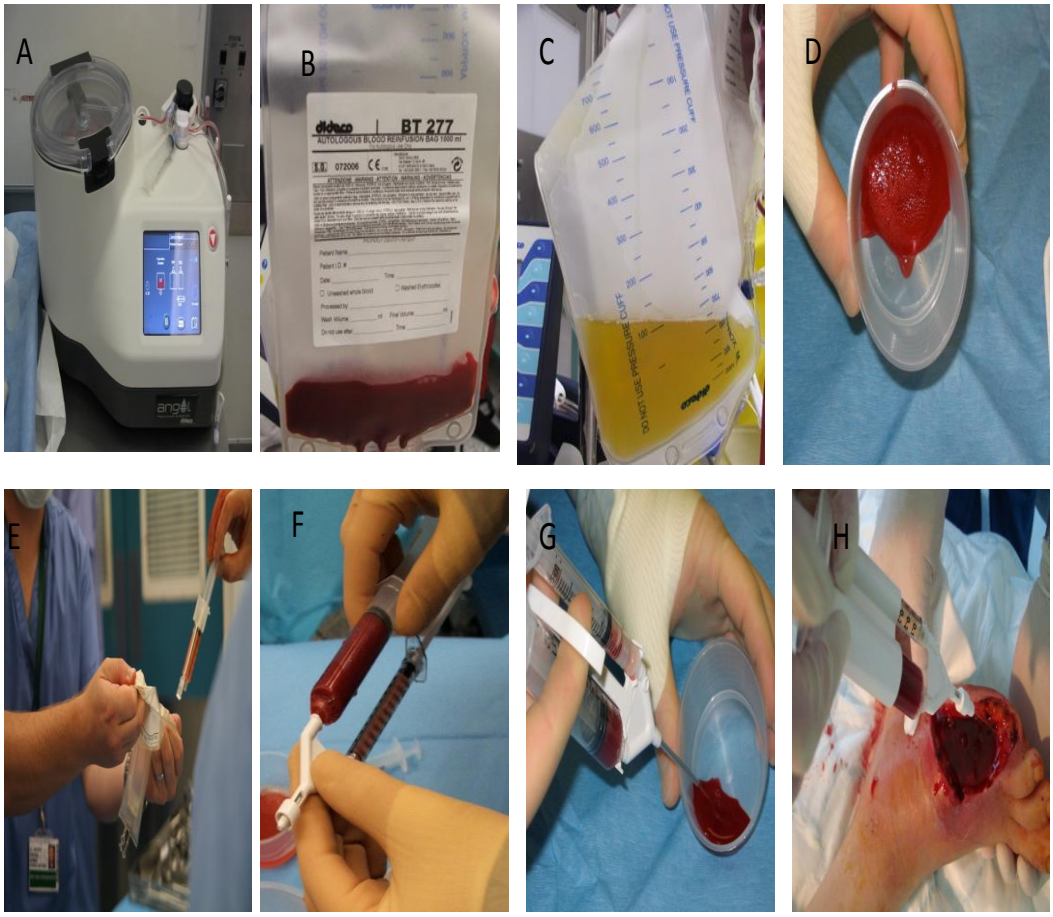


Figure 6.1 Represents the process involved in processing Autologous derived platelet- gel. A) Represents the automated centrifuging machine called the platelet separating system, which has 2 collecting bags connected to it; **B)** represents the separated red blood cells and after the 2 cycles of centrifuging and **C)** is the separated fresh plasma. **D)** The platelet concentrate from the process; **E)** shows the process of fresh thrombin production. **F)** The mixing of platelet concentrate with freshly prepared thrombin in 2 separate mixing chambers; **G)** the final product is the autologous derived platelet rich plasma (APG). H) Demonstrates the process of application of AGP to a debrided wound.

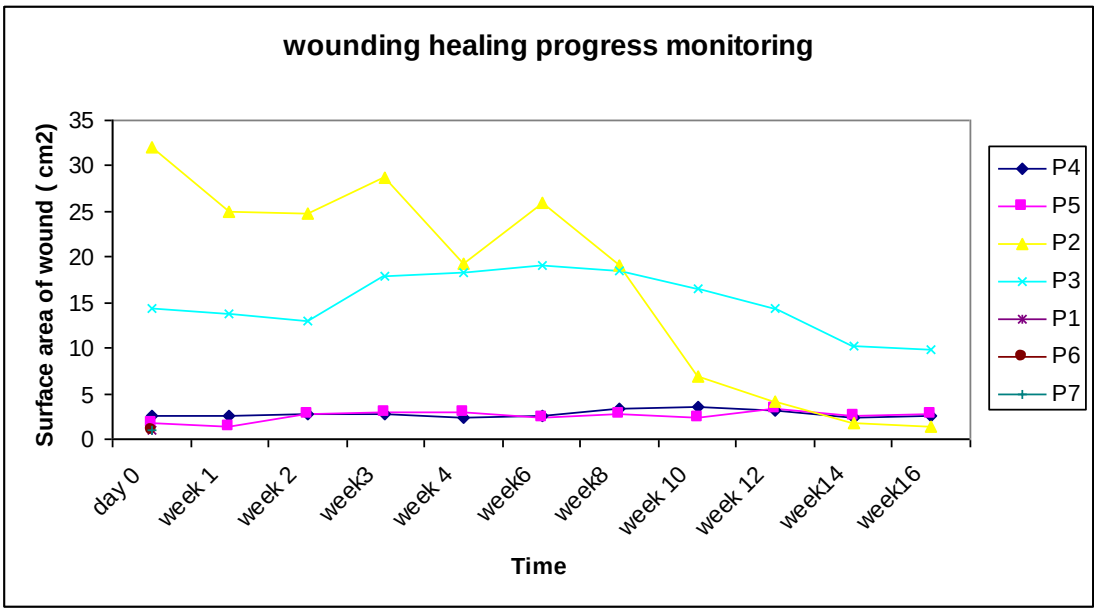


Figure 6.2 Graphical illustration of the wound healing rate **for all the groups of patient** treated during the follow up period of 16 weeks. P1-P3 achieved complete re-epithelisation, whereas P4-P7 did not heal during the study period.

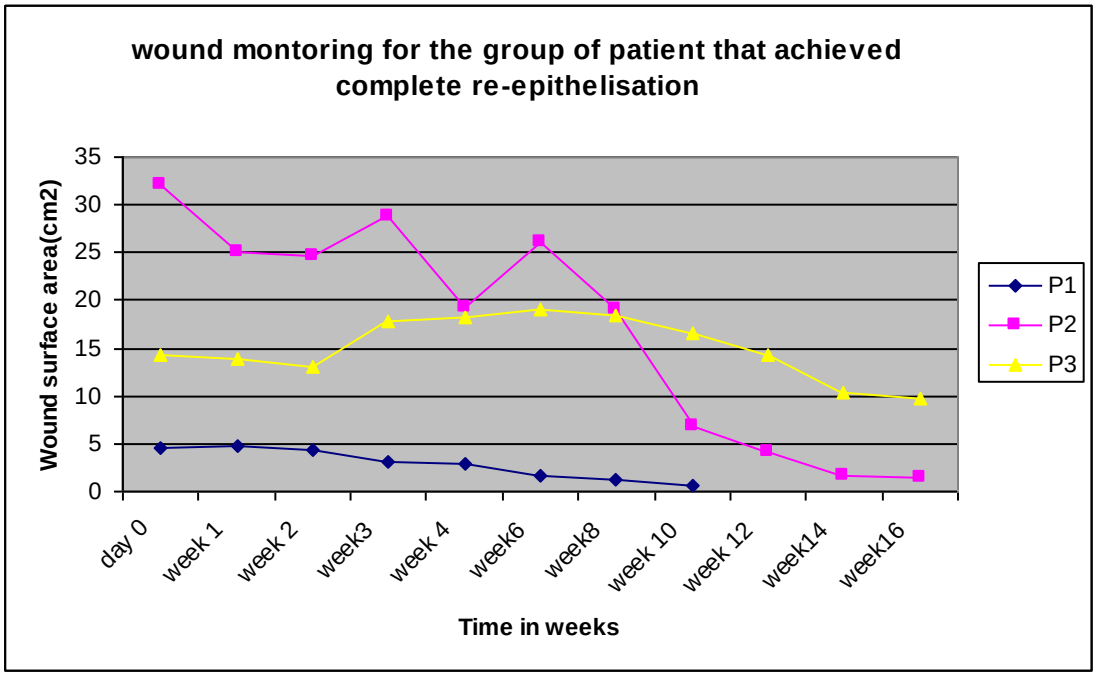


Figure 6.3 Graphic representation of the rate of re-epithelisation shows that P1 achieved complete re-epithelisation at 10 weeks, whereas P2 and P3 were completed healed at 16 weeks.

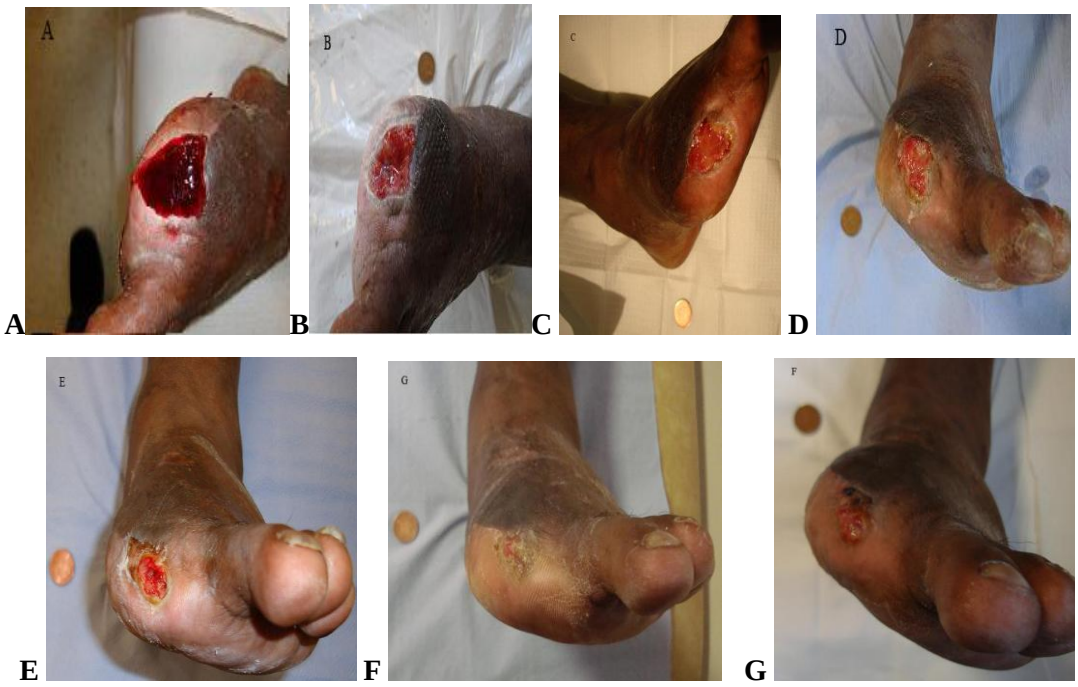


Figure 6.4 Patient I achieved complete re-epithelisation following single treatment with PRP after 8 weeks: **A)** debrided ulcer with platelet gel therapy at day 0, **B)** a day 8, **C)** week 1, **D)** week 3, **E)** week 4 , **F)** week 8 and **G)** week 10.

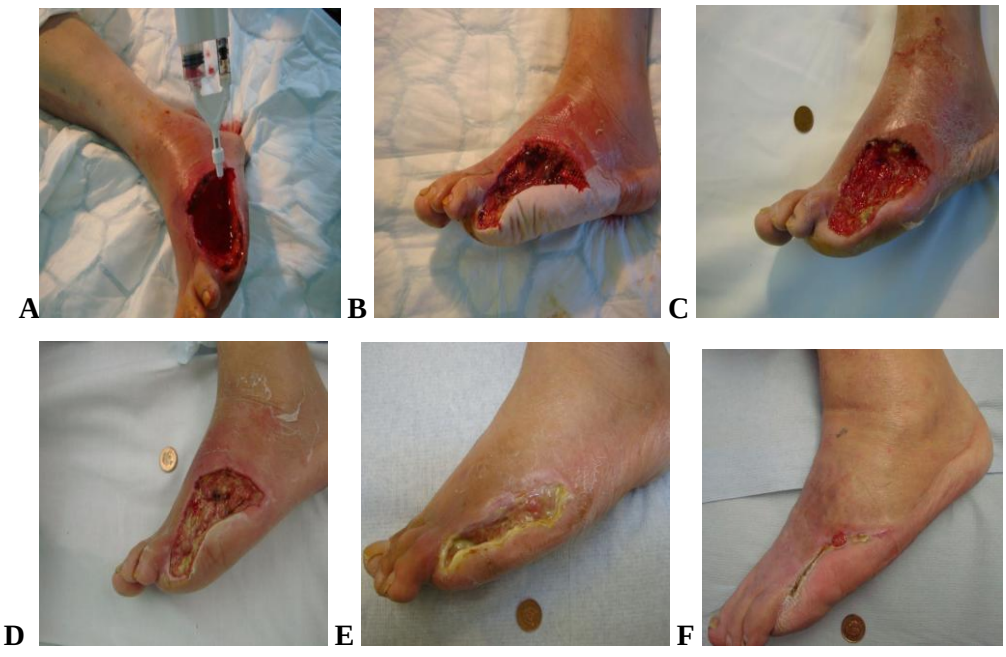


Figure 6.5 Patient II; evolution of a typical ulcer treated with PRP: **A)** debrided ulcer with PRP treatment; **B)** at week 1, **C)** week 4, **D)** week 6, **E)** week 10, and **F)** week 16

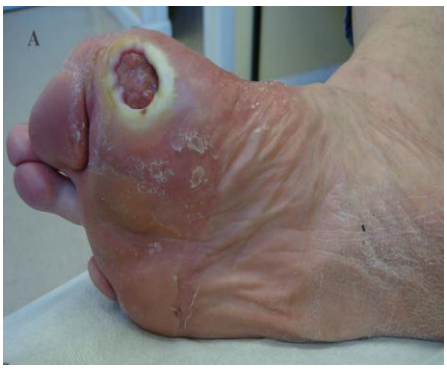
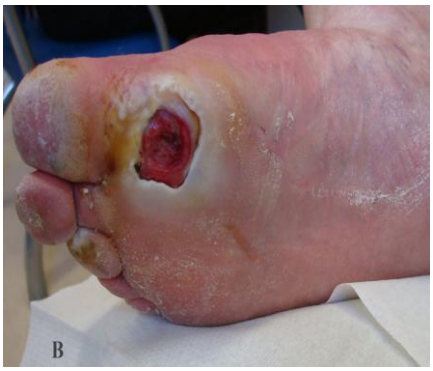
A  B 

Figure 6.6 photographic representations of Patient IV and V ulcers that were unable to heal despite treatment with PRP and good standard wound care. The common feature in these patients was that the ulcer was on the weight bearing point (base of the 5th Metatarsal). Biomechanical pressure off-loading was a major challenge with these patients, they were both fully ambulatory, and orthosis prothesis booth was used as a means of pressure offloading devise. Clinical feature seen from ulceration includes hypergranulosis, hyperkeratosis and raised wound edges; these features are in keeping with recalcitrant ulcers

6.9 DISCUSSION

In spite of available evidence that both recombinant PDGF (becaplermin) and platelet-derived growth factors administered as autologous platelet gel, can be effective in treating chronic cutaneous wounds (Crovetti et. al, 2004 and Papanas, 2007) and that this treatment can be cost effective (Dougherty, 2008), the consensus on the therapeutic use of PG is still controversial. The difficulty in recruiting large number of chronic diabetic foot ulcer patients' with reasonably comparable lesions is the main handicapping situation that have resulted in setbacks in organising randomised controlled clinical trials in this peculiar disease entity (Martinez-Zapata et.al). The main challenge with the clinical use of PRP is not whether it can be used in the treatment of cutaneous ulcer, but at what time-point and how often in the treatment course. Furthermore, the elaborate organisation involved in the processing of PG and also the need for repeated application of the therapy in most cases, in an already physiologically compromised patient is a major rate limiting step in its implementation.

Based on the above considerations, we planned a pilot study aimed at validating the production and characterization of growth factors derived from PRP **(details in chapter 3).** In addition, we assessed its safety and efficacy amongst diabetic patients with difficult to treat ulcers. In carrying out this study the following issues were pivotal to our study: **1)** to validate production of PRP (aliquots of

concentrated platelets) that would satisfy several requirements of routine process of periodic blood collection and processing of varying blood components; **2)** to maintain closed and aseptic conditions through the entire process; **3)** to ensure a reproducible and predictable quality and quantity of PRP produced; **4)** to ascertain that the PRP used is able to deliver adequate level of GF to promote wound healing and finally to report any adverse reactions directly or indirectly related to the use of PRP during this study. Although our study sample was small, our experience supports the feasibility and safety of our operational model; no undesired side effect related to the treatment occurred.

All seven male patients were treated with PRP, over a mean follow up period of 12weeks. Most ulcers were treated with a single application of PRP; however 2 patients were treated twice, making a total of 9 different applications. It was not possible to establish a clear correlation between number of applications and reduction in wound size, but this could be due to the small sample size of the study. The result from our pilot study could be summarized thus **(Table 6.1);** 7 patients were treated. All patient received the initial treatment with Platelet rich plasma gel after wound debridement. Furthermore, 5 patients later underwent surgical debridement and digital amputation and the remaining 2 others with plantar ulcers were debrided non-surgically. It is worth mentioning that all of our treated patients had ABPI of greater than one, which could be a reflection of the ongoing diabetic process and possibly vessel calcification, which by itself compromises blood supply to the lower limb. None of our treated patient had treatable peripheral vascular disease at the time of treatment.

Most of our patients received single treatment with PRP (71.4%), whilst 28.6% were treated twice. Furthermore, continuous non–surgical debridement were carried out in most of the patients at the outpatient clinic, with a cautious use of short term antibiotic for patients that appeared to have clinical and microbiological evidence of raging wound infection, two (28.6%) patients required antibiotic in the course of the follow up. Two (P6 & P7) patients (28.6%) were withdrawn from the study at 4 and 5 weeks respectively, because the ulcers became grossly infected and both required transmetatarsal amputation and application of vacuum assisted closure device, as a result of the large wound cavity. At the end of the 16 weeks study, 3 patients (42.8 %) of the 7, achieved complete re-epithelisation. If the 2 patients that were withdrawn from the study were to be adjusted for, we would have achieved 60% complete wound healing rate. Bearing this in mind, our result can be compared to results from Anitua et.al 2008. In their pilot study on 14 ulcers in nine patients, it was reported that 72.9% of the lesions which were treated with autologous platelet derived factors healed. Whereas only 21.7% of the lesions treated conventionally healed during the same period of 8 weeks. Similarly Mc Aleer , 2006, reported 20 (60%) out of 33 chronic ulcers lasting for 6 months achieved complete closure in a mean period of 11.1 weeks after treatment with APG. In a series of 32 patients with diabetic foot ulcers, 68.4% treated with PRP achieved complete closure compared to 42.9 % of the ulcers under conventional treatment (Driver. et.al, 2006).

Other confounding factors that was considered when analysing the outcome from this treatment were patients age, duration of ulcer before debdridement, size of ulcer post debridement, glycaemic control using glycosylated haemoglobin which is a reflection of the glycaemic control 2-3month before treatment, presence of systemic co morbidity such as renal impairment and the platelet count before concentration and the Wagner classification as shown in **Tables 6.1**. In view of the small sample size, we could only make observational remarks from our study and no significant statistical

inference could be drawn from it. It appears that patients with grade 4 Wagner classifications and long standing type 2 diabetes did not achieve complete re-epithelisation. The 2 patient with plantar ulcers did not achieve wound closure despite the fact that they had input from the podiatrist team. In addition, they were given adequate foot care to off load the biomechanical pressure off the big toe with the aid of appropriate footwear **(Figure 6.6).** A graphic illustration of the wound size-time course can be seen in **Figure 6.1**.

This study has its inherent pit falls. As stated earlier, it was a small pilot study aimed at demonstrating the safety and efficacy of using APG. In addition, although the sample size was small, it gave us the opportunity to study the immuno-histochemistry behaviour and cellular alterations that occur in diabetic ulcers. This would have been impossible with a larger series. Premature withdrawal of patients from the study and difficulties encountered in recruiting patients into the control arm of the study are part of the major limitations of our pilot study. The study was designed originally to recruit 5 patients into each arm of the study comprising of the active group (treatment with PRP) and control group (treatment with PPP). This was however not achievable, because of patient's preference even though we had originally obtained ethic approval for this small comparative pilot study. The patients that failed to heal after the 16 weeks of follow up were treated appropriately by the principal investigator. The 3 patients that achieved complete epithelisation were also followed up for a further 1 month in our joint multidisciplinary clinic to ensure that the wound did not breakdown again.

Although, in our small pilot we did not look at cost-effectiveness analysis, Dougherty, 2008 reported, that the use of PRP gel resulted in improved quality of life and lower cost of care over a 5-year period than other treatment modalities for non-healing diabetic foot ulcers. PRP represents a potentially attractive treatment alternative for health centre providers to address the cost burden and health effects of non-healing diabetic foot ulcers. In another study Mazzucco et al. observed that treatment of dehiscent sternal wounds with platelet gel reduced the complete healing rate (3.5 vs. 6 weeks) and hospital stay in 21 days when compared with conventional treatment. (Mazzucco et al, 2004).

However, some other studies on the efficacy of platelet rich products have no reported beneficial effects on the treated ulcers. One of such example, Stacey et al. compared 42 patients receiving a platelet lysate preparation with 44 patients having conventional treatment. The results showed that the platelet lysate product had no influence on the healing of chronic ulcers. These discrepancies may lead to a general controversy about the potential benefits of platelet-rich products. However, this result should be interpreted in the context of the aetiopathogenesis and the heterogeneicity of the patients' ulcers studied.

Weibrich, 2004 showed that platelet derived growth factor (PRGF) contains moderately elevated platelet concentration (2–3-fold increase), which has been reported to induce an optimal biological

benefit. In our study, we achieved about 6 fold increase in platelet concentrate. The platelet concentration and separation system is designed in such a way to avoid contamination of PRGF with leukocytes. However, it has been noted that neutrophil contamination could be as high as 1-3% during PRP production. PRGF without contamination improves the homogeneity of the product and equally reduces the donor-to-donor variability. Another disadvantage of neutrophil contamination is that neutrophils are an important source of MMP-8 and -9 and secrete other proteases, such as elastases (Yager et al., 1996) that are destructive for growth factors and release reactive oxygen species deleterious for cell survival Zimmermann, et al. (2001), showed that the lowest concentration and the highest activation of platelets, as well as, the highest WBC concentration in platelet concentrate occur from tube technique.

It has been observed that the plantar pressure is higher in subjects with callus formation (Pitei, 1999). There appears to be a relationship between callus and reduced tissue thickness. It is suggested that callus reduces the cushioning effect of plantar skin thickness which protects the skin from breaking down. Increased pressures under the metatarsal head (MTH) have also been shown prospectively to predict ulcer development (Pham, 2000). Preliminary data from diabetic and rheumatoid arthritic patients suggested that plantar foot pressures are strongly associated with reduced plantar tissue thickness under the MTHs (Young, 1995). Diabetic patients, especially those with foot ulcers, have been reported to have reduced plantar soft tissue thickness compared with non-diabetic subjects, indicating that this could be an important contributing factor in the development of foot ulceration (Brink, 1995).

Debridement is aimed at ensuring physiological progression of chronic wounds through the normal stages of healing, provided that they are maintained in an environment conductive to anabolic metabolism. Surgical wound debridement remains the most crucial step in enhancing the healing process of all chronic wounds, irrespective of the aetiopathogensis of the wound. In this study, wound debridement appears to be the commonest denominator to the 2 categories of the wound treated (the healed ulcer and the recalcitrant ulcer). Wounds may require additional measures to promote healing when they fail to progress despite adequate debridement or the progress of debridement has left vital structures (bone, tendon or neurovascular bundle) exposed that can not be left to heal by secondary intention. Also, when the wound closure rate is less than 10 to 15% per week, negative pressure therapy may be used to assist closure. Negative pressure wound therapy is an excellent tool that stimulates formation of granulation tissue as well as decreasing local oedema and controlling bacterial proliferation (Defranzo et al. 2001).

It was recently reported that fluid exudates from long-standing venous ulcers inhibit experimental angiogenesis (Drinkwater, 2002). This inhibiting effect could be related to presence of anti-angiogenic agents such as tissue inhibitors of metalloproteinase-1 (TIMP-1) in wound fluids, which counteract the proangiogenic effect of growth factors contained in topical platelet products (Herouy et al., 2000). Fibroblasts isolated from venous ulcers are more senescent compared with fibroblasts isolated from healthy skin, and have decreased proliferative ability, dependent on ulceration (Stanley, 2000). Indeed, chronic wound fibroblasts generally exhibit a decreased mitogenic response to PDGF-AA, PDGFBB, bFGF, TGFβ1, and EGF, compared with normal or acute wound fibroblasts (Agren, 1999). This may explain the poor healing response to topical recombinant growth factors (Agren 1999) and to platelet products of long-standing venous ulcers. The absence of the healing effect of platelet products on venous ulcers could also be due to destruction of its components by wound proteases. High levels of proteases are found in wound fluids from chronic

venous ulcers, impairing the healing process by degradation of exogenous added growth factors, such .

6.10 CONCLUSION

In summary, this study allows us to draw a number of conclusions; **1)** the preparation of well standardized PRP is feasible within a routine surgical practice; **2)** After adjusting for withdrawals, 60% of our patients achieved complete re-epithelisation. The healing observed can not be said to be entirely as a result of PRP treatment. However, it is not unreasonable to conclude that a well organised frequent standard wound care with appropriate foot care in conjunction with platelet gel therapy can facilitate healing of chronic diabetic foot ulcers. Furthermore, we can say that PRP is a safe and potentially reasonable adjunct in the treatment of chronic diabetic foot ulcers; **3)** The safety of APG was not in question as there were no adverse events reported during this study period (howbeit a small sample size). Many other studies support the fact that PRP is responsible for actively extruding growth factors, which initiate soft tissue healing and recruitment of stem cells. In the light of all the confounding factors and difficulties encountered in conducting this study, we proposed that a multicentre clinical randomised control trial with adequate statistical power might provide the missing link and answer some of the questions raised in this study. This will be the beginning of standardizing the use of PRP as an adjunct for treating chronic diabetic foot ulcers. Sharp wound debridement and a combination of many other discussed treatment modalities hold a promising future.

6.11 Overall Summary of the Thesis

The initial work in this project began by establishing a standard protocol for the production of autologus–derived Platelet rich plasma (PRP) and Platelet poor plasma gel (PPP) through the use of Platelet collecting and concentrating system (PCCS) {Sorin-Angel}. Through series of repeated production, we were able to establish a consistently high quality PRP/PPP gel. The next step was to characterise the expression of growth factors (GFs) which have been implicated in wound healing process. Furthermore, two groups of patients were recruited for the characterisation of GFs from PPP/PRP; 14 healthy volunteer donors and 6 diabetic patients, with the aim of comparing the expression of the different GFs from PRP/PPP.

Chapter 3 focussed on the 2 different immunoassay technique employed in measuring the expression of the GFs and the rationale for using the techniques. The GFs were measured using immuno-assay technique; Platelet derived growth factor (PDGF-AA), Fibroblast growth factor-2

(FGF-2), Transforming growth factors-beta1 (TGF-β1), vascular endothelia growth factor (VEGF), epidermal growth factor (EGF), Thrombospodin-1(TSP-1) and Platelet factor-4 (PF-4). The scientific basis for generating GFs from Platelet were founded on the principle that, alpha-granule of Platelet contains secretory proteins which on activation releases GFs. In our study, we used the Platelet count from the diabetic patient group as a prototype to demonstrate that the PCCS used in this research as the ability to increased Platelet concentration to an accepted published level. The average Platelet baseline value ranged from 130,000 to 500,000 platelet /µl. The PCCS increased the platelet count on the average from 334,000±117,000platelet/ µl to 1,995,000±805,000 platelet/µl; this is a 6-fold increase. The PRP group was significantly higher in platelet number compared to the baseline Platelet count, with a significance value of $p > 0.0005$.

Furthermore, a comparison was made between the various GFs expressed from PRP/PPP and the 2 groups. The details of the actual values were described in **chapter 3**. However it will suffice to mention that a 5-fold increase in EGF, 68-fold of PDGF-AA, 4-fold of VEGF, 28-fold of TGF-β1, 2-fold of P-Selectin and a 3-fold increase of TSP-1 was found when PRP and PPP was compared in the diabetic group. On the other hand, there was no significant increase in the expression of FGF-2, IGF and PF-4 in the diabetic group. Similarly, the same pattern of GFs expression was observed with the healthy donor with the exception of 69-fold increase in PDGF-AA and 38-fold increase in TGF-β1. It has been postulated that by increasing the concentration of Platelets, the expression of GFs released will invariably increase. There are existing controversies surrounding these postulations; from our study, by using the more expressed GFs as a prototype (PDGF-AA, VEGF and TGF-β1) from PRP derived from both the diabetic and healthy volunteers, we were unable to establish any correlation between platelet counts and amount GFs expressed.

We went a step further in **Chapter 4**, by obtaining supernatant from PRP/ PPP (from both the healthy volunteers and group diabetic patients) and were preserved in -80^0C. Primary human cells (fibroblast and keratinocyte) were extracted and cultured from redundant plastic surgery procedures such as foreskin, face lift. The cells were maintained in tissue culture and passage to established cells that were used for the purpose of different experiments. In addition, human umbilical endothelia cell (HUVEC) was commercially procured and used in the study. Other cell line used in the study were the NEB-1 cell, fibroblast cell line (mouse 3T3) and EA.hys cell, all of which were either used to maintain primary cells in culture or in optimizing experimental protocol **(See Chapter 2 and 4 for detailed description).**

In **chapter 4**, we used experimental model through tissue culture assay to test our research hypothesis. The preferred assay and cells used in this study were those with direct involvement with wound healing process. The hypothesis been tested were to assess (i) the proliferative effect of

PRP/PPP on HDF, HEK, NEB-1, EA.hys and HUVEC; (ii) to assess migratory effect of PRP/PPP on all the cell line mentioned above and finally (iii) to assess angiogenic effect of PRP/PPP on HUVEC. Proliferation assay was assessed through Alamar blue fluorescence technique, a positive proliferative effect of PRP/PPP was observed on all the cell types following experimental repeats. The proliferative effect observed with HUVEC, EA.hys and HDF were very similar, with 5% PRP showing the most prominent effect over other varying concentration of PRP (10%, 25% and 50%), PPP and the positive control (Media with 1% FCS or 1% BSA). It is worth mentioning that this effect also reached statistical significance. It is interesting to also note that a decreasing dose response phenomenon was observed in all 3 cell types mentioned above.

A further experiment will be needed to elucidate and explain this phenomenon **(see section on future work).** HEK and NEB-1 cell showed a slightly different pattern, in that the, the positive control showed a better proliferative effect over the varying concentration of PRP/PPP. Although, the positive control showed the most positive effect, the decreasing dose response effect was still preserved with the PRP/PPP treated cells. To further ascertain that the effect observed was not dependent on the type of the culture media used, the experiments were repeated with HDF/HEFK using Bovine serum albumin (BSA) and Fetal calf serum as the medium, it was interesting to note that proliferation for both cell types were better in BSA. In another experiment, the effect of serum was eliminated from fibroblast proliferation, we observed that all the varying concentration of PRP and PPP produced a far better proliferative effect than the control experiment which was a serum starved, in the 5% PRP produced the most positive effect. We therefore concluded that, the decreasing dose response phenomenon was possibly due to either the presence of an inhibitory growth factor in the constitution of PRP or there exist an antagonist effect of the GFs amongst each another. Furthermore, we suggested that presence of TSP-1 and PF-4 in PRP/PPP which are believed to possess anti-proliferative and anti-angiogenic effect might be responsible for this effect. BSA produced the optimal serum condition for sub-culturing fibroblast and keratinocytes and in addition PRP/PPP is capable of maintaining cell in tissue culture without the serum protein.

The migratory assay was aimed at evaluating the chemotactic effect of PRP/PPP on the different cell types through the use of modified Boyden chamber (Transwell migration assay) and wound/scratch assay technique. The details of how the two assays were conducted can be found in **Chapter 4.** A positive transwell migratory effect was observed with HUVEC, EA.hys and HDF when treated with varying concentration of PRP/PPP, with a statistically significant effect over the control treatment. A similar pattern of decreasing dose response which was initially observed with proliferation assay was also seen with transmigration assay, 5% PRP showing the most migratory effect. NEB-1 cell and

HEK also behaved in a similar pattern to their proliferation assay, with the control treatment producing the most positive migratory response.

Several attempts at conducting a wound/scratch assay with HUVEC were unsuccessful. The wound assay with HDF was only possible without mitomycin treatment. A statistically significant migratory effect was observed with 5% PRP over the control, and also with the decreasing dose response maintained. On the contrary, HEK and NEB-1 cell were both tested with and without mitomycin; we observed that there were no statistical difference after comparing the mitomycin treated with the non-treated group. The positive control showed a better migratory effect over the PRP/PPP treatment. In conclusion, some inference could be drawn form our study; i) the decreasing dose response seen with at 5% PRP could not have been an observational error, ii) the decreasing dose response is consistent with HUVEC and HDF across 3 different assay types, iii) we may infer that PRP dose not have a strong enough proliferative and migratory effect on HEK, or perhaps, the age and the site of keratinocyte extraction may down play the biological effect of PRP on HEK.

Finally, **in chapter 4,** we evaluated the angiogenic effect of PRP on HUVEC cell through matrigel angiogenic assay. Details of the 2 angiogenesis assay technique employed (Collagen/ matrigel) was discussed in full details in **chapter 4.** Following adequate literature review we identified that morphometric and topometric method of quantifying certain parameters from the angiogenesis assay may represents a better quantification method as oppose to either purely descriptive of semi-quantitative analysis. Hence, we developed our own macro-system which analyses micro-photographic images obtained at the end of the assay. This system analyses 2 morphometric parameter (nodal and mesh formation) and 2 topometric parameters (length of the skeletinised network and cellular area). The details and rationale for proposing this method has been discussed in great details in **Chapter 2 and 4.**

We observed that PRP had a positive and statistically significant angiogenic effect on HUVEC over the positive control. We can categorically say that nodal and mesh formation are reliable parameters with direct correlation to the degree of angiogenic activity. Measuring the length of the skeletinised capillary network, appear promising from our result, but we believe a repeat of the experiments may produced a more reliable quantification result. Surprisingly, one would expect that measuring the cellular area will give a better index of angiogenic activity, but to our surprise the soft programme was unable to distinguish between the properly formed capillary network and clump of cells. For example, PPP demonstrated a very weak angiogenic effect on matrigel assay, but formed large area of clumpy cells, during analysis, the cellular area under PPP larger as compared with PRP 5% which had a better angiogenic effect morphologically. It is worth mentioning that the formation obtained from cellular area is inversely proportionally to the angiogenic effect, and must be interpreted with

caution. Once again, it is interesting to note that 5% PRP produced the most angiogenic effect over other concentration of PRP/PPP and the positive control. Although, this method needs validating through experimental repeats with different endothelial cell types and angiogenic agents, we proposed that this may be a simpler, less cumbersome and more effect way of quantifying angigogenic properties of potential compounds.

In chapter 5, we endeavour to understand the possible alterations and histological changes that might be occurring between day 0 (post debridement) and week 8 (when complete re-epithelisation is in view or guaranteed). Wound biopsies taken from the wound margin of chronic ulcers of diabetic patient's after been treated with PRP at different time points (day 0, day 8, weeks 4 & 8) were subjected to both histological and immuno-histochemistry analysis. Details of the histological and immunohistochemistry techniques and method of tissue preservations employed were discussed in **Chapter 2 & 4.** A histological comparison was made between the patients' that achieved complete re-epithelisation and non-healed group. The cellular activities of the epithelia layer were monitored and measured at day 0, day 8 and week 4 & 8 respectively. In general, the thick and nucleated corneum layer in the non-healed group is characteristic of a recalcitrant ulcer. There was a gross distortion in the structural architecture of the epithelial layer. Furthermore, a general decrease in cellular activity in the granulosum and spinosum layer from day 0 to week 8 as evident by decrease in the size of the layer as the wound progress towards normal healing in the group that achieved complete wound healing. On the contrary, the cellular activities in the non-healed group remained perpetually high.

There are striking differences in the immunostaining patterns for keratins K14, K1/K10, Ki67 when comparing chronic ulcer margin to the unwounded normal skin. A suprabasal expression of K14 in all the keratinocyte layers was seen from day 0 through week 8 for both the healed and non-healed group; in contrast to the unwounded skin it remained at the basal layers. K1/K10 showed a purely limited pattern of expression to the granulosum layer on the immediate post debridement sample but as the wound progresses towards week 8, the expression becomes suprabasal. K16 showed an up-regulation of its expression in a suprabasal layer from the day 0 until week 8, this expression is similar in both categories of patients. This is a sign of hyperproliferation in chronic ulcers. Ki67 showed a decreasing trend in its expression as the wound progresses towards normal re-epithelisation, on the contrary the expression of Ki67 remained static in the non-healing group. The expression of beta-1 integrin and CD 44 was also observed to be up-regulated in both categories of

patient ulcers which could both represent a sign of ongoing inflammatory changes in the wound. The proinflammatory role of CD44 remains debatable in chronic wounds.

The restoration of Collagen VII has been shown as a hall mark of true re-epithelisation. From our study we were able to clearly demonstrate that collagen VII was completely restored as major basement membrane component in the group that achieved complete re-epithelisation whereas it remained distorted in the non-healed group. There no changes in the alignment of collagen I and III either from the 2 categories or during the wound healing process. The main limitation of this study was the fact that we were unable to recruit patients into the control arm of the study; hence the changes observed were not directly attributable to PRP treatment. To better understand the effect of PRP on the molecular mechanism involved in keratinocyte migration, basement membrane restoration and changes occurring in extraceullar matrix during healing, a control group (treated with PPP) and or a group receiving standard care for diabetic ulcer will be required for comparison. Immunohistochemistry technique to analyze the expression of various chemotactic and growth factors and their receptors in the margin of diabetic foot ulcers and in normal non diabetic foot skin might in give a better insight to molecular changes at the wound edges.

In **Chapter 6,** we evaluated the treatment outcomes of diabetic patients with chronic foot ulcers after been treated with PRP. The initial protocol and ethic approval allowed recruited of patients into 2 arms of treatment; the PRP group (active) and the PPP group (placebo), however due to patient's preference we were unable to recruit into the placebo arm. The details of the inclusion and exclusion criteria, the treatment protocol for PRP and the routine post treatment standard care of the patients have been discussed in **chapter 2 and 6.** The clinico-demographic data of the 7 patients recruited is detailed in **chapter 6**. Three of the 7 patients achieved complete re-epithelisation; two patients were withdrawn mid week into the study. Both required below knee amputations because of worsening foot sepsis. The other two patients with recalcitrant plantar ulcer we followed up to 16 weeks. It is very difficult to categorically attribute both the success and failure of this wound to PRP treatment, this because of the so many irresolvable confounding factor in the process and the patients themselves. This further illustrates the difficulty that other researchers are encountering in the planning of a randomised control trial which is the way forward in standardising the use of PRP. We are of the opinion that PRP is able to deliver constellation of GF to wounds which are believe to have potentials to accelerate the process of wound healing.

6.12 Future work

The biological and cellular mechanisms of impaired healing in chronic diabetic foot ulcers are not well known, due to a lack of appropriate experimental animal models. Previously reported studies have focused on analysis of wound fluids, and also on biopsied tissues, but sufficient specimen samples needed for research studies are difficult to obtain for practical and ethical reasons. Several concepts have recently emerged from the molecular and cellular analysis of the chronic wound environment. Chronic diabetic foot ulcers exhibit a long-standing inflammatory cellular infiltrate in tissues, and elevated levels of proinflammatory cytokines. Measurement of wound pro-inflammatory cytokines and anti-angiogenic agents from wounds treated with PRP and placebo may begin to provide new insight to the cellular mechanism of chronic wounds. Fibroblasts isolated from chronic diabetic foot ulcers are more senescent compared with fibroblasts isolated from healthy skin, and have decreased proliferative ability and exhibit a decreased mitogenic response to recombinant growth factor treatment. Proteases present in chronic wounds are believed to destroy growth factors delivered to wound. This suggests that delivery of PRP in combination with protease inhibitors, by means of a polymeric device to control release of the growth factors and prolong the biological effects of the various components, may provide a solution on the early degradation of PRP.

In a study conducted by Birc et al., 2009, they used gelatin hydrogel as a sustained release carrier for growth factors in PRP to investigate its angiogenic properties in wild type C57BL6 mice after inducing hind limb ischemia by excising right femoral artery. The conclusion from the study was that sustained release of PRP containing potent angiogenic growth factors helps restore blood perfusion presumably by stimulating angiogenesis, arteriogenesis, as well as vasculogenesis in mouse hind limb ischemia. A similar clinical trial may be tried in humans to see if the same results can be obtained.

Furthermore, understanding of how fibroblasts respond to keratinocyte–derived stimuli is important in understanding the role of TGF-β activity in the transformation of fibroblast to myofibroblast. This is crucial for the formation and regulation of quality granulation tissue. A coculture experiment (fibroblasts and keratinocytes), comparing messenger RNA (mRNA) expression pattern obtained from non-diabetic human wound edges should be compared with that from human diabetic wound edges. A similar study conducted by Werner et. al 2007, which compares the RNA expression of co cultured and control fibroblast, revealed a differential expression of growth factors, extra cellular matrix, protease, and intracellular structural protein transcripts. The RNA expression showed that several gene characteristics of TGF- β signalling were up regulated; for example smooth muscle acting alpha and collagen type 1. To further understand the role of PRP in wound healing and the

kinetics involved in this process, a study to understand the role of autologous derived platelet-rich plasma at the micro-circulatory level may answer specific questions on how PRP influence micro-vascularity at the capillary bed. A similar study was conducted using a mice model by Eberhard et al., 2003. Microvascular perfusion can be measured by using laser Doppler perfusion imager, which will provide information on red blood cell influx. Intravital fluorescence microscopy will provide information on microcirculatory changes such as the inner diameter of arterioles and venules, as well as the number of rolling and adherent leucocytes in post capillary venules.

Most of the study published on PRP has mainly been to investigate its role in tissue reparation. More research will be needed in the area of tissue regeneration if the potentials of PRP were to be fully explored. Understanding the role of PRP at the embryological stages (pre-differential and progenitor cellular level), will open a new horizon for its use. In a study conducted by Wen-Cheng Lo et. al., 2009, the group investigated the potential use of fibroblasts in combination with platelet-rich plasma (PRP) to achieve bone regeneration in osteoporotic mice. In the study, a senescence-accelerated mouse (SAM) model was developed to depict a pathophysiologic scenario of senile osteoporosis. NIH3T3 embryonic fibroblasts were used for osteoinductive cell transplantation and served as tracers for this study. PRP medium was used to stimulate proliferation and differentiation of NIH3T3-G cells into osteoblast-like cells before transplantation. The result demonstrated that transplantation of PRP treated NIH3T3-G cells into ovariectomized SAMP8 mice induced bone regeneration and significantly reversed osteoporosis not only at the implantation site, but also in other regions of the skeleton.

The future role of platelet- rich plasma is hanging in the balance and to some extent it remains at the potential and anecdotal level at this present time. Many more large controlled clinical studies will be required to establish clear cut treatment benefit significance in specific groups of patients and conditions. The dose-dependent relationship and time of its application when treating such conditions will need to be made more specific. In addition, there is need to conduct a cost-effectiveness analysis study on the potential economic benefit of an autologous, platelet-rich plasma (PRP) gel to alternative therapies in treating non-healing diabetic foot ulcers. This study will centre on economic models using peer-reviewed data to simulate clinical and cost outcomes and quality-adjusted life-years (QALYs) associated with PRP gel and other treatment modalities. A comparative cost analysis study looking at other modalities of treating chronic diabetic foot ulcers particularly in patients with neuropathic ulcer versus platelet rich plasma therapy, will provide a very useful data on the use of PRP .

Appendix 1 – Materials

Transport Medium (TM)

Dulbecco's modified eagles medium (DMEM) supplemented with 5% FCS (v/v) +antibiotic/ antimycotic solution of 600 units/mL Penicillin-G, 600mg/nl Streptomycin sulphate, 250 mg/mL Gentamycin (Sigman-Aldrich Company Ltd, Dorset, UK.) and 2.5 mg/mL Fungizone (Invitrogen, Paisley, UK.)

E4 Medium

Dulbecco's modified eagle's medium (DMEM) 4.5g/L glucose, supplemented with 10% FCS (v/v) and 1% glutamine

Add the following to 400mL to Dulbecco's modified eagles medium (DMEM)

· 5mL Penicillin (10,000 units/mL)/Streptomycin (10mg/mL) (1%)

· 5mL L-Glutamine (1%)

· 50mL FCS (10%)

Penicillin/Streptomycin contains 10,000 units/mL penicillin and 10mg/mL streptomycin in 0.9% sodium chloride.

RM+ Medium

3 parts DMEM + 1 part HAMS F12 medium + 10% FCS + 1% Penicillin/Streptomycin + 1% L-Glutamine + 1% RM+ supplement

Add the following to 400mL of 3 parts Dulbecco's modified Eagle medium (DMEM) and 1 part Ham's F12 medium:

· 40mL FCS (10%)

· 5mL Penicillin (10,000 units/mL)/Streptomycin (10mg/mL) (1%)

· 5mL L-Glutamine (1%)

· 5mL RM+ supplement (1%)

RM+ supplement contains the following mitogens: 0.4mg/mL hydrocortisone, 10-10 M Cholera toxin, 5mg/mL transferrin, 2-11 M lyothyronine, 1.8-4 M adenine, 5.5mg/mL insulin.

Antibiotic/Antimycotic solution600 units/mL Penicillin-G, 250 mg/mL Streptomycin sulphate, 250 mg/mL Gentamycin(Sigma-Aldrich Company Ltd, Dorset, UK) and 2.5 mg/mL Fungizone (Invitrogen, Paisley, UK)

Freezing medium

Add 5mL DMSO (Dimethyl Sulfoxide) to 45mL FCS

Reagent	Manufacturer	Catalogue No.	Lot No.
Dulbecco's PBS(1X) without Ca& Mg	PAA, Cell Culture company, UK	H15-002	H00208-1984
Trypan Blue (0.4%)	Sigma-Alrich Company Ltd, Dorset	T8154	107K2360
Dimethyl Sulfoxide	BDH Laboratory Supplies, Poole UK	23500260	07L190523
Trypsin (0.25x)	PAA, Cell Culture company, UK	L11-004	L00408-1885
Versene(1x)	Invitrogen ,paisley, UK	15040-066	473989
DMEM/F12 Modified	PAA, Cell Culture company, UK	T15-276	T27608-1982
DMEM High Glucose(4.5g/L without L-glutamate with sodium pyruvate	PAA, Cell Culture company, UK	E15-011	E001108-1017
Paraformaldehyde (10%)	VWR International, UK	361367L	N/A

Table showing the material used for tissue culture and the manufacturer

Antigen Unmasking Solutions for Immuno-histochemistry

Vector antigen unmasking solution pH 6

Ready to use solution; prepared by mixing 15 ml of the stock antigen unmasking solution with 1600mL of distilled water

Tris-EDTA- Citrate Unmasking Solution pH8.1

This is at X10 strength stock solution in bottles; 900mL of distilled water is added to the stock before use. The stock is prepared from 15g EDTA, 7.5 g Tris Base, 9.6g Sodium Citrate and 3000ml distilled water. Sodium hydroxide is added to it to achieve the desired pH of 8.1

Tris-EDTA Unmasking Solution pH9.0

This is at X10 strength stock solution in bottles; 900mL of distilled water is added to the stock before use. The stock is prepared from EDTA 11.1 g, Tris Base 36.3g, and distilled water 3000ml. The pH is checked with a pH-meter.

Appendix 2 – Protocols

Cell culture

3T3 (NIH) Fibroblasts

Passaging

3T3 cells should be passage at 75% confluence, higher confluence may result in Mitomycin-C resistant cells.

1. Aspirate E4 medium and add 5mL of PBS to wash flask
2. Aspirate off PBS
3. Add 5mL versene/EDTA to flask and leave for one minute
4. Aspirate off
5. Add 5mL trypsin-EDTA 0.05% and leave for a 10- 15 minute, watch for cell detachment
6. Tap flask gently and confirm cell detachment by looking under a microscope. Once all cells are detached; add 5mL of E4 medium to neutralize trypsin-EDTA
7. Using a pipette transfer contents to falcon tube
8. Check flask once more under microscope for any remaining cells, if present, wash flask with E4 and retrieve remaining cells and add to existing tube.
9. Depending on confluence of flask and requirement of cells split e.g. 1:5 (1 part returned to flask, 4 parts used for experiments)
10. Add 15mL E4 medium to flask to a T75 Flask
11. Label flask with date, name, and cell type and passage number
12. Put flasks back into incubator (10% CO2), check confluence daily

Mitomycin -C Treatment

3T3 cells are treated with Mitomycin C to produce 3T3 "Feeder" cells as described by Rheinwald and Green, these cells become incapable to proliferation after treatment and therefore can be used to grow keratinocytes.

1. Cells obtained from passaging should be centrifuged at 1200 revolutions per minute (RPM) for 5minute and resuspended in 10mL E4 medium in a falcon tube
2. Add 20microgram /ml of Mitomycin C to resuspended cells in media. (Mitomycin-C is light sensitive, so vials should be wrapped in aluminium foil)
3. Attach lid to tube and mix gently
4. Place in incubator for 2 hours (10% CO2) with gentle mixing every 15 minutes (to ensure all 3T3s are exposed to treatment)
5. Centrifuge for 5 minutes at 1200 RPM

6. Aspirate off liquid, being careful to leave remaining cell pellet
7. Resuspend pellet in 10mL PBS thrice allowing pellet to settle for 5minute between each of the washes and mix well with pipette
8. Repeat steps 6-7 two more times (i.e. 3x PBS wash)
9. Resuspend cells in 10mL E4

Irradiation treatment of 3T3

1. A cell density of 1 million per mL prepared from subculturing,
2. A total of 40million / ml were resuspended in equal volume of media
3. The falcon tube was left on ice and transfer to the gamma radiation room
4. The cells were irradiated at 6,000gyrad for 40minute.
5. Following irradiation, the cells were used for the experiment as described earlier (2 million feeders/ T-75 flask) and the remaining were cryopreserved.

Keratinocytes

Extraction (Done under sterile condition in clean Tissue culture hood)

1. Using a dis*posable* scalpel, cut out an appropriate size of redundant abdominal skin or face fit. The forceps and scissors used are sterile in 70% ethanol during extraction
2. Using forceps transfer to a large Petri dish a portion of the sample and add enough versene (EDTA) to cover
3. Using scalpel, forceps and scissors aim to remove as much fat from the underlying dermis as Possible. The more fat removed, the greater the keartinocyte yield. Alternatively a 1-2mm thick dermatome can be used to isolate epidermal sheets.
4. Repeat step 2 as necessary to keep the skin clean
5. Once the bulk of fat is removed, focus on using the scalpel to scrape off any remaining subcutaneous fat
6. Cut the skin into small squares (~5mm x 5mm) and place into a 50mL falcon tube
7. Add 20mL of 0.25% trypsin and swirl tube
8. Place tube into an incubator at 37°C 10% $C0_2$ for 1-2 hours until the cells have detached (Regular checking of the medium to see when media go cloudy and the skin pieces will (begin to float to The surface). Check every 15 minutes.
9. Place skin pieces back into a new Petri dish and fill with culture medium (E4) to inhibit trypsin action.
10. Using two needle tips carefully scrape off the epidermis from the dermis. Use one needle to fix the piece to the dish, whilst scraping with the remaining needle. Keep the dish organised, i.e. separate scraped from non-scraped skin pieces.
11. Transfer dish contents back into a falcon tube

12. Pour contents through sterile gauze into a new falcon tube leaving behind scraped skin pieces i.e. dermis. The remaining dermis can be used for fibroblast extraction.
13. Centrifuge falcon tube at 1200 RPM, for 5 minutes
14. Aspirate off supernatant, being careful to leave pellet behind. Resuspend in an appropriate volume of medium e.g. 20mL RM+
15. Cell count with trypan blue
16. Seed cells into a T75 flask containing 2x106 3T3 mitomycin treated / or irradiated feeders
17. Incubate at 37°C/10% CO2, media change after 24hours
18. After 48 hours, add more feeders if necessary and change media. Use solutions (wash solutions, trypsin solutions etc) that is low in calcium, or calcium and magnesium free. Calcium exposure encourages differentiation and decreases growth.

Passaging

Keratinocytes should be passage at 75% confluence; higher confluence may result in decreased growth or failure to adhere to the flask.

1. Aspirate RM+ medium and add 5mL of PBS to wash flask
2. Aspirate off PBS
3. Add 5mL versene/EDTA to flask
4. Check every minute under the microscope to confirm 3T3 feeder detachment, tap flask as necessary to encourage detachment
5. Once all feeders are detached, aspirate off versene/EDTA
6. Add 5mL trypsin EDTA 0.05% and leave for a 10-15 minute
7. Tap flask gently and confirm cell detachment by looking under a microscope, cells will become rounded. Once all cells are detached, add 5mL of RM+ medium to neutralize trypsin
8. Using a pipette transfer contents to falcon tube
9. Check flask once more under microscope for any remaining cells, if present, wash flask with RM+ and retrieve remaining cells and add to existing tube.
10. Depending on confluence of flask and requirement of cells split e.g. 1:5 (1 part returned to flask, 4 parts used for experiments)
11. Add 30mL RM+ medium to split flask
12. Label flask with date, name, cell type and passage number
13. Put flasks back into incubator (10% CO2), check confluence daily

General

Media changes

Media changes should be performed every 3 days to maintain optimal growth conditions.

1. Attach pipette to aspirator, aspirate out old medium
2. Add 5mLL PBS to wash flask, aspirate
3. Add appropriate type (E4/3T3, RM+/KC) and volume (15mL / T75 flask, 30mL /T175 flask)

Cell counting

The most effective method for cell counting is to use a haemocytometer. This instrument is composed of a thickened glass slide containing a small grid chamber in the centre. Cells are counted with trypan blue dye, which allows cell viability to be assessed; dead cells take up the dye appearing blue on the haemocytometer.

1. Add 20µL cell suspension to 20µL Trypan blue dye into an eppendorf™ Microcentrifuge tube.
2. Leave for one minute and prepare haemocytometer by cleaning and drying off ethanol. Place a coverslip onto the haemocytometer and look for Newton rings to confirm correct setup.
3. Pipette 10 µL of cell suspension/trypan blue solution onto haemocytometer channel
4. Place under microscope and count all 4 large square quadrants.
5. Obtain average.
6. Double average to obtain number of cells (n): n x 104/mL.
7. Depending on volume of cell suspension, factor in the total e.g. 20mL cell suspension is n x 20.

Cryopreservation and cell recovery

Cell preservation and recovery follow the same procedure but different cell have varying concentration of DMSO/ Fetal Calf Serum/Medium

1· Determine cell count and then resuspend $2x10^6$ cells/1mL freezing medium per cryovial
2· Label the cryovials with name, date, cell type and passage number
3· Wrap cryovials in paper towels and use tape to make a parcel
4· Store in freezer at -80°C
5· For long term storage (>28 days) transfer to liquid nitrogen storage
6· To retrieve cells warm cryovials in waterbath, once thawed, pipette cell suspensioninto a falcon tube and add equal volume of culture medium (i.e. E4/3T3 or RM+)
7· Centrifuge 1200RPM/5 minutes
8· Resuspend in culture medium

Appendix 3

Macro progamme for analysing angiogenesis assay image

```
# mesh.mcr SEG April May 2010
# read angiogenesis images and do topological anbalysis according
# Guidolin, D et al. Microvascular Res, 67, (2004) 117 - 124

DBdelete "database"
DBnew "database",5
DBsetcolumn "database",1,"Specimen","String","<none>"
DBsetcolumn "database",2,"No_of_nodes","Int","<none>"
DBsetcolumn "database",3,"No_of_meshes","Float","<none>"
DBsetcolumn "database",4,"Skel_length","Float","micron"
DBsetcolumn "database",5,"Cell_area","Float","micron"

imgsetpath "c:\ks400\conf\images\femi\angiogenesis_assay"
# imgload "c:\ks400\conf\images\femi\angiogenesis_assay\ag100007.jpg",1
MSload "femi_node"                              #min area only 1 pixel instead of default 4

thresh[6] := 1              #storage for  2 sets of colour threshold values

##########################Calibration##############################
#All images x10 objective. Previously calibrated by Femi using 250 micron blob on my calibration
slide
MSsetprop "SCALEX",0.9328         # micron/pixel
MSsetprop "SCALEY",0.9328
MSsetprop "UNIT","micron"
###################################################################

# Make colour threshold database, get previously stored values and store in thresh array
DBnew "thresholds",1
DBsetcolumn "thresholds",1,"thresh_val","Int","<none>"
DBcopy "mesh_thresholds","thresholds",0,1
DBfirstline "thresholds"
for i=1; i <= 6; i = i+1
   DBgetvalue "thresholds","1",temp
   thresh[i] = temp      #for some reason, can't write va directly into an array
   DBnextline "thresholds"
   # write i, thresh[i]
endfor

another = "yes"
while another == "yes"
   Gclear 0

   #get image and extract name for later saving of modified image
   ! imgload in_file_name,1
   imgstatus 1,_sizex,_sizey   #needed for editbina, to edit images of any size
   entire_path = imgload_1
   write entire_path
   in_file_name = imgload_1
```

```
   # Knock off dot plus suffix for outfile name only
   len_in_file_name_s= flength(in_file_name) - 4
   in_file_name_s := ""
   pos = 0
   for i2 = 1; i2 <= len_in_file_name_s;i2 = i2 + 1
      in_file_name_s = in_file_name_s + "x"
      pos = pos + 1
      z = (in_file_name[i2])
      in_file_name_s[i2] = z
   endfor

# Initial processing according to Guidolin, D et al. Microvascular Res, 67, (2004) 117 - 124
   imgdisplay 1

   showwindow "Messages", 1
   write "Threshold image. Don't forget to click on left thumbnail"
   dislevrgb 1,2,1,0,thresh[1],thresh[2],thresh[3],thresh[4],thresh[5],thresh[6],10,"RGB"
   !dislevrgb 1,2,1,0,thresh[1],thresh[2],thresh[3],thresh[4],thresh[5],thresh[6],10,"RGB"
   write "@"
   binscrap 2,3,0,200,0

   # thresh values for later writing in case they've been changed
   thresh[1] = dislevrgb_5
   thresh[2] = dislevrgb_6
   thresh[3] = dislevrgb_7
   thresh[4] = dislevrgb_8
   thresh[5] = dislevrgb_9
   thresh[6] = dislevrgb_10

   passes = 8
   satisfied = _FALSE
   while satisfied ==_FALSE
      read passes,  "How many passes?"
      ! binclose 3,4,7,passes

      # Generate skeleton in image 9 and count nodes by Paul Sheperd's method (email 26-03-10)
      binthinning 4,5,3,158
      binscrap 5,6,0,30,0
      lowpass 6,7,3,1
      binand 6,7,8            # Not in Paul's method but necessary because low pass filter generates lines
> 1 pixel wide
      dislev 8,9,112,113,1
      imgdisplay 6
      Gclear 0

      node_count = 0
      RGnew 9,9
         RGfirstregion
         while _STATUS == 1
            MSmeasregion 1
            node_count = node_count + 1
            MSdrawregion
```

```
        RGnextregion
      endwhile
   RGdelete

   # Overlay skeleton onto original image then add dilated nodes, result in image 14
   Gextract 6,128,253,11
   imgclear 10,0
   Gmerge 10,255
   Gclear 0
   binthinning 10,11,3,0
   bindilate 11,12,7,1
   imgdisplay 12
   Gextract 12,128,255,10
   add 1,6,14,2                          # display net on original image
   Gmerge 14,255
   Gclear 0

   # Generate meshes in image 17 and count them
   binfill 6,15
   binxor 6,15,16
   binerode 16,17,7,1   # count won't work if mesh boundaries are only 1 pixel wide

   mesh_count = 0
   RGnew 17,17
      RGfirstregion
      while _STATUS == 1
         MSmeasregion 1
         mesh_count = mesh_count + 1
         MSdrawregion
         RGnextregion
      endwhile
   RGdelete

   Gclear 0
   imgdisplay 14

   MByesno "Satisfied?"
   satisfied = _STATUS
endwhile

# Measure length of thinned skeleton (img. 18 thinned from img. 6)
binthinning 6,18,1,1

RGnew 18,18
   RGfirstregion
   while _STATUS == 1
      MSmeasregion 1
      MSgetvalue "PERIM",length
      MSdrawregion
      RGnextregion
   endwhile
RGdelete
```

```
   # Measure area of filled cells (img. 4)
   RGnew 4,4
      RGfirstregion
      while _STATUS == 1
         MSmeasregion 1
         MSgetvalue "AREA",area
         MSdrawregion
         RGnextregion
      endwhile
   RGdelete

   #Write values into database
   DBaddline "database"
   DBsetvalue "database","Specimen", in_file_name_s
   DBsetvalue "database","No_of_nodes", node_count
   DBsetvalue "database","No_of_meshes", mesh_count
   DBsetvalue "database","Skel_length",length/2.0
   DBsetvalue "database","Cell_area", area
   # Save orig imag showing skeleton outline
      MByesno "save  image"
      if (_STATUS == _TRUE)
         outname = in_file_name_s + "m.jpg"
         ! imgsave 14,outname
      endif

   DBflush "database"
   datalist "database",0,1
   MByesno "Another?"
   if (_STATUS == _TRUE)
      another = "yes"
   else
      Valid = 1
      another  = "no"
   endif
   write "@"
endwhile   another

# This stores the colour threshold database on disk
DBfirstline "thresholds"
for i=1,i <= 6,i = i+1
   DBsetvalue "thresholds","thresh_val",thresh[i]
   DBnextline "thresholds"
endfor
DBcopy "thresholds","mesh_thresholds",0,1

datalist "DATABASE",0,0
pause "Don't forget to paste"
write "@"
showwindow "Messages",0
showwindow "Display",0
```

APPENDIX- 4 – Protocol for Imunohistochemistry

Histology and Immuno-histochemistry

Note: all reagents and consumables are purchased from VWR labs Ltd UK unless otherwise stated.

Tissue processing for paraffin sections

1. Tissue was fixed for a minimum of 24 hours in 10% formal saline or longer is need be
2. Tissues were processed on a Shandon Excelsior ES™ Tissue Processor (See below)
3. Specimens were embedded in paraffin wax
4. Blocks were then cut on a rotary microtome (Leitz), sections cut at 3µm and sections picked up on APES coated slides
5. Slides were dried either at 45°C overnight for immunohistochemistry staining. Slides were stored at 37°C if not used immediately.
6. Shandon Excelsior ES™ Tissue Processor Programme Step Reagent Temperature

Shandon Excelsoir ES™ Tissue Processor Programme

Step	Reagent	Temperature (^{0}C) Hold/Use	Time (Hr)	Vacuum
1	10% Formalsaline	Abmient/45	1:30	NO
2	Industrial methylated spirit (IMS)	30	1	Yes
3		30	1	Yes
4		30	1	Yes
5		30	1	Yes
6		30	1	Yes
7		30	1	Yes
8		30	1	Yes

9		30	1	Yes
10		30	1	Yes
11		62	1:20	Yes
12		62	1:20	Yes
13		62	1:20	Yes

Tissue processing for frozen sections

1. Fill dewar with liquid nitrogen
2. Fill small container with approximately 2 inches of isopentane
3. Suspend isopentane container from clamp on ring stand and lower the container into the liquid nitrogen. The base of the container should just touch the top of the Liquid nitrogen and cause it to bubble as it cools the container. If you insert the container too far into the liquid nitrogen the isopentane will freeze. If this happens, just remove the container and allow the isopentane to thaw and then place it back in the liquid nitrogen.
4. Label a cryomold with the identifying data of the tissue. Make sure to use a marker resistant to organic solvents
5. Place a small amount of Cryo-M-Bed in the bottom of the cryomold
6. Remove the tissue to be frozen
7. Place the tissue oriented with the side you are interested in at the bottom of the cryomold
8. Fill the cryomold with Cryo-M-Bed compound. Use caution not to move the tissue when adding the Cryo-M-Bed and be sure to remove any bubbles that form near the tissue with a forceps as these will cause problems with sectioning (cracking and fragmentation)
9. Place the cryomold into the isopentane. Use caution not to tip the cryomold and dislogding the tissue. A little preparation here and ordering a pair of long handled forceps that can open wide enough to grasp the entire cryomold help here quite a bit
10. The Cryo-M-Bed will freeze into a solid white color. The tissue should be covere and not visible as you look down on the mold. Allow approximately 1 minute for freezing of the entire block

11. Transfer the tissue to and ice bucket with dry ice and continue with the rest of the tissues

12. Place tissues in plastic bag or other airtight container and store at -80ºC

R.T.U Vectastain Universal Elite Procedure

Immuno-cytochemistry Manual Version 1 - Courtesy of Queen Mary University, ICSM Core Pathology Group (for staining paraffin)

1. The following controls should be used.

Positive control section for each primary antibody used.

Negative control section [omitting primary antibody]

2. Place the sections into a black plastic staining rack

3. De-wax the sections in xylene for 2 changes of 2 minutes each

4. Remove the xylene in Alcohol for 2 changes of 2 minutes each

5. Place the sections into the sink and wash in tap water for 5 minute If the particular antibody you are going to use does not require antigen retrieval then continue with the next step. Otherwise perform antigen retrieval technique first.

6. Prepare the endogenous peroxidase blocking solution by adding 6mL of hydrogen peroxide to 194mL of distilled water in a black staining trough.

7. Place the sections into the endogenous peroxidase blocking solution for 15 minutes.

8. Remove the rack and place into the sink and wash in tap water for 15 minutes

9. Wash out the black plastic staining trough previously used for the endogenous block with distilled water and fill it with wash buffer.

10. Place the rack of slides into the wash buffer and leave to soak for 5 minutes.

11. Prepare an immuno-staining tray by adding a few drops of wash buffer to it to create a moist chamber.

12. Taking each slide in turn, wipe around the section to remove excess wash buffer and using a PAP pen draw a line across the slide above and below the section.

13. Place the section onto the rack in the immuno-staining tray and cover with wash buffer. Do allow the sections to dry out.

14. When all of the sections are on the rack tip off the wash buffer.
15. Apply sufficient drops of normal blocking serum (Normal horse serum – yellow bottle from the R.T.U. Kit) to cover each section
16. Place the lid on the immuno-staining tray and leave for 20 minutes
17. Prepare the primary antibody at the appropriate dilution using antibody diluent.
18. Tip off the normal blocking serum.
19. Apply the primary antibody to each test section and the positive control section.
20. Apply antibody diluent alone to the negative control section.
21. Replace the immuno-staining tray lid and leave for 40 minutes.
22. Rinse off the primary antibody with wash buffer.
23. Cover each section with wash buffer, replace the immuno-staining tray lid and leave for 2minutes. Rinse with wash buffer and cover each section again and leave for a further minutes.
24. Tip off the wash buffer.
25. Apply drops of the Universal biotinylated secondary antibody (Biotinylated AntiRabbit/mouse – blue bottle from the R.T.U. kit) to cover each section.
26. Replace the immuno-staining tray lid and leave for 30 minutes.
27. Rinse off the secondary antibody with wash buffer.
28. Cover each section with wash buffer, replace the immuno-staining tray lid and leave for 2minutes. Rinse with wash buffer and cover each section again and leave for a further 2 minutes.
29. Tip off the wash buffer.
30. Apply sufficient drops of the Vectastain Elite ABC Reagent (Grey bottle from the R.T.U. kit) to cover each section.
31. Replace the immuno-staining tray lid and leave for 30 minutes.
32. Rinse off the ABC reagent with wash buffer.
33. Cover each section with wash buffer, replace the immuno-staining tray lid and leave for 2minutes. Rinse with wash buffer and cover each section again and leave for a further 2 minutes.
34. Into a yellow topped tube place 1mL of substrate buffer from the Biogenex Two Component

DAB kit.

35. To this, add 1 drop of the DAB Chromogen from the kit and mix well.
36. Tip off the wash buffer from the slides and apply the DAB solution to each of the slides, leave for 5 minutes.
37. Wash the slides in tap water for 5 minutes.
38. Load the slides into a machine staining rack ensuring that all of the sections are facing in the direction of the arrow on the rack.
39. The remainder of the technique is carried out using machines and involve staining the sections with the dye Haematoxylin. This stains the nuclei a light- blue colour, giving contrast against the brown immuno-cytochemical staining. The sections are then dehydrated in alcohol to remove water and then washed in Xylene before having a glass cover slip placed over the section to protect it and produce a permanent preparation.

Staining procedure for frozen sections

Immediately before staining, fix sections with acetone or appropriate fixative for the antigen. Antigen retrieving step is not needed for frozen sections. For endogenous peroxidase blocking 3% of hydrogen peroxide with distilled water for 15 minutes or 0.3% peroxide in methanol for 30 minute is used. Continue with step 8 -39 as described above.

Embedding of Specimens Procedure

The following procedure is observed for processing paraffin embedded wax tissue after obtaining the tissue biopsy sample

1. The cassette basket is lifted from processing machine and the excess wax allowed to drain. It's then taken to the Embedding centre in a plastic tray.
2. The cassettes are carefully tipped into the storage tray in the embedding centre that is kept at 60^0C to prevent the specimen solidifying.
3. The processing basket and the lid are then placed into the plastic tray ready to receive the used cassettes lids.

4. Cassettes are taken one at a time from the storage tray and the metal lid carefully removed ensuring that the tissues does not fall out. There must never be more than one cassette at a time with its lid removed.

5. The lids are checked to make sure that the tissue is not attached to it's before discarding it into basket or in the case of plastics lids into the waste bin.

6. Very small specimens may be enclosed either in speciwrap or between two pieces of foam biopsy pad. If enclosed in paper it is essential that the paper is kept on the hot part of the embedding centre to prevent the wax with which it is impregnated from solidifying. If enclosed in biopsy pads, the top pad should carefully be removed and checked to ensure that the specimen is not attached to it. The bottom pad can either be left in the cassette or removed and placed on the hot area with specimen uppermost.

9. A mould of appropriate size is selected and partially filled with wax from the built in dispenser.

10. The tissue is removed from the cassette /speciwrap/ biopsy pad using warm forceps, placed in the mould and oriented.

10. It is important that the tips of forceps are checked each time they are used, to avoid any carry over which may be attached to the forceps from the previous specimen.

11. The mould is then carefully moved to the 'cold spot' and a film wax allowed to form on the bottom sufficient to hold the specimen in place.

12. The plastic cassette is placed on the mould and completely filled with wax.

13. The mould is then carefully moved to the cold plate and left until solid.

14. Once set, the block can be removed from the mould and placed into a plastic box ready for cutting.

15. The basket lid are placed in the tray in the wax oven to allow the wax to drain. Any excess wax is cleaned out using a tissue. Accumulated wax in the tray should be cleaned out when the cassettes are removed for cleaning.

16. The cassettes are finally cleaned by placing in the processing machine on an extended flush cycle, after which they dried in the oven.

17. The cassette is returned to the embedding centre tray

18. The cassette centre was cleaned down and the wax reservoir topped up.

APPENDIX – 5

Invitation to participate in a Research Project

Title: Study to elucidate the role of autologous platelet rich Plasma gel (APG) in treating chronic non-ischemic Diabetic foot ulce

Investigator: Mr Costas Kyriakides and Prof. Harry Navsaria.

We invite you to take part in a research project, which we think may be important. The information, which follows, tells you about it. It is important that you understand what is in this leaflet. It says what will happen if you take part and what the risks might be. Try to make sure you know what will happen to you if you decide to take part. Whether or not you do take part is entirely your choice. Please ask any questions you want to about the research and we will try our best to answer them.

Part 1

Invitation

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relative and your GP if you wish. Ask if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

1. Part 1 tells you the purpose of this study and what will happen to you if you take part.
2. Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. The study is sponsored by Barts and The London Hospitals NHS Trust.

Introduction to the study

Diabetic foot ulcers are a condition that diabetics need to handle with every care. Treatment of these ulcers, which range from superficial to deep, can take months to years to heal in some patients, while others can quickly resolve.

With the help of medical research and biotechnology techniques, we are now able to successfully process various parts of human blood to make new potential treatments for these ulcers

Diabetic foot ulcers result mainly from two causes: poor circulation and peripheral neuropathy. Poor circulation in the feet will result in a diminished ability to heal. Peripheral neuropathy results in localised nerve damage, which causes a loss of sensation in the feet and further contributes to inadequate circulation and increases the likelihood of injury.

Because of the difficulty the body has in delivering blood supply to the diabetic foot, finding a way for us to copy the normal healthy healing could be an important factor in treating these ulcers.

Most diabetic ulcers can be traditionally managed by bandages, dressing and controlling infection with antibiotics; but a large proportion will not respond to these conventional treatments. With our increasing understanding of normal wound healing systems in the body, it has been shown that growth factors are necessary for effective wound healing.

Platelets and Growth Factors

Several kinds of cells contribute to the wound healing process. Among them are blood cells called platelets. Platelets are cells essential to clotting, and are the first cells to gather at the wound site. They start the healing process by releasing various growth factors.

Growth factors are proteins that act as signals between cells to control communication. They summon useful cells and proteins to the wound site; promote cell movement and growth of new skin.

This new method uses the patient's own blood to produce the treatment gel. Blood is collected from a vein in the arm, just like a blood test. The blood is then processed into its different parts using a portable machine. The amount of blood collected depends on the size of the ulcer. The gel is made when a clotting chemical and the processed blood are mixed; this component contains platelets and other growth factors. We gently spray the gel into the wound until the gel-like substance fills the area. This is then dressed as per usual.

This treatment is currently used by the Barts & The London NHS Trust for some patients with non-healing ulcers. This study wishes to understand more about this new treatment and also to prove how well it works.

Benefits of the treatment

- Your own blood will be used for your treatment.
- The gel can be applied as many times as required on the wound.
- Your treatment may help heal your ulcer quicker.
- The treatments will be carried out in Out Patient clinics.

The purposes of the study are:

To study the natural role of platelet rich plasma gel in treating non-healing diabetic foot ulcers. We also want to determine the rate of wound healing and how many wounds will heal during the study period.

Furthermore, the study will enable us to understand the cell changes in the wound that take place in the diabetic ulcer and healing process.

Why have I been ask to participate?

This is because you have a non-healing diabetic ulcer and we believe that you may benefit from this relatively new treatment option. We hope to recruit twenty patients for this pilot study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. If you do not wish to take part, your Doctor will discuss with you other options for trying to treat your ulcer.

You are still free to withdraw at anytime and without giving a reason.

Before you can begin the study

You may read the full study protocol as well as this information, which gives you many details about the study. The recruiting investigator will tell you more about the study. You will be given adequate information about the study and your involvement. You are encouraged to ask questions to clarify anything you are not clear about.

Screening Visit

You will be examined by the study doctor, who will take a detailed medical history and conduct a physical examination. The Doctors will also review your medical records. We will ask you for a sample of your urine (40mL) for routine safety analysis.

Study Protocol

On the first day of the study, you will be allocated into one of two groups after you have been found eligible during the Screening.

The choice of group will be random, (like tossing a coin) with ten patients in each group. You will not be told which group you are in. These groups will receive slightly different treatments. Group (1) will be treated with a gel containing your platelets and growth factors and Group (2) will be treated with a gel, which contains some growth factors but no platelets. All other treatments and care will be the same for both groups.

The study doctor, will review your ulcer by taking photographs of it and measuring its size. This photograph will not identify you as a person, but the picture may be use in educational meetings or for publications. At this time, we will measure the blood flow to your legs and around the ulcer. These measurements are entirely non-invasive and painless.

You will have a needle inserted into a vein of one of your arms. A single blood sample will be immediately collected from this needle. A total amount of blood will be determined by the size of your wound (about 10 times the estimated size). This blood will be carefully labelled with your details and made into the treatment gels. At this time we will also collect about a 15mL blood sample for routine safety tests. This total blood collection will always be less than half a

standard blood donation, which in your case is not a medically significant amount.

If medically indicated, you might undergo surgical wound debridement; which is a surgical procedure under general anaesthetic to adequately remove all the dead tissue in and around you foot ulcer. You may have already had this treatment in the past. We will keep this material to make measurements in the laboratory to learn more about your ulcer and healing processes.

We will be collecting fluid from your wound; this will be done with the help of a device called vacuum assisted closure therapy (VAC). Which simply means applying a small portable machine with a dressing device on the wound for up to one hour to collect the fluid? This is procedure should not be painful, but it may cause some discomfort.

We will also be taking a small punch biopsy (6mm) of your wound before applying the gel, so that we can measure any healing that occurs during the study. The biopsy should not hurt you, but if necessary we can use a topical anaesthetic cream to numb the area. After the biopsy, the gel will be applied into the ulcer and the normal dressings applied.

You will be given an appointment for your wound review 4 days after the first treatment, at one week and then weekly at clinic for the first one-month and then every two weeks until the wound has completely healed. The details of the tests, treatments and the frequency of your visits to clinic are shown in the table at the end of this Information Sheet.

Routine measurement of the wound and photographs will be taken at every clinic visit before and after the gel application. This will give us a guide to how well your wound is healing.

Depending on the response of the wound to the gels; if the wound is not improving, another gel treatment will be applied but not until after 14 days from the initial application.

Further wound fluids and biopsies will also be taken from the wound on the 4th day, week 4 and week 8 respectively. At the same time, blood flow around the ulcer will also be measured as before. The exact timings will depend upon your clinic visits and the stage of the healing.

If it has not healed before the end of the study (16 weeks), your Doctor will discuss with you other treatment options to treat your ulcer.

You will be encouraged to get involved in the care of your ulcer and follow routine instructions from the Podiatric and Vascular nurses.

Expenses

Transportation arrangements will be made to transport patients outside their routine clinic attendance. The Research team will meet all reasonable travel expenses.

What do I have to do?

You will need to come to the treatment clinic at the times requested and remain there for the duration of time required. If you are unable to make any of the appointments, kindly contact us to make an alternative appointment.

You are requested not to participate in any other form of research or study whilst you are involved with this research.

What are the other possible risks of taking part in the study?

There are no known risks from the autologous platelet gel in our experience. But if you encounter any unusual reaction, please report it to your G.P or contact us immediately.

There are very few risks involved in inserting a cannula into a vein in the arm. You may feel some discomfort and sometimes there may be a small bruise around the area, which may be sore and last for a couple of days.

Taking of a wound biopsy should not be painful in your case, but local anaesthetic may be applied to further numb the area. The wound may bleed slightly, but should stop quickly after application of gentle pressure. There is also a small risk of infection; however, but we will regularly and closely review your wound during the study.

The use of vacuum assisted closure therapy device to collect wound fluids should also not be painful, but if any discomfort is experienced the procedure

will be discontinued immediately. This device is used routinely in our wound care clinics and is very safe.

PART 2

What if relevant new information becomes available?

If new information becomes available during the research period about the treatment, your research doctor will discuss these issues with you and will advise you on the best available option about your involvement.

Also on receiving new information your research doctor might consider it to be in your best interests to withdrawal you from the study. He will explain the reasons and arrange for your care to continue.

If the study is stopped for any other reason, you will be told and your continuing care will be arranged.

What will happen if I don't want to carry on with the study?

If you withdraw from the study, we would like to use the data collected up to withdrawal date. Any blood or tissue samples that can still be identified as yours will be destroyed if you wish.

If your participation in the study is discontinued after you have received the study treatment you may be asked to undergo a final examination for your own safety. If the reason for ending the study is, for example, an adverse event or a side-effect of the treatment, you will be asked to give information on these in order to protect the other patients taking part in this clinical study.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm will be addressed. If you have a concern about any aspect of this study, you should ask to speak with the Research Team who will do their best to answer your questions.
Mr. Costa Kyriakides, telephone: 0207 3777134
Mr. Femi Akingboye, telephone 07859062928

Complaints:

If you remain unhappy and wish to complain formally you can do this by contacting the National Health Services complaint session, which be done locally through Patient Advisory Liaison Service (PALS), Telephone 020 7377 6355 or email pals@bartsandthelondon.nhs.uk.

You may make a formal complaint via Jarrard O' Brien, Quality Development, Barts and The London NHS Trust, Healthcare Governance Directorate, 3rd Floor, Prescott Street, tel. 0207480 4857, or email jarrard.obrien@bartsandthelondon.nhs.uk

Will my taking part in this study be kept confidential?

All the information obtained about you in the course of the study is confidential and will be kept in a secure locked room. The investigators performing the study and a study Monitor will have access to the data collected in this study. They may also be looked at by representatives of regulatory authorities and by authorised people from Barts and the London NHS Trust to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

If you consent to take part in the research, the research team will abide by the Data Protection Act 1998, and the rights you have under this Act.

Involvement of the GP / Family doctor

With your permission we will contact your General practitioner to advise them of your potential participation in the study.

What will happen to any samples I give?

All your blood, tissue and urine samples taken for safety screening will be tested immediately in the clinic and once the results obtained, they will be destroyed. Other samples taken will be stored until they are tested. The samples will be anonymised and will contain only your unique study number, time and date of the sample. Once the results have been obtained they will be destroyed.

All blood and tissue samples collected during the research will be analysed with Barts and the London Hospital NHS Trust and the Institute of cell and molecular science, Queen Mary Medical School, London

Will there be genetic testing?

We shall not be performing genetic screening during this study period, but proteins involved in wound healing process will be evaluated.

What will happen to the result of the research study?

The results of this study may be published or presented at meetings. You will not be identified in any report/ publication or presentation.

Who is organising and funding the research?

This research is being Sponsored and funded by Barts and the London NHS Trust.

What happens if you are worried or if there is an emergency?

You will always be able to contact an investigator to discuss your concerns and/or to get help

Mr. Costa Kyriakides and or Femi Akingboye

Vascular Surgery Unit,

Department of General Surgery,

The Royal London Hospitals NHS Trust,

White Chapel,

London. E1 1BB.

Or contact Surgery department via switchboard for the on call Vascular Surgeon on 0207 3777000.

What happens if something goes wrong?

We believe that this study is basically safe and do not expect you to suffer any harm or injury because of your participation in it. However, we carry insurance to make sure that if your health does suffer as a result of your being in the study, then you will be compensated. In such a situation, you will not have to prove that the harm or injury, which affects you, is anyone's fault. If you are not happy with any proposed compensation, you may have to pursue your claim through legal action.

Who has reviewed the study?

This study has been reviewed and approved by National Research Ethics committee.

Thank you for taking the time to read this information sheet.

Invitation to Donate Blood for a Research Project

Title: Study to elucidate the role of autologous platelet rich Plasma gel (APG) in treating chronic non-ischemic Diabetic foot ulcers

Investigator: Mr Costas Kyriakides and Prof. Harry Navsaria.

We invite you to take part in a research project, which we think may be important. The information, which follows, tells you about it. It is important that you understand what is in this leaflet. It says what will happen if you take part and what the risks might be. Try to make sure you know what will happen to you if you decide to take part. Whether or not you do take part is entirely your choice. Please ask any questions you want to about the research and we will try our best to answer them.

Part 1

Invitation

You are being invited to donate blood that will be used for a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss with us if you have any doubt. Take time to decide whether or not you wish to take part

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. The study is sponsored by Barts and The London Hospitals NHS Trust.

Introduction to the study

Platelets are cells essential to clotting, and are the first cells to gather at the wound site. They start the healing process by releasing various growth factors. They summon useful cells and proteins to the wound site; promote cell movement and growth of new skin. Blood is collected from a vein in the arm, just like a blood test. The blood is then processed into its different parts using a portable machine. The gel is made when a clotting chemical and the processed blood are mixed; this component contains

platelets and other growth factors. This gel produced will be analysed and used for laboratory experiment.

The purposes of the study are:

To study the effect of platelet rich plasma gel on cells involved with wound healing in the laboratory, which have a processed and experimental relationship with human wound. Furthermore, the study will enable us to further understand changes that occur in the cells that are involved in wound healing process.

Why have I been ask to participate?

This is because we believe you might be able to donate your blood voluntarily for the purpose of the study

Do I have to take part?

It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form.
You are still free to refuse to participate without giving a reason.

If you decide to participate

At this time, blood will be collected from your arm vein, which will be about 300ml in total, less than the total amount of blood donated for regular blood donation.

There are very few risks involved in inserting a cannula into a vein in the arm. You may feel some discomfort and sometimes there may be a small bruise around the area, which may be sore and last for a couple of days.

What will happen to the blood donated?

Any blood samples taken from you will be processed into the platelet gel, part of the gel will be analysed immediately for presence of different growth factors and the remaining will be stored and use for the laboratory experiment.

The samples will be anonymised and will contain only your unique study number, time and date of the sample. Once the study is over any blood

products remaining will be destroyed. All blood samples collected during the research will be analysed with Barts and the London Hospital NHS Trust and the Institute of cell and molecular science, Queen Mary Medical School, London.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm will be addressed. If you have a complaint, please contact the following person in the first instance, Mr. Costa Kyriakides 0207 3777134.

Complaints:

If you remain unhappy and wish to complain formally you can do this by contacting the National Health Services complaint session, which be done locally through Patient Advisory Liaison Service (PALS), Telephone 020 7377 6355 or email pals@bartsandthelondon.nhs.uk.

If the patient wishes to make a formal complaint: Jarrard O' Brien, Quality Develeopment, Barts and The London NHS Trust, Healthcare Governance Directorate, 3rd Floor, Prescot Street, tel. 0207480 4857, or email jarrard.obrien@bartsandthelondon.nhs.uk

Will there be genetic testing?

We shall not be performing genetic screening during this study period but proteins involved in wound healing process will be evaluated.

What will happen to the result of the research study?

All the information obtained about you in the course of the study is confidential and will be kept in a secure locked room. The investigators performing the study and a study Monitor will have access to the data collected in this study. They may also be looked at by representatives of regulatory authorities and by authorised people from Barts and the London NHS Trust to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

If you consent to take part in the research, the research team will abide by the Data Protection Act 1998, and the rights you have under this Act.

The results of this study may be published or presented at meetings. You will not be identified in any report/ publication or presentation.

Who is organising and funding the research?

This research is being Sponsored and funded by Barts and the London NHS Trust, London.

Who has reviewed the study?

This study has been reviewed by National Research Ethics committee, and has been given approval by the local ethics committee.

Thank you for taking the time to read this information sheet.

Patient Information Sheet (Tissue donation)
Study Number : APG-1

Study title : A study to elucidate the role of autologous platelet rich plasma gel (APG) in treating chronic non-ischemic diabetic foot ulcers

Investigators : Prof H. Navsaria & Mr. Constantinos Kyriakides

Sponsor : Barts and The London Hospital, NHS Trust, London

You are being asked to take part in this study because you are an adult who is about to undergo a <u>planned</u> surgery procedure on your foot to treat your diabetic ulcer.

<u>Part 1</u>

Invitation

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.
Barts and The London Hospitals NHS Trust, is the sponsor of this research.

What is the purpose of the study?

Diabetic foot ulcers are a condition that diabetics need to handle with every care. Treatment of these ulcers, which range from superficial to deep, can

take months to years to heal in some patients, while others can quickly resolve.

With the help of medical research and biotechnology techniques, we are now able to successfully process various parts of the patient's own blood to make new potential treatments for these ulcers.

Platelets or thrombocytes are the cell fragments circulating in the blood that are involved in cellular mechanisms which are essential for clotting, and are the first cells to gather at the wound site. They start the healing process by releasing various growth factors. They summon useful cells and proteins to the wound site; promote cell movement and growth of new skin.

We are conducting experimental studies to enable us understand how this process, together with how healing may be accelerated and improved, through special techniques which colour the cells and identify areas of interest.

To further enable these studies, we would like to collect a skin sample which will be removed from diabetic patients undertaking routine surgical debridement operations (cleaning of the wound and removal of dead tissue). This skin is removed as part of your normal surgery and it is generally discarded and incinerated.

Why have I been chosen?
You are being asked to take part in this study because you are a diabetic adult who is about to undergo a **planned** surgical procedure on your foot. We are requesting that you donate a small piece (about 1cm) of the skin which is going to be removed from your foot.

Do I have to take part?
No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason.

Before you can begin the study

You may read the full study protocol as well as this Information sheet, which gives you more details about the study. The research doctor will tell you about the study and any adverse events, which could occur. You will be

told exactly what the study entails and what will be required of you. You are encouraged to ask questions of the Investigators conducting the study, until you are satisfied that you fully understand the nature of the study and the requirements.

What do I have to do?

If you agree to take part and to sign the research section of your *Consent Form,* we will review your medical records and collect details relating to your health, diagnosis and planned treatment.

In relation to the study, we only request that you donate a piece of the skin left over from surgery, which would otherwise be destroyed in the clinical waste. Participation in the study will have no impact on your planned treatment or health.

What are the alternatives for diagnosis or treatment?

This study is not related to your planned surgery. Your surgeon will have already discussed your treatment with you. If you have any concerns about your planned treatment, please discuss them with the surgery team.

What are the other possible disadvantages and risks of taking part?

We do not believe that there are any disadvantages or risks in you taking part.

Expenses and payments:

There will be no payment in exchange for your participation in this study or for the donation of your skin sample.

What are the possible benefits of taking part?

None, although you will be contributing to our understanding of the reasons behind diabetic wounds and this knowledge may help others such as you in the future.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in part 2.

If you have a complaint please contact the following in the first instance: Mr. C. Kyriakides or Mr. Femi Akingboye

If you are employed by Barts & The London NHS Trust or Barts & The London School of Medicine & Dentistry, non-participation or dropping out of the study will not affect your training or career.

Any complaint about the way you have been dealt with during the study the

detailed information on this is given in Part 2.

A contact number for complaints will be given.

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact Details:

If you require any further information please contact:

Mr. Constantinos Kyriakides

Vascular Surgery Department,

The Royal London, White Chapel

London, E1 1BB

Tel Number: 02073777020

Or:

Mr. Femi Akingboye

Vascular Surgery Department,

The Royal London, White Chapel

London, E1 1BB

Tel Number: 02073777020, 07859062928

PATIENT RECRUITMENT INFORMATION

A STUDY TO ELUCIDATE THE ROLE OF AUTOLOGOUS PLATELET RICH PLASMA GEL (APG) IN THE TREATMENT OF CHRONIC NON-ISCHAEMIC DIABETIC FOOT UCLERS

Ethnics Approval by the East and City of London Ethnics Committee Local REC Number: 08/H0704/17

Study is Sponsored by :

Barts & The Royal London Hospitals NHS Trust,

Joint Research Office, 3rd Floor, Rutland House, 42 – 46, New Road,

The Royal London Hospital, White chapel, London. E1 1BB.

Principal Investigator: Mr. C. Kyriakides (Consultant Vascular Surgeon)

Clinical Research Fellow: Femi Akingboye

INCLUSION CRITERIA

In order to be eligible to enter the study, patients must meet the following criteria.

- A patient with documented Type I or Type II diabetes mellitus of not less than 2 years duration.
- Only patients that are on pharmacotherapy treatment for diabetes will be used in the study.
- Only one ulcer on the foot of the Lower limb (medial malleolus to the toes).
- The ulcer should have a surface area of between 4cm2 –40cm$^{2.}$
- The treatment is specific to clinically non-infected full thickness wounds
- Ulcer present for a minimum of 4 weeks
- The wound must extend through the dermis and may involve tendon, muscle or bone exposure.
- The wound must have failed to respond to intensive conventional treatment.
- The patient should be competent to give consent and have an appropriate support system to facilitate his/her full participation in the follow-up care associated with the treatment of the wound.
- A case control method will be applied to counter the effects of variables that might affect the study outcome.
- An ankle brachial pressure index value of ≥0.7 and transcutaneous oxygen tension on the foot with the ulcer of ≥ 40mmHg

EXCLUSION CRITERIA

- Patients with clinical evidence of platelet dysfunction and thrombocytopenia < 100,000.
- Patients with documented malignant disease and connective tissues disease.
- Patients with who are not ambulant from other reason other than the poor mobility due to the ulcer, except inpatients, will not be included.
- Any other significant pathology, which might impair healing other than diabetes such as end stage renal impairment.
- Patients with clinically documented Hepatitis B & C infection and HIV.
- Patients with documented MRSA wound infections.
- Patients with poor prognosis associated with other disease processes.
- Ulcers from any cause other than diabetes will be excluded such as pressure sore.

Flow chart illustrating events that will occur from recruitment to the end of the study

Interval		**Day 0**	**Day 4**	**Wk 1**	**Wk 2**	**Wk 3**	**Wk 4**	**Wk 6**	**Wk 8**	**Wk 10**	**Wk 12**	**Wk 14**	**Wk 16**
SCREEN	↓		↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
Consent	☼												
Demo	◙												
Urinalysis	◘						◘						◘
STUDY													
Haematology		●					●						●
Biochemistry		♦					♦						♦
Microvascular		♥	♥				♥						♥
Photography		♣		♣	♣	♣	♣	♣	♣	♣	♣	♣	♣
Planimetry		♠	♠	♠	♠	♠	♠	♠	♠	♠	♠	♠	♠
Debridement		◄											
APG treatment		▼											
APG analysis		►											
Biopsy		▲	▲				▲		▲				
Wound Fluid		▓					▓		▓				
Dressing		▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒

Kindly contact Mr. Femi Akingboye, Vascular research Fellow on 07859062928 for patient recruitment.

Title of research proposal: A PILOT STUDY ON THE ROLE OF AUTOLOGUS PLATELETRICH PLASMA (APG) FOR THE TREATMENT OF DIABETICFOOT ULCERS

Name of Researchers: Mr. Costa Kyriakides & Prof H.Navsaria

Name of Patient / Volunteer (Block Capitals)**:**

Address:

SCREENING CONSENT FORM (Initial boxes to agree with statement)

1. I confirm that I have read and understand the information sheet dated ..... , (**Platelet Plasma Gel**) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation of the ***SCREENING*** is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals from Queen Mary University of London Medical School and from regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to my GP being informed of my participation in the study and subsequently informed any significant information relevant to my participation.

5. I agree to take part in the above study.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Title of research proposal: A study to elucidate the role of autologous platelet rich plasma gel (APG) in treating chronic non-ischaemic diabetic foot ulcers

Name of Researchers: Professor Harry Navsaria & Mr. Constantinos Kyriakides
Name of Patient / Volunteer (Block Capitals)**:**

Address:

DONATION OF REDUNDANT TISSUE CONSENT FORM (Initial boxes to agree with statement)

1. I confirm that I have read and understand the information sheet **dated 10th Jan 2009 version 1.0**, for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation of the ***DONATION*** is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I understand that relevant sections of any of my data generated during the study, may be looked at by responsible individuals from Queen Mary University of London Medical School and from regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Title of research proposal: Study to elucidate the role of autologous platelet rich plasma gel (APG) in treating chronic non-ischaemic diabetic foot ulcers

Name of Researchers: Professor Harry. Navsaria & Mr. Constantinos Kyriakides
Name of Patient / Volunteer (Block Capitals)**:**

Address:

BLOOD DONATION CONSENT FORM

(Initial boxes to agree with statement)

1. I confirm that I have read and understand the information sheet **dated 15th Jan 2009 version 1.1**, for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation in the ***DONATION*** is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I understand that relevant sections of any of my data generated during the study, may be looked at by responsible individuals from Queen Mary University of London Medical School and from regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.
4. I agree to take part in the above study.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

APPENIX 6- Procotol for PRP preparation

WOUND DRESSING PROTOCOL FOR PATIENTS TREATED WITH AUTOLOGUS PLATELET GEL

Barts & The London Standard Operating Procedure – Specialist wound care services in conjunction with the Vascular Research team.

1. Patient with chronic diabetic foot ulcers who have been found suitable will have their wound treated according to the platelet gel treatment protocol.

2. Autologous platelet gel therapy is believed to promote wound heeling. There are no documented severe adverse effects from the treatment. This is because patients own blood is used in healing patients wound.

3. The treatment is carried out under an aseptic technique either in operating room or treatment room.

4. Graft/gel is produced by a group of clinical perfusionists, who are trained personnel in this field.

5. To allow the graft/gel to stay on the wound, we recommend the following wound dressing protocol, after the initial application of the platelet gel graft

6. Gentle application of damp mepitel on the wound (This will prevent disruption of the germinal layer of the wound).

7. Apply 2 layers of damp sterile gauze (normal saline) on the mepitel. This is to prevent absorption of the gel into the dressing material.

8. Then apply 4 layers of dry sterile gauze on the damp gauze. This is to further protect the wound.

9. Apply an Opsite on the dry gauze, which will serve as an adhesive dressing material.

10. Gently apply a crepe bandage, which should not be too tight to keep all the dressing material in place.When appropriate patient should be given medics shoe to promote the wound and to encourage patient ambulation.

11. The first change of dressing after gel/graft therapy will be carried out by the research team.

12. The initial dressing after gel application is left on for 96hrs (4days). This is preventing distortion and good take of the gel, the gel is believed to be a form of haemopoetic team cell. Wound biopsy will be taken during this change of dressing

13. Wound will be reviewed weekly during the first month of the treatment and then alternate weeks until the end of the treatment.

14. If worried about any deterioration in wound condition kindly contact the research team, (Femi Akingboye 0 785906 2928), or the wound specialist nurse at the wound clinic in the Outpatient department of the Royal London Hospital, White Chapel. The contact person is Sis. Deborah Conway 02073777788.

15. District Nurses will be invited to attend our autologus platelet gel treatment and wound dressing sessions to allow for continuity of care in the community

Blood Sequestration Protocol.

Blood sequestration is used to separate different components of the blood for use in autologous blood therapies. Blood drawn (directly from the patient or via a donated bag) can be divided into 3 separate bags of: - 1) Platelet rich plasma (PRP)

2) Platelet poor plasma (PPP)

3) Red blood cells (RBC)

The machine used to sequestrate the blood is the Dideco Electa Cell Saver. It can be used with a variety of bowl sizes but information given relates to the 125ml and 55ml bowls.

Blood should be taken from the patient at the earliest opportunity to counter the activation of the clotting cascade, which starts from the insertion of the first cannular in the anaesthetic room. Care must be taken to remove blood in the least aggressive manor possible in order not to activate the platelets with the donated blood. Where possible the use of a large bored lumen is advocated, (such as those used for venous canulation of the neck vessels).

Enough blood must be taken to insure that the bowl size selected can be completely filled with packed red blood cells. It is possible to ascertain the amount needed from details in the patients notes and use of the table located in the 'blood volume for platelet gel' section of this protocol'.

Blood should only be collected into an appropriate sterile collection unit. To stop coagulation of the collected blood, it should be drawn directly into pre-anticoagulated bag. (CPDA-1 Single Blood Collection System, Baxter, ref R8101). Each of these bags is pre-filled with 63ml of the anticoagulant solution and to this a maximum of 450ml of whole blood can be harvested. This gives a total of 493ml volume into the bag. If more volume than this is required (see blood volume table), then a second bag should be used to avoid the risk of under anticoagulation.

Specific care should be taken with all aspects of health and safety when handling any body fluids including universal precautions.

Initial Set Up

- Set up cell saver as for normal cardiac case (see cell saver protocol), but do not add saline bags to the end of the wash lines.
- Change the program to PRP2
- Change set up of the cell saver using Dideco COD. 150 BLOOD COMPONENTS COLLECTION SET (ref 04025).
- Remove the bags and 4-way adapter from the collection set packaging and close the roller clamps on the plasma collection bags.

- Connect the plasma collection bags to the lines of the 4-way adapter.
- Hang the plasma collection bags on the I.V pole ensuring the inlet lines are face up (i.e. hanging upside down) **WARNING: POSITIONING THE BAG WITH THE INLET LINE FACE DOWNDOES NOT PERMIT CORRECT EXECUTION OF THE PROCEDURE.**
- Disconnect the connection between the bowl outlet and the waste bag inlet.
- Connect the inlet of the 4-way adaptor to the outlet male connector of the bowl.
- Connect the inlet of the waste fluid bag to the single remaining line from the 4-way adaptor.
- If necessary disconnect the blue (wash) line from the blood connection reservoir and connect it to the drawing line found in the blood components collection set.
- Attach the spike adaptor to the end of this line and connect it to the collected blood.

Sequestration Procedure.

- Before starting check that the line between the bowl and the collection bag is open and the lines between the bowl and the waste bags are closed.
- Carefully spike donated autologous blood collecting bag.
- Press the BLUE prime button to start the 'PRIME PHASE' and follow the on screen instructions.
- Blood will slowly fill the centrifuge until packed red cells fill the bowl. The displaced plasma is collected in one of the plasma collection bags.
- When the bowl is full of packed red cells, the program will pause for approximately 60 seconds before automatically changing into the 'SPILL PHASE'.
- You will be asked to close the PPP bag line and open the PRP bag line.
- Further 'spilling' will now occur with the platelets being collected into the PRP bag. During this spill, it is possible to increase the spill volume by 5ml from 30ml to 35ml. This dips further into the red cell region of the blood to ensure maximum yield of platelets.
- Great care should be taken of the PPP and PRP bags, so as not to activate the platelets or damage the collected product.
- The red cells are pumped into the remaining blood collection bag (for re-infusion to the patient), and the process may be restated in necessary. Once a sufficient quantity of blood has been sequestrated the bags may be closed and the products removed ready for application.
- EACH BAG SHOULD BE CAREFULLY LABELLED WITH THE CORRECT PATIENTS DETAILS, INCLUDING THEIR NAME, DATE OF BIRTH AND HOSPITAL NUMBER.

APPENDIX 6

RAW DATA FROM ALL THE EXPERIMENT IN THE THESIS

Data from quantification of growth factors from Platelet rich and Platelet poor plasma as graphically represented in Chapter 3 of the main thesis

Patient ID	HPPP EGF	HPPP FGF-2	HPPP PDGF-AA	HPPP VEGF	HPPP TGFb1	HPPP IGF	HPPP P-Sel
P1	32.	32.000000	1068.559000	0.000000	344.676700	154.284800	55.999100
P2	32.	0.000000	2023.134000	291.218200	724.270400	103.316700	56.481250
P3	32.	0.000000	466.890000	476.456000	803.489500	141.818800	33.388360
P4	32.	32.000000	778.406700	154.653000	1529.222000	142.449000	49.610210
P5	32.	0.000000	1254.991000	56.799660	172.430700	112.195100	319.465900
P6	32.	211.668900	1041.089000	273.205000	142.814000	159.756400	348.579200
P7	32.	0.000000	1076.121000	0.000000	2224.928000	114.278200	408.501800
P8	32.	62.956070	2044.020000	0.000000	1412.898000	150.971100	55.900880
P9	32.	32.000000	1163.501000	170.773700	162.734800	117.920400	38.982190
P10	32.	0.000000	1463.833000	0.000000	347.785700	105.461100	61.783010
P11	32.	32.000000	1965.068000	82.919290	3526.818000	124.319900	48.463070
P12	32.	32.000000	4775.592000	0.000000	127.099400	101.283600	67.477350
P13	32.	0.000000	1290.483000	32.000000	64.000000	116.033700	41.690680
P14	32.	86.590760	722.012500	56.799660	79.808840	154.041600	64.252700

Table 1 . The raw data from the quantification of growth factors of platelet poor plasma (PPP) from health volunteers using the immunoabsorbent technique. Concentration of all the growth factors was measured in ng/mL

Patient ID	HPRP EGF	HPRP FGF-2	HPRP PDGF-AA	HPRP VEGF	HPRP TGFb1	HPRP- IGF	HPRPP- Sel
P1	459.22670	101.9610	97767.020	1327.1980	2454.8530	149.66240	308.52180
P2	523.4370	173.27190	109620.10	1057.4620	28558.780	114.78920	425.10590
P3	565.12570	377.8320	157814.0	3186.4160	78634.53	137.43730	387.56140
P4	517.75340	0.000000	147228.10	765.99560	109560.40	137.97060	319.46590
P5	769.57070	309.11940	190001.60	334.01230	10337.780	158.24590	319.46110
P6	917.36160	231.2810	167508.00	230.42160	11138.250	121.49170	221.29220
P7	698.16910	0.000000	183361.90	586.83620	18266.790	135.31680	391.0410
P8	553.48580	204.52430	275348.70	1006.7520	47962.460	130.69350	334.90160
P9	485.50780	117.65320	222237.20	304.21530	34282.750	98.75160	347.00740
P10	61.63388	0.000000	40302.630	105.80710	9395.3310	92.935730	103.91490
P11	63.12603	0.000000	42657.850	239.23030	13051.390	107.75110	118.83720
P12	177.61730	0.000000	49813.820	314.07420	5501.3350	109.85440	136.38280
P13	146.48810	32.000000	8551.2610	132.76840	4118.7020	96.034070	104.83600
P14	544.70840	32.000000	37982.380	0.000000	8161.1840	127.37730	333.27790

Table 2. The raw data from the quantification of growth factors from platelet rich plasma (PRP) from healthy volunteers using the immunoabsorbent techniuque. Concentration of all the growth factors was measured in ng/Ml

Patient ID	DPPP FGF-2	DPPP PDGF-AA	DPPP VEGF	DPPP TGFb1	DPPP- IGF	DPPPP-Sel
P1	0.	260.000000	113.000000	599.000000	97.000000	64.173100
P2	0.	744.211900	74.983260	64.000000	75.914570	66.538180
P3	32.	761.303900	119.767100	2195.280000	109.857600	74.304000
P4	32.	620.411500	56.799660	389.135900	68.192550	51.238020
P5	32.	4216.464000	90.395170	6980.553000	106.081200	72.747730
P6	0.	148.327400	546.402100	1518.187000	179.055300	25.154690

Table 3. The raw data from the quantification of growth factors from PPP from Diabetic patients using the immunoabsorbent technique. Concentration of all the growth factors was measured in ng/ml

Patient ID	DPRP EGF	DPRP FGF-2	DPRP PDGF-AA	DPRP VEGF	DPRP TGFb1	DPRP IGF	DPRP P-Sel
P1	32.000000	32.000000	23758.0000	104.000000	14792.0000	127.000000	131.254800
P2	580.513400	32.000000	215261.3000	828.443400	35284.29000	72.518970	384.656500
P3	230.442300	32.000000	27986.07000	178.252600	32349.16000	121.35120	192.111700
P4	294.019800	0.000000	112601.8000	1058.524000	37076.07000	81.966420	131.300700
P5	240.904300	62.956070	247629.000	2816.444000	45755.68000	121.06700	247.968400
P6	32.000000	0.000000	1504.464000	343.672600	5047.802000	166.38780	39.677700

Table The raw data from the quantification of growth factors from PRP from Diabetic patients using the immunoabsorbent Technique Concentration of all the growth factors was measured in ng/ml

Data for standard plot for Thrombospodin -1 (TSP-1)

Conc (ng/ml)	Mean optical density
0.000000	0.0810
7.810000	0.2190
15.600000	0.2890
31.300000	0.4810
62.500000	0.8105
125.000000	1.2310
250.000000	1.8760
500.000000	2.4755

Table showing the optical density and pre-determined concentration of TSP-1 for determining the standard plot

Data for standard plot for Platelet factor -4 (PF-4)

Conc (ng/ml)	Mean optical density
0.0	0.0855
0.5	0.7030
1.0	1.2850
2.0	1.9930
5.0	3.1330
10.0	3.7260

Table showing the optical density and pre-determined concentration of PF-4 for determining the standard plot

Patient ID	Conc (ng/ml)	Patient ID	Conc (ng/ml)

HV1 PRP	**1581.32768**	**HV1 PPP**	**534.06736**
HV2 PRP	**1526.42528**	**HV2 PPP**	**593.20784**
HV3 PRP	**1496.95136**	**HV3 PPP**	**419.1576**
HV4 PRP	**1521.03136**	**HV4 PPP**	**543.892**
HV5 PRP	**1461.5056**	**HV5 PPP**	**395.65552**
HV6 PRP	**1576.51168**	**HV6 PPP**	**403.16848**
HV7 PRP	**1566.87968**	**HV7 PPP**	**363.09936**
HV8 PRP	**1527.19584**	**HV8 PPP**	**619.5032**
HV9 PRP	**1447.82816**	**HV9 PPP**	**487.83376**
HV10 PRP	**1445.32384**	**HV10 PPP**	**557.47312**
HV11 PRP	**1439.352**	**HV11 PPP**	**555.83568**
HVI2 PRP	**1424.904**	**HVI2 PPP**	**647.82128**
HVI3 PRP	**1223.78784**	**HVI3 PPP**	**467.51024**
HV14 PRP	**1432.03168**	**HV14 PPP**	**324.86032**

Table represents the mean values of TSP-1 from PRP amd PPP from healthy volunteers (HV)

Patient's ID	Conc (ng/ml)	Patient's ID	Conc (ng/ml)
P1 PRP	1359.4064	P1 PPP	372.73136
P2 PRP	1423.55552	P2 PPP	342.87216
P3 PRP	1405.44736	P3 PPP	618.15472
P4 PRP	997.62848	P4 PPP	131.44976
P5 PRP	1406.21792	P5 PPP	405.38384
P6 PRP	1303.34816	P6 PPP	243.66256

Table represents the mean values of TSP-1 from PRP amd PPP of Diabetic patients

Patient ID	Conc (ng/ml)	Patient ID	Conc (ng/ml)
HV1 PRP	107.8999408	HV1 PPP	106.1364308
HV2 PRP	113.4085738	HV2 PPP	108.0897623
HV3 PRP	105.6978558	HV3 PPP	110.7009436
HV4 PRP	100.0801923	HV4 PPP	103.8286804
HV5 PRP	98.26233002	HV5 PPP	103.8907138
HV6 PRP	104.7611536	HV6 PPP	97.53992172
HV7 PRP	98.20202595	HV7 PPP	108.85074
HV8 PRP	93.85059684	HV8 PPP	97.90078754
HV9 PRP	103.4568754	HV9 PPP	98.26233002
HV10 PRP	100.2629089	HV10 PPP	106.3874586
HV11 PRP	101.8535391	HV11 PPP	92.61736751
HVI2 PRP	113.343721	HVI2 PPP	93.61505695
HVI3 PRP	104.0769264	HVI3 PPP	102.7769901
HV14 PRP	166.3166437	HV14 PPP	104.1390349

Table represents the mean values of PF-4 from PRP and PPP from healthy volunteers (HV)

Patient's ID	Conc (ng/ml)	Patient's ID	Conc (ng/ml)
P1 PRP	103.3949736	P1 PPP	103.9528
P2 PRP	110.9573847	P2 PPP	98.32265
P3 PRP	40.63717655	P3 PPP	40.98318
P4 PRP	95.74574244	P4 PPP	102.4687
P5 PRP	89.2543672	P5 PPP	90.4647
P6 PRP	84.21685524	P6 PPP	83.10532

Table represents the mean values of PF-4 from PRP and PPP from Diabetic patients

RAW-DATA FROM PROLIFERATION ASSAY EXPERIMENTS AS REPRESENTED GRAPICALLY IN CHAPTER 4 FROM THE MAIN THESIS

Cell Density/ well	4hrs	16hrs
1,000	198	1050
3,000	274	1414
4,000	343	1810
6,000	863	2871
8,000	1092	4290

10,000	**1192**	**4792**
20,000	**1754**	**5573**
40,000	**4423**	**6506**
80,000	**5955**	**7084**
100,000	**6332**	**7130**
200,000	**7186**	**7541**

Table represents data from standardization of human dermal fibroblast with alamar blue. The cell density were measured with spectrometry (plate reader). The data represents the mean fluorescence of three readings from each well plate

Treatment	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Cont 10%	**0.521137**	**0.526406**	**0.861687**	**1.118666**	**1.175634**	**1.306982**
PRP 10%	**0.440331**	**0.372725**	**0.861687**	**0.938643**	**0.947486**	**1.201527**
PRP 5%	**0.498876**	**0.391294**	**0.751412**	**0.796107**	**1.037223**	**1.089046**
PPP	**0.551951**	**0.467411**	**0.890793**	**0.863293**	**1.147179**	**0.995886**

Table represents proliferation of HDF with 10% FCS as the diluents media over 5 days Proliferation measured as redox reaction of Alamar blue dye. The redox reaction was measured as absorbance with the plate reader.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	5536.00	4798.	4788.	4733.333	4992.6670	4688.3330	4990.0
EXP 2	5362.667	4876.	4879.	4239.00	4943.3330	4887.3330	4803.667
EXP 3	4869.333	4876.	4765.	4733.3330	4992.6670	4688.3330	4990.0

Table represents the mean value of three readings for proliferation assay of human dernal fibroblast (HDF) for each experiment (EXP), with experiment done in triplicate at day 0. PRP/PPP from diabetic patient, with 1% BSA as the diluent media. Proliferation measured as fluorescence of Alamar blue .

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	15225.670	17896.	15832.	14823.0	11934.330	6268.6670	629.0
EXP 2	16619.670	16798.	14956.	13922.0	11249.670	5943.0	670.66670
EXP 3	12948.670	15468.	11946.	8852.333	7951.6670	734.33330	116.66670

Table represents the mean value of three readings for proliferation assay of HDF for each experiment (EXP), with experiment done in triplicate at day 5. PRP/PPP from diabetic patient, with 1% BSA as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp. Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	0.8220652	2.557255	1.297512	0.9736841	0.2113268	0.4681818	0.1254093
EXP 2	0.9903786	1.369905	1.112543	0.9852337	0.8599617	1.077666	0.7255532
EXP 3	1.299008	1.299119	1.530721	1.026845	1.026550	0.5382216	0.05412615

Table represents N- fold increase (derived from data from day 0 and day 5 above) of HDF proliferation assay with PRP/PP from diabetic patient with 1% BSA as the diluents media.

Treatment	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Cont 10%	0.521137	0.526406	0.861687	1.118666	1.175634	1.306982
PRP 10%	0.440331	0.372725	0.861687	0.938643	0.947486	1.201527
PRP 5%	0.498876	0.391294	0.751412	0.796107	1.037223	1.089046
PPP	0.551951	0.467411	0.890793	0.863293	1.147179	0.995886

Table represents proliferation of HDF with 10% FCS as the diluents media over 5 days Proliferation measured as redox reaction of Alamar blue dye.

Exp. Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	5536.0	4798.0	4788.0	4733.3330	4992.6670	4688.3330	4990.0
EXP 2	5362.667	4876.0	4879.0	4239.0	4943.3330	4887.3330	4803.667
EXP 3	4869.3330	4876.0	4765.0	4733.3330	4992.6670	4688.3330	4990.0

Table represents the mean value of three readings for proliferation assay of HDF for each experiment (EXP), with experiment done in triplicate at day 0. PRP/PPP from healthy volunteers, with 1% BSA as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	15225.670	17896.0	15832.0	14823.0	11934.330	6268.667	629.0
EXP 2	16619.670	16798.0	14956.0	13922.0	11249.670	5943.0	670.6667
EXP 3	12948.670	15468.0	11946.0	8852.3330	7951.6670	734.3333	116.6667

Table represents the mean value of three readings for proliferation assay of HDF for each experiment (EXP), with experiment done in triplicate at day 5. PRP/PPP from healthy volunteers, with 1% BSA as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	2.750302	3.729887	3.306600	3.131620	2.390372	1.337078	0.1260521
EXP 2	3.099143	3.445037	3.065382	3.284265	2.275726	2.301801	0.1396156
EXP 3	2.659229	3.172272	2.507030	1.870211	1.592669	1.696054	0.0233801

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of HDF proliferation assay with PRP/PP from healthy volunteers with 1% BSA as the diluents media.

Exp. Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	14039.670	12782.0	12547.670	13129.0	13033.0	12816.0	12466.
EXP 2	14149.00	12891.330	12657.0	13238.330	13142.33	12925.330	12930.

Table represents the mean value of three readings for proliferation assay of HDF following serum starvation for each experiment (EXP), with experiment done in triplicate at day 0. PRP/PPP from diabetic patient, with 1% BSA as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	1839.7780	26919.78	16287.440	19966.890	19910.0	12694.670	7547.2220
EXP 2	1716.7780	26769.78	16164.440	19843.890	10868.0	12571.670	7424.2220

Table represents the mean value of three readings for proliferation assay of HDF following serum starvation for each experiment (EXP), with experiment done in triplicate at day 5. PRP /PPP from diabetic patient, with 1% BSA as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	0.1310414	2.106070	1.298045	1.520823	1.527661	0.9905329	0.6054245
EXP 2	0.1213356	2.076572	1.277115	1.498972	0.8269462	0.9726382	0.5741858

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of HDF proliferation assay with no serum (serum starvation assay) with PRP/PP from healthy volunteers with 1% BSA as the diluents media.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	495.50	512.33330	502.50	506.66670	515.00	513.33330	509.00
EXP 2	1443.4440	1489.7220	1448.833	1444.7780	1475.3890	1437.7780	1433.3890
EXP 3	11721.0	11720.00	11689.00	11622.330	11625.670	11647.00	11621.00

Table represents the mean value of three readings for proliferation assay of HDF following serum starvation for each experiment (EXP), with experiment done in triplicate at day 0. PRP/ PPP from diabetic patient, with 1%FCS as the diluents media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	407.33330	1310.1670	652.00	493.33330	108.83330	240.3333	63.83330
EXP 2	1429.5560	2040.7780	1611.8890	1423.4440	1268.7780	1549.4440	1040.0
EXP 3	15225.670	19225.70	17892.60	14823.00	11934.330	6268.6670	629.00

Table represents the mean value of three readings for proliferation assay of HDF following serum starvation for each experiment (EXP), with experiment done in triplicate at day 5. PRP /PPP from diabetic patient, with 1%FCS as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	0.9919342	1.086145	0.771805	0.7889527	0.6556302	0.3568295	0.5570968
EXP 2	0.8220652	2.557255	0.7160861	0.9736841	0.3407767	0.4681818	0.1254093
EXP 3	1.570052	2.163292	1.589769	0.9548309	1.783480	1.324648	0.880870

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of HDF proliferation assay from PRP/PP from Diabetic patient with 1% FCS as the diluents media.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	1570.3330	1580.6670	1544.0	1547.8330	1545.3330	1558.1670	1550.0
EXP 2	495.50	512.33330	502.5	506.66670	515.00	513.3330	509.0
EXP 3	1573.8330	1547.3330	1521.5	1564.500	1571.8330	1516.00	1533.330

Table represents the mean value of three readings for proliferation assay of HDF following serum starvation for each experiment (EXP), with experiment done in triplicate at day 0. PRP/PPP from healthy donor, with 1%FCS as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	1557.6670	1716.8330	1191.6670	1221.1670	1013.1670	556.00	863.50
EXP 2	407.33330	1310.1670	359.83330	493.3330	175.50	240.3330	63.83330
EXP 3	2471.0	3347.3330	2418.8330	1493.8330	2803.3330	2008.1670	1350.6670

Table represents the mean value of three readings for proliferation assay of HDF following serum starvation for each experiment (EXP), with experiment done in triplicate at day 5. PRP /PPP from healthy donor, with 1%FCS as the diluents media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	2.885078	2.557255	1.297512	0.9736841	0.2113268	1.070346	0.275049
EXP 2	0.2821954	1.369905	1.112543	0.9852337	0.8601885	0.1671561	0.04453315
EXP 3	1.299008	1.653728	1.539502	1.268115	0.5348692	0.5382216	0.05412615

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of HDF proliferation assay from PRP/PP from healthy donor with 1% FCS as the diluents media.

RAW DATA FROM HUMAN KERATINOCYTE PROLIFERATION ASSAY

Treatment	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Cont 10%	0.277379	0.444046	0.583561	0.592119	0.553609	0.399544
PRP 10%	0.331287	0.366328	0.519991	0.452106	0.505873	0.426165
PRP 5%	0.338445	0.23643	0.443991	0.43135	0.417512	0.358521
PPP	0.342773	0.374817	0.492153	0.495471	0.357425	0.402465

Table represents proliferation of Human epithelial keratinocyte (HEK) with 10% FCS as the diluents media over 5 days Proliferation measured as redox reaction (measured as absorbance) of Alamar blue dye.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	7782.6670	7370.8330	8013.1670	7770.1670	8034.1670	8065.1670	7785.50
EXP 2	3704.3330	3762.1670	4026.50	3431.0	3710.8330	3720.1670	4014.8330
EXP 3	10007.00	10000.0	9988.0	9901.333	9998.0	9986.3330	9888.0

Table represents the mean value of three readings for proliferation assay of HEK following each experiment (EXP), with experiment done in triplicate at day 0. PRP /PPP from diabetic patient, using 1% FCS as the diluents media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	8276.1670	9347.333	3097.6670	3270.833	3493.1670	631.66670	399.16670
EXP 2	8584.1670	6519.00	6890.1670	2424.0	2130.0	128.50	127.83330
EXP 3	43961.330	9754.000	5452.00	2968.6670	1229.6670	1085.3330	876.0

Table represents the mean value of three readings for proliferation assay of HEK following each experiment (EXP), with experiment done in triplicate at day 5. PRP /PPP from diabetic patient, with 1% FCS as the diluents media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	1.063410	1.268152	0.3865721	0.4209476	0.4347889	0.07832035	0.05127053
EXP 2	2.317331	1.732778	1.711205	0.7064996	0.5739951	0.03454146	0.03184025
EXP 3	4.393058	0.975400	0.545855	0.299825	0.1229913	0.1086818	0.08859223

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of HEK proliferation assay from PRP/PP from diabetic patient with 1% FCS as the diluents media

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	3088.1670	2772.50	2974.3330	2980.3330	3055.3330	2716.50	2719.0
EXP 2	857.83330	1261.833	948.16670	965.0	854.83330	1032.1670	902.66670
EXP 3	1628.6670	1877.0	1699.1670	1685.6670	1662.3330	1643.6670	1506.1670

Table represents the mean value of three readings for proliferation assay of HEK following each experiment (EXP), with experiment done in triplicate at day 0. PRP /PPP from healthy donor, with 1% FCS as the diluents media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	2836.0	2148.0	2080.3330	2004.6670	1169.8330	1370.330	1660.0
EXP 2	2323.1670	1382.0	1279.1670	899.66670	860.33330	37.83330	35.3330
EXP 3	3381.1670	1435.1670	1263.0	1058.500	1429.6670	1701.6670	129.0

Table represents the mean value of three readings for proliferation assay of HEK following each experiment (EXP), with experiment done in triplicate at day 5. PRP /PPP from healthy donor, with 1% FCS as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeat	Cont1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	0.918344	1.135437	0.6994284	0.6726319	0.382882	0.504448	0.6105186
EXP 2	2.708180	1.095232	1.349095	0.9322971	1.006434	0.0366543	0.0391433
EXP 3	2.076033	0.764607	0.7433054	0.6279414	0.860037	1.035287	0.0856479

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of HEK proliferation assay from PRP/PP from healthy donor with 1% FCS as the diluents media.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	12647.330	12645.9	12706.	12732.000	12503.000	12647.000	12600.
EXP 2	6391.333	6400.0	6456.	6501.333	6398.667	6466.333	6400.
EXP 3	6044.667	6074.0	6098.	6120.000	6165.330	6217.000	6200.

Table represents the mean value of three readings for proliferation assay of HEK following each experiment (EXP), with experiment done in triplicate at day 0. PRP /PPP from healthy donor, with 1% BSA as the diluent media. Proliferation measured as fluroscence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP

EXP 1	**16935.70**	**17863.0**	**16543.0**	**15284.0**	**8816.333**	**6081.3330**	**3000.0**
EXP 2	**18279.67**	**16754.0**	**13245.0**	**2109.333**	**899.667**	**938.6670**	**543.0**
EXP 3	**33969.00**	**4532.0**	**3214.0**	**2891.660**	**1809.000**	**976.6667**	**543.0**

Table represents the mean value of three readings for proliferation assay of HEK following each experiment (EXP), with experiment done in triplicate at day 5. PRP /PPP from healthy donor, with 1% BSA as the diluent media. Proliferation measured as fluroscence of Alamar blue dye.

Exp.Repeats	**Cont 1%**	**PRP 5%**	**PRP 10%**	**PRP 25%**	**PRP 50%**	**PPP**	**unaPPP**
EXP 1	**1.395156**	**0.889989**	**0.6786143**	**0.301643**	**0.1229246**	**0.1086818**	**0.1107161**
EXP 2	**1.985956**	**1.098433**	**0.7348731**	**0.3170366**	**0.1346806**	**0.1323955**	**0.1333333**
EXP 3	**2.194840**	**0.9074096**	**0.6358422**	**0.270973**	**0.1443678**	**0.2176334**	**0.2091807**

Table represents N- fold increase (derived data from day 0 and day 5 measurement above) of HEK proliferation assay from PRP/PP from healthy donor with 1% BSA as the diluents media.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP

EXP 1	**10007.0**	**9999.0**	**9988.0**	**9901.33**	**9998.0**	**9986.333**	**9845.0**
EXP 2	**9091.0**	**9001.0**	**9222.0**	**9102.00**	**9236.667**	**9099.00**	**9000.0**
EXP 3	**8643.0**	**8435.0**	**8543.0**	**8654.00**	**8700.0**	**8620.0**	**8605.0**

Table represents the mean value of three readings for proliferation assay of HEK following each experiment (EXP), with experiment done in triplicate at day 0. PRP /PPP from diabetic patient, with 1% BSA as the diluents media. Proliferation measured as fluorescence of Alamar blue dye.

Exp.Repeats	**Cont 1%**	**PRP 5%**	**PRP 10%**	**PRP 25%**	**PRP 50%**	**PPP**	**unaPPP**
EXP 1	**13961.33**	**8899.0**	**6778.0**	**2986.667**	**1229.0**	**1085.333**	**1090.0**
EXP 2	**18054.33**	**9887.0**	**6777.0**	**2885.667**	**1244.0**	**1204.667**	**1200.0**
EXP 3	**18970.00**	**7654.0**	**5432.0**	**2345.000**	**1256.0**	**1876.000**	**1800.0**

Table represents the mean value of three readings for proliferation assay of HEK following each experiment (EXP), with experiment done in triplicate at day 5. PRP /PPP from diabetic patient, with 1% BSA as the diluents media. Proliferation measured as fluorescence of Alamar blue dye.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	1.339073	1.412553	1.301983	1.200440	0.7051374	0.4808518	0.2380952
EXP 2	2.860072	2.617812	2.051580	0.3244462	0.1406023	0.1451622	0.08484375
EXP 3	5.619664	0.7461311	0.5270581	0.4724935	0.293415	0.1570961	0.08758064

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of HEK proliferation assay from PRP/PP from diabetic donor with 1% BSA as the diluents media.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	1.339073	1.412553	1.301983	1.200440	0.7051374	0.4808518	0.2380952
EXP 2	2.860072	2.617812	2.051580	0.3244462	0.1406023	0.1451622	0.08484375
EXP 3	5.619664	0.7461311	0.5270581	0.4724935	0.293415	0.1570961	0.08758064

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of NEB-1 cell proliferation assay from PRP/PP from diabetic patient with 1% FCS as the diluents media.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP
EXP 1	30249.67	31345.0	29887.7	29998.0	31000.0	30008.0
EXP 2	41882.67	38972.0	37876.0	41222.0	41000.0	40231.0

Table represents the mean value of three readings for proliferation assay of NEB-1 cell following each experiment (EXP), with experiment done in triplicate at day 0. PRP /PPP from healthy donor, with 1% FCS as the diluents media. Proliferation measured as fluorescence of Alamar blue dye.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP
EXP 1	79653.0	36789.0	25123.0	24137.33	16332.70	1359.0
EXP 2	73207.0	31000.0	29876.0	28230.0	11993.67	2211.0

Table represents the mean value of three readings for proliferation assay of NEB-1 cell following each experiment (EXP), with experiment done in triplicate at day 5. PRP /PPP from healthy donor, with 1% FCS as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	2.633186	1.173680	0.8405799	0.8046313	0.5268613	0.1107161	0.04528792
EXP 2	1.747907	0.7954429	0.7887844	0.6848285	0.2925285	0.1333333	0.05495762

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of NEB-1 cell proliferation assay from PRP/PP from healthy donor with 1% FCS as the diluent media

RAW DATA FOR HUMAN UMBILICAL VEIN ENDOTHELIA CELL (HUVEC) PROLIFERATION ASSAY

Cell density / well	Mean fluor at 4hrs	Mean fluor at 16hrs
10000	3450.5	934
40000	6951	6470.25
80000	7236.75	5871
100000	8132.75	6963.25
400000	7646.75	10411
800000	7238	11532

Table represents mean fluroscence of three seperate triplicate reading for standard optimization plot of HUVEC proliferation at 4hrs and 16hrs respectively.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	1351.50	1214.50	1228.1670	1198.50	1236.167	1079.333	1056.0
EXP 2	1351.50	1214.50	1228.1670	1198.50	1236.167	1079.333	1045.0
EXP 3	1491.3330	1589.833	1498.50	1414.50	1527.0	1249.333	1200.0

Table represents the mean value of three readings for proliferation assay of Ea.Hys cell following each experiment (EXP), with experiment done in triplicate at day 0. PRP /PPP from diabetic patient, with 1% FCS as the diluents media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	3081.0	1555.3330	2193.1670	2293.0	1936.0	444.8330	340.0
EXP 2	4355.8330	3340.8330	8071.1670	4408.80	5150.0	957.6670	579.0
EXP 3	9174.50	913.16670	7099.1670	6744.330	7340.1670	98.0	89.0

Table represents the mean value of triplicate readings for proliferation assay of Ea.Hys cell following each experimental (EXP), with experiment done in triplicate at day 5. PRP /PPP from diabetic patient, with 1% FCS as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	2.497878	2.418869	2.907276	2.933071	2.083313	1.389751	1.361212
EXP 2	3.273142	5.271666	5.391776	4.372150	3.377024	0.9713662	0.8701304
EXP 3	1.400537	3.036481	1.085896	1.139566	0.1125828	0.5481893	0.4247505

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of Ea.hys cell proliferation assay from PRP/PPP from Diabetic patient with 1% FCS as the diluents media.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	1218.1670	1308.3330	1362.1670	1310.3330	1381.50	1418.0	1243.50
EXP 2	1496.1670	1389.8330	1498.50	1414.50	1527.0	1449.330	1244.8330
EXP 3	993.66670	1031.8330	984.0	1024.50	954.8330	869.8330	1252.500

Table represents the mean value of three readings for proliferation assay of Ea.Hys cell following each experiment (EXP), with experiment done in triplicate at day 0. PRP /PPP from healthy donor, with 1% FCS as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	3042.8330	2725.6670	3995.330	3809.50	3341.6670	1770.6670	1692.6670
EXP 2	4897.1670	4693.50	6551.6670	7626.67	8049.8330	1407.8330	2783.1670
EXP 3	1391.6670	116.16670	1121.3330	1112.50	1899.3330	476.8330	532.00

Table represents the mean value of three readings for proliferation assay of Ea.Hys cell following each experiment (EXP), with experiment done in triplicate at day 5. PRP /PPP from healthy donor, with 1% FCS as the diluents media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	2.497878	2.418869	2.907276	2.933071	2.083313	1.389751	1.361212
EXP 2	3.273142	5.271666	5.391776	4.372150	3.377024	0.9713662	0.8701304
EXP 3	1.400537	3.036481	1.085896	1.139566	0.1125828	0.5481893	0.4247505

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of Ea.hys cell proliferation assay from PRP/PPP from healthy donor with 1% FCS as the diluents media.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	1351.50	1214.50	1228.1670	1198.50	1236.1670	1079.3330	1222.0
EXP 2	1351.50	1214.50	1228.1670	1198.50	1236.1670	1079.3330	1190.0
EXP 3	1491.3330	1589.8330	1498.50	1414.50	1527.0	1249.3330	1340.0

Table represents the mean value of three readings for proliferation assay of HUVEC cell following each experiment (EXP), with experiment done in triplicate at day 0. PRP /PPP from diabetic patient, with 1% EMG as the diluent media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	3081.0	1555.3330	2193.1670	2293.0	3936.0	444.8330	234.0
EXP 2	3355.8330	3340.8330	5071.1670	4408.830	5150.0	957.6670	456.0
EXP 3	7174.50	913.16670	5099.1670	6744.330	7340.167	98.0	80.00

Table represents the mean value of three readings for proliferation assay of HUVEC cell following each experiment (EXP), with experiment done in triplicate at day 5. PRP /PPP from diabetic patient, with 1% EMG as the diluents media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	2.279689	3.184036	1.913225	1.785724	1.280636	0.4121372	0.1914894
EXP 2	2.483043	4.166104	3.678626	4.129053	2.750789	0.8872764	0.3831933
EXP 3	4.810797	4.806920	4.767998	3.402848	0.574379	0.07844186	0.05970149

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of HUVEC cell proliferation assay from PRP/PPP from diabetic patient with 1% EMG as the diluents media

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	1218.1670	1308.3330	1362.1670	1310.330	1381.50	1418.0	1243.50
EXP 2	1496.1670	1389.8330	1498.50	1414.50	1527.00	1449.330	1244.8330
EXP 3	993.66670	1031.8330	984.0	1024.50	954.8330	869.8330	1252.50

Table represents the mean value of three readings for proliferation assay of HUVEC cell following each experiment (EXP), with experiment done in triplicate at day 0. PRP /PPP from healthy donor, with 1% EMG as the diluents media. Proliferation measured as fluorescence of Alamar blue dye

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	3042.8330	2725.6670	3995.3330	3809.50	3341.6670	1770.6670	1692.6670
EXP 2	4897.1670	4693.50	6551.6670	7626.667	8049.8330	1407.8330	2783.1670
EXP 3	1391.6670	116.16670	1121.3330	1112.500	1899.3330	476.8330	532.0

Table represents the mean value of three readings for proliferation assay of HUVEC cell following each experiment (EXP), with experiment done in triplicate at day 5. PRP /PPP from healthy donor, with 1% EMG as the diluents media. Proliferation measured as fluorescence of Alamar blue

Exp Repaets	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP	unaPPP
EXP 1	2.497878	2.418869	2.907276	2.933071	2.083313	1.037142	0.5570301
EXP 2	3.273142	5.271666	3.270885	2.370148	1.938003	0.9713662	0.4684701
EXP 3	1.400537	3.036481	1.085896	1.130589	0.1125828	0.5481893	0.4247505

Table represents N- fold increase (derived from day 0 and day 5 measurement above) of HUVEC cell proliferation assay from PRP/PPP from healthy donor with 1% EMG as the diluents media

RAW DATA FOR SCRATCH ASSAY WITH HEK AND NEB-1 CELL

Exp Repeat	Cont 1%	PRP 5%	PRP 10%	PPP
Exp1	404.6033	457.4217	451.0634	384.9325
Exp 2	360.7825	223.6500	319.6775	186.1067
Exp3	225.5800	457.4217	364.0550	425.3258

Table represents triplicate reading from each expriemental repeats with NEB-1 cell after been treated with mitomycin. The wound gap was measured in micron meter (µm).

Exp Repeat	Cont 1%	PRP 5%	PRP 10%	PPP
Exp1	108.5863	95.1557	147.0213	65.6550
Exp 2	225.5800	457.4217	364.0550	425.3258
Exp3	332.1392	213.5884	208.2942	105.9109

Table represents mean triplicate reading from each experimental repeats with NEB-1 cell without mitomycin treatment. The final wound gap was measured in micron meter (µm).

Exp Repeat	Cont 1%	PRP 5%	PRP 10%	PPP
Exp1	53.0	230.17830	139.7550	117.86250
Exp 2	263.25050	298.30740	317.14620	296.12330
Exp3	365.95170	396.99670	411.74720	363.97330

Table represents triplicate reading from each experimental repeats with HEK after been treated with mitomycin. The wound gap was measured in micron meter (μm).

Exp Repeat	Cont 1%	PRP 5%	PRP 10%	PPP
Exp1	53.52750	190.17830	139.75500	117.86250
Exp 2	133.66620	122.56170	130.33940	89.88889
Exp3	133.66620	122.56170	130.33940	89.88889

Table represents mean triplicate reading from each experimental repeats with NEB-1 cell without mitomycin treatment. The final wound gap was measured in micron meter (μm).

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP
EXP 1	30.31929	156.52	122.7914	50.62143	45.71714	26.90214

Table represents mean triplicate reading from each experimental repeats with HDF cell without mitomycin treatment. The final wound gap was measured in micron (µm).

RAW DATA FROM TRANSWELL MIGRATION ASSAY

Exp Repeat	Cont 1%	PRP 25%	PRP 50%	PPP
Exp1	711.33330	1445.0	1326.0	1535.3330
Exp 2	405.00	706.0	641.66670	709.33330
Exp3	405.33330	1035.0	643.66670	376.33330

Table represents mean triplicate reading from each experimental repeats with EA.hys cell in transwell migration assay. Migration cell was quantified with Alamar blue cell quantification technique (measured as fluorescence of alamar blue in the redox reaction)

Exp Repeat	Cont 1%	PRP 25%	PRP 50%	PPP

Exp1	**61.580540**	**18.508810**	**19.371920**	**17.063610**
Exp	**58.054260**	**29.910090**	**26.557900**	**24.096190**
Exp3	**1.442500**	**2.930200**	**2.688900**	**3.113400**

Table represents mean triplicate reading from each experimental repeats with NEB-1 cell in transwell migration assay. Migration cell was quantified with Alamar blue cell quantification technique (measured as % absorbance of alamar blue in the redox reaction)

Exp.Repeats	**Cont 1%**	**PRP 5%**	**PRP 10%**	**PRP 25%**	**PRP 50%**	**PPP**
EXP 1	**208.833300**	**538.00**	**422.166700**	**251.00**	**203.500**	**174.50**
EXP 2	**228.500**	**455.666700**	**252.00**	**239.16670**	**213.83330**	**179.0**
EXP 3	**162.833300**	**430.666700**	**202.00**	**95.666660**	**80.666660**	**109.0**
Exp 4	**289.545400**	**960.8700**	**799.636400**	**730.63640**	**196.27270**	**146.090**

Table represents mean triplicate reading from each experimental repeats with HUVEC in transwell migration assay. Migration cell was quantified with Alamar blue cell quantification technique (measured as fluorescence of alamar blue in the redox reaction)

RAW data for angiogenesis ASSAY

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP
EXP 1	73.4	105.0	71.6	57.75	56.350	10.20
EXP 2	81.0	96.4	61.4	71.80	52.550	13.40
EXP 3	70.2	69.2	102.6	59.55	58.947370	14.15

Table represents the mean value of six separates analysis of each micrograph from an angiogenesis well plate for Nodal formation during HUVEC angiogenesis. Each experimental was performed in triplicate.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP
EXP 1	42.50	59.625	41.6	25.2	23.850	2.55
EXP 2	46.30	58.750	33.1	28.5	24.100	4.25
EXP 3	40.20	50.000	40.0	26.7	24.738	6.05

Table represents the mean value of six separates analysis of each micrograph from an angiogenesis well plate for Mesh formation during HUVEC angiogenesis. Each experimental was performed in triplicate.

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP
EXP 1	6963.8710	9198.7700	6234.3160	4446.9350	9170.540	2422.1580
EXP 2	8526.4220	9033.3850	5449.4870	5170.5400	7070.7710	3207.1230
EXP 3	6905.5560	8228.2060	8444.0880	9217.9000	1004.5460	4119.0220

Table represents the mean value of six separates analysis of each micrograph from an angiogenesis well plate for skeletal length measurement (measured in micron) during HUVEC angiogeneis. Each experimental was performed in triplicate

Exp.Repeats	Cont 1%	PRP 5%	PRP 10%	PRP 25%	PRP 50%	PPP
EXP 1	203375.40	294780.40	472445.90	550849.10	613908.30	3095.720
EXP 2	293639.30	303633.30	393469.10	670918.50	459896.80	6499.1820
EXP 3	297071.90	353114.70	380821.50	543891.50	614472.90	4929.6940

Table represents the mean value of six separates analysis of each micrograph from an angiogenesis well plate for cellular are cover (measured in micron square) during HUVEC angiogeneis. Each experimental was performed in triplicate.

Quantification of microvessel density from biopsied chronic diabetic wound using CD 31 through immunonistochemistry technique (Graphic representation in Chapter 5)

Patient's ID	Day 0	Day 8	Week 4	Week8
P1	77.25	60	44	23
P2	51.25	40.5	35.5	29
P3	47	35	31.5	28.75

Table represent the mean mircovessel density from 4 separates counts under rectangular area per high power fields. P1-P3 represents patients that achieved complete re-epithelisation

Patient's ID	Day 0	Day 8	Week 4	Week 8
P4	26.75	24.75	20.25	15.75
P5	25.25	20.25	18	14
P6	39.25	31.0	27.5	nil
P7	29.5	26.75	nil	nil

Table represents the mean microvessel density from 4 separates counts under specified rectangular area per high power fields. P4—P7 represents patients that were unable to achieved complete re-epithelisation. Patients P6 and P7 dropped out of the study before the nd of the 16 weeks follow up.

Biochemical parameters of the Diabetic patients with chronic foot ulcers treated with PRP Gel

Patients ID	Age	Gender	HB g/dl	WBC	PLT count	PLT conc	WBC in PLT conc
P1	45	M	10.9	10.1	310	1210	31.1
P2	58	M	13.7	9.4	120	1100	20.5
P3	59	M	12.7	10.3	358	2300	30.4
P4	66	M	12.2	6.4	280	1230	16.9
P5	68	M	13.1	12.1	323	2430	49.9
P6	53	M	7.4	10	293	1500	34
P7	50	M	12.6	9.1	273	2757	23.6

Table represents biochemical parameters from patients before commencing treatment, M- male, HB – Haemoglobin (g/dl), WBC –white cell count (x1000/ml), PLT- platelet count (x1000/ml), PLT conc- Platelet count after concentration through the platelet separate and concentrating system, WBC in PRP conc- white cell count in concentrated Platelet.

Patient's ID	day 0	day 8	Wk2	Wk4	Wk6	Wk8	Wk10	Wk12	Wk14	Wk16
P1	4.6	4.8	4.4	2.9	1.7	1.6	1.2	0.4	0.2	0
P2	32.1	25	24.7	19.2	26	19	6.8	4.1	1.7	1.2
P3	14.3	13.8	13	18.2	19	16	16	10.3	9.8	9
P4	2.5	2.6	2.8	2.3	2.6	3.3	3.5	3.2	1.4	4.1
P5	1.8	1.3	3	3	2.4	2.8	2.4	3.3	2.5	2.7
P6	4.6	3.6	3.5	3.7						
P7	16.6	16.2	15.8							

Table represents measurement of wound area (cm^2) using computerized wound tracer (vistrak). Graphic representation can be found in Chapter 6. Patients P 6 and P7 dropped out of the study before the end of the 16 weeks follow up.

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Application of Autologous Derived-Platelet Rich Plasma Gel in the Treatment of Chronic Wound Ulcer: Diabetic Foot Ulcer

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Abstract: The treatment of chronic wounds remains problematic, despite new insight into the cellular and molecular basis of wound healing. Although the aetio-pathogenesis of chronic wounds is said to be multi-factorial, it is evident from literature that effective and adequate wound debridement has produced the most consistent effect in chronic wound treatment. There is a growing body of evidence that suggests that wound healing in chronic diabetic foot ulcers is growth factor dependent and that the therapeutic delivery of these growth factors to wounds topically, has the potential ability to accelerate wound healing in conjunction with conventional wound care. Autologous derived platelet concentrate is activated to release growth factors that are stored in the platelet granules. These secretory proteins include cytokines and growth factors such as transforming growth factor–beta, vascular endothelia growth factor, platelet derived growth factor, and so on. The enhancement of soft tissue healing by the application of autologous derived platelet rich plasma gel (APG) is supported by basic science and some clinical studies. This review article will attempt to provide a concise report of current concepts on the use of APG in treating chronic ulcers.
Keywords: autologous derived platelet rich plasma, diabetic foot ulcer, growth factors, wound healing. *JECT. 2010;42:20–29*

Chronic wounds pose a major management problem in the health sector often requiring a multi-disciplinary approach. The majority (90%) of chronic wounds result as a progression from diabetic wounds, venous ulcers (chronic venous insufficiency ulcers), and pressure sores. Diabetic foot ulcers (DFU) seem to account the most for the global burden of the disease, especially with the attending complications of recurrence, chronicity, and the resultant lower limb amputation (1).

There is a global increase in the prevalence of diabetes mellitus, particularly type II diabetes mellitus, which is directly linked with obesity and a sedentary life style. It is believed that 20% or more of the United Kingdom adult population over the age of 65 years suffer with type II diabetes mellitus (2). Two studies from North European countries reported the annual incidence of foot ulcers in the general population to be just more than 2% (3). The annual incidence rates in diabetic neuropathic individuals vary from 5–7% (4). The lifetime risk of a person with diabetes having a foot ulcer could be as high as 25% (5). Of this population, 15% will either suffer foot or limb amputation (5). It is likely that the cumulative lifetime incidence of foot ulcers may be as high as 25% (5). Shearer et al. confirmed that diabetic patients with neuropathic risk factor incur five times more direct medical costs for ulcers and amputations, and live for 2 months less than individuals without neuropathy (6). Lower limb amputation is performed 15 times more frequently among diabetics, as compared with non-diabetic patients (7).

The American Diabetic Association did a cost analysis of medical expenditure for the United States population with and without diabetes in 2002, which was based on national health care survey data. The direct and indirect expenditure attributable to diabetes was estimated at 132 billion U.S. dollars. The direct medical expenditure alone totaled 91.8 billion U.S. dollars (23.2 billion U.S. dollars for diabetic care, 24.5 billion for chronic complications, and 44.1 billion U.S. dollars for excess prevalence of general medical conditions). The attributable indirect expenditure

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resulting from lost workdays, restricted activity days, mortality, and permanent disability due to diabetes totaled 39.8 billion U.S. dollars (8,9).

Fundamentally, the development of diabetic foot ulcers and the resultant chronicity could be said to have resulted from peripheral vasculopathy, peripheral neuropathy, and immunopathy. There are other contributory factors which stem from these three major risk factors. These include repetitive trauma to areas of the foot exposed to moderate to high pressure, reduced resistance to infection, immunologic disturbances, and microcirculation alterations.

Different authors and researchers have attempted to classify diabetic wounds from the view point of aetiopathogenesis and failure to demonstrate significant improvement following good clinical care as a guide to determine poor outcome. Presently, there are no early predictive factors to guide clinicians to differentiate patients who will heal readily from those who will have a protracted course of treatment. The American Diabetic Association have attempted to offer a lasting solution by clarifying the definition of a chronic wound, which they defined as "failure to show continuous progress towards healing. These are wounds that remained unhealed after 4 weeks, were a source of concern and were associated with worse outcomes, including amputation" (10). The decision to change to advanced therapeutic intervention should not be based solely on timing, but also in conjunction with careful monitoring of the percentage wound closure. A reduction in wound area of 10–15% per week represents a normal healing process and should not necessitate a change in wound management strategy or re-evaluation (11).

Other authors have demonstrated that chronic diabetic wounds lack significant growth factors which are stimulatory agents for wound healing. These growth factors play important roles in the tissue-remodeling phase through mesenchymal cell recruitment and extra matrix synthesis. This understanding has led to the development of recombinant platelet derived growth factor-BB (becaplermin) that has been approved for use and has shown a degree of success in treating DFU (12). Some authors have shown improved rate of wound healing in acute and chronic wounds following the use of autologous platelet rich plasma gel (Table 1). This review article aims at examining the role of the delivery system of multiple growth factors in healing chronic diabetic foot ulcers.

Aetio-Pathogenesis of Diabetic Foot Ulcers

The physiological alteration in diabetic foot ulcers could partly explain the chronicity and/or the recurrence of the ulcers. The triad of neuropathy, vasculopathy, and immunopathy forms the cardinal point on which the chronicity of diabetic wounds rest (1,13). The neuropathic effect on the foot produces a biomechanical abnormality, which could predispose areas of concentrated pressure to significant risk of ulceration, repetitive trauma, vasodilatation, and decreased sweating (14). Atherosclerotic plaque deposit contributes to the development of diabetic peripheral vasculopathy resulting in either occlusion or stenosis of the vessels. This causes decreased blood flow and ultimately ischemia will ensue. Infection has more deleterious effect in this group of patients, whose impaired immunity increase their risk for local and systemic infections. The wound infections may either be superficial or deep and are commonly poly-microbial in etiology. Superficial wound infections may be treated with antibiotic therapy only whilst deep-seated wound infections might require surgical debridement which may be followed by antibiotic and or other wound care adjunct.

Physiological Changes in Diabetic Foot Ulcers

Aggressive sharp wound debridement is believed to convert chronic wounds to acute ones and allows growth factors to function more effectively. This allows the wound to progress through the normal phases of wound healing. Regular and frequent wound debridement removes collagenase, matrix metalloproteinase, and elastase which are inhibitors of wound healing (15,16).

The cellular and molecular alteration has a direct link with the major physiological changes mentioned earlier and there is evidence to support hyperglycaemia—related

Table 1. Summary of clinical studies investigating the use of PRP and PPP in treating chronic non-healing wounds.

Authors	Study Design	Results
Knighton DR, et al.	32 patients, prospective randomized, controlled, blinded, crossover study	By 8 weeks, 81% of treated group had 100% epithelialization, 15% of the controls did ($p < .0001$); All of the remaining 75% in the placebo group achieved complete healing after crossover (57).
Ganio C, et al.	Conventional treatment of patients, 171 patients presented with 355 wounds of average duration of 75 weeks	100% epithelization achieved at 10 weeks, with 78% limb salvage rate for at-risk patients (58).
Driver, et al.	RCT, blinded, multicentre study involving 72 patients treated over 12 weeks	13 of 19 patients treated with PRP had total re-epithelization; 9 of 21 of the control (42%) also re-epithelized (65)
Yuan, et al.	A plot study of 13 refractory diabetic dermal ulcers were treated with PRP	69.2% were cured; significant reduction in ulcer size after 3 weeks of initial treatment

deleterious molecular and cellular alteration in the cellular environment. Hyperglycemic non-enzymatic glycosylation has an inhibitory effect on structural and enzymatic proteins. In addition, glycosylated collagen is resistant to enzymatic degradation and less soluble than the normal protein products, which makes connective tissue inelastic (17). High deposition of sorbitol has been implicated in diabetic vasculopathy. This is associated with increase in dermal vascular permeability and results in peri-capillary albumin deposition (18), which impairs oxygenation and nutrient distribution (19). Bennett et al. demonstrated increased destruction of growth factors by elevated levels of proinflammatory cytokines and metallo-matrix proteins following repeated trauma and infection (19,20).

Most chronic wounds are characterized by increased protease levels, particularly matrix metalloproteinase's (MMPs) and neutrophil elastases. Furthermore, tumor necrosis factor–alpha (TNF-alpha) has been shown to increase the production of MMPS, whilst inhibiting the production of tissue inhibitory metalloproteinase (21,22). The response mounted from the host immune system following critical wound colonization by bacteria is followed by polymorph nuclear leukocyte reaction that releases protease and oxidant species. This is followed by degradation of cytokines and extra-cellular matrix which distort the internal cellular milieu, thereby contributing to the non-healing of wounds. The cellular response to injury is poorly coordinated in diabetic wounds, most of which are complex and inter-related biochemical activities.

Molecular Mechanisms Involved in Chronic Wounds

The understanding of the molecular mechanisms involved in chronic wounds holds the key to solving the huge problem posed by this disease entity. Several efforts made at understanding this process have been undermined by the absence of an easily reproducible animal model that mimics the human chronic wound. Keratinocyte biology attempts to explain the mechanism involved in chronic wound healing through cellular injury. Keratinocytes are the first cells to respond to injury; they are programmed to maintain skin integrity based on their specific attachment to extracellular matrix and vice versa. The absence of normal migration and proliferation of cells caused by failure of keratinocyte activation during wound healing results in non-healing (23). The activation of C-myc affects epidermal biology directly and its relevance to wound healing. C-myc is required for transition from the G1 to S1 phase of the cell cycle and it also promotes proliferation of transit amplifying cells. Deregulation of c-myc results in depletion of epidermal stem cells thereby making the tissue non-responsive to injury (24–26).

Stojadinovic and colleagues discovered that the stabilization of β-catenin (a subtype of keratinocyte adhesion molecule) inhibits keratinocyte migration and wound healing of human skin in organ culture (26). This is done through different mechanisms: (a) activation of c-myc, (b) blocking of epidermal growth factor (EGF) effects and by synergizing with glucocorticoids. β-catenin also participates in glucocorticoid signaling and repression of keratin genes, which are involved in cytoskeletal network and keratinocyte migration. The main features of chronic wound such as persistent inflammation, decreased activity of growth factors, and angiogenesis may be affected by the activities of β-catenin and c-myc. Other authors suggested that c-myc was found to inhibit the expression of platelet derived growth factor (PDGF)-BB and its receptors (28), whereas fibroblast growth factor (FGF)-Basic and EGF were shown to induce c-myc expression (29,30). In contrast to their stimulatory effects, these growth factors may also stimulate negative feedback in chronic wounds by sustaining c-myc expression.

Physiology of Platelets in Wound Healing

Platelets are formed from megakaryocytes and are synthesized in bone marrow by pinching off pieces of the cytoplasma. Platelets are small discoid blood cells, with the average platelet count ranging from $1.5–3.0 \times 10^{-5}$/ML of the circulating blood, and with an average half-life of about 7 days. They have a trilaminar cell membrane with a glycoprotein receptor surface overlying and are partially interspersed with a penetrating bilayer of phospholipids and cholesterol. Platelets lack nuclei but contain organelles and structures such as mitochondria, microtubules, lysosomes, and granules (α, δ, λ). The cytoplasma contains an open canalicular system that increases the effective surface area for intake of stimulatory agonists and the discharge of effectors' secretions. The dense granules contain adenosine diphosphate adenosine triphosphate, serotonin, and calcium. The α-granule contains clotting factors, growth factors, and other proteins. Platelets are normo-thrombogenic and require a trigger, before they become potent and active in hemostasis and wound healing. They are found intravascularly and are concentrated in the spleen.

Hemostasis is the balanced interaction of platelets, endothelia cells, and plasma clotting proteins. Platelets are responsible for initiating the immediate and most important step in coagulation. Platelet adhesion occurs through the interaction of the damaged vessel wall and exposes the subendothetial collagen, which binds with von Willebrand factor, with a resultant change in the platelet structure, thus, making it possible for its adherence to blood vessels. This effect is accomplished through the activities of glycoprotein 1b, and 11b/111a receptors on the platelet membrane. After aggregation, the granular contents are then released; serotonin probably assists with vasoconstriction. Adenosine diphosphate (ADP) promotes release of granules, which further encourages platelets aggregation.

Phospholipase A_2, when activated, results in release of arachidonic acid, which is converted into thromboxane A_2, resulting in further platelet aggregation and growth factor release. The process described attempts to produce a platelet plug—the primary hemostasis. The platelet plug acts as a barrier to micro-organism invasion of wounds, with the help of highly concentrated leukocyte buffer. The clotting cascade is also activated along with the complement system, producing the secondary hemostasis with a resultant fibrin network formation. Degranulation of α-granules results in the fusion of platelet cell membrane during which some of the secretory proteins (e.g., PDGF) are transformed to a bioactive state by the addition of histones and carbohydrate side chain (52). These active proteins then bind to the transmembrane receptors of target cells such as mesenchymal stem cells, osetoblast, and fibroblast, endothelial and epidermal cells. Once bound to the transmembrane receptors, intracellular signal proteins are activated, resulting in expression of gene sequence that directs cellular proliferation, matrix formation, and initiation of collagen synthesis (52). These proteins are secreted within 10 minutes of clotting, with more than 95% of the pre-synthesized growth factors secreted within 1 hour.

Platelet Derived Growth Factors in Wound Healing

Wound healing is a well-orchestrated and complex series of events involving cell-cell and cell-matrix interactions, with growth factors acting as messengers to regulate the various processes. The role of growth factors in wound healing has been well described in acute wound healing; growth factors are stored in the form of α-granules in platelets and when these platelets are activated, they in turn release a multiplicity of growth factors. After the formation of platelet coagulum, the activated platelets are interspersed among the fibrin strands forming a matrix within the clot, which helps to keep the growth factors within the mesh. They eventually diffuse out into the surrounding tissue. Growth factors act locally to recruit undifferentiated cells to the injury site by chemoattraction and also stimulate mitosis in the undifferentiated cells. Stem cells are attracted to areas of high concentration of growth factors and cellular movement occurs by forming attachments to the matrix/scaffold. Growth factors attach to receptors on the stem cell membrane, thereby activating genes controlling cell division. They also attach to cell receptors and control the genetic expression of stem cells via the modulation of signal transduction pathways of secondary proteins, resulting in cellular division and differentiation. Mitosis occurs via a signal transduction pathway through the tryosinase kinase located on the cell membrane. Receptor activation leads to the activation of secondary messenger proteins, which enter the cell nucleus and influence the expression of the genes responsible for triggering mitosis, angiogenesis, and macrophage activation (31).

The growth factor that was first identified was the platelet derived growth factor, which has other isomers (αα, ββ, αβ). PDGF has been synthesized through recombinant DNA technology and has been used topically for treating chronic diabetic foot ulcers. The first efficacy study documented showed complete healing of non-ischemic foot ulcers in 25% of the placebo-treated group and 48% in the PDGF-treated group ($p < .01$) (32). The second efficacy study found complete healing in 35% of the placebo-treated group, and 50% of the PDGF-treated group, which represented a 43% improved healing ($p < .007$). The PDGF-treated group also showed a reduced healing time by 6 weeks ($p < .013$) (33). Other identifiable growth factors include transforming growth factor (TGF-β), which has β1 and β2 as its isomers; platelet derived angiogenesis factor; platelet derived epidermal growth factor; fibroblast growth factor; keratinocyte growth factor; insulin like growth factor; interleukin-1; vascular endothelial growth factor; epidermal growth factor; osteocalcin; osteonectin; fibrinogen; and fibronectin. The summary of the roles of the super-family growth factors in wound healing cascade is shown in Table 2. Growth factors have special roles they play at different phases of the wound healing, and they also appear to have synergistic effects on one another. The other groups of growth factors not discussed in the table are collectively called the adhesive proteins. These include fibrinogen, fibronectin, and thrombospondin. They are known to participate in thrombus formation and some mitogenic action (55). More recently, Kubota et al. (49) described a new platelet growth factor known as connective tissue growth factor (CTGF). They showed in their experiment that CTGF is released after the activation of platelet rich plasma. They are said to be expressed along with the platelet coagulation process. The expression of CTGF following autologous derived platelet rich plasma gel (APG) activation is said to be more than a 20-fold increase when compared with any other platelet growth factors.

Rationale for the Use of Autologous Platelet Gel

In the late 1970s the importance of growth factors within the wound-healing cascade was first identified. These began with platelet-derived growth factor and the subsequent identification of many other growth factors now known to be important within the different stages of the wound healing cascade. These growth factors aid the three phases of wound healing; inflammatory, proliferative, and remodeling phases. These phases involve complex paracrine mediated growth factors which influence mitogenic and cellular differentiation activity.

Chronic wounds are thought to have increased proteases, increased proinflammatory cytokines, decreased protease inhibitors, and decreased growth factor activities. Cooper et al. showed that a number of growth factors were markedly reduced in wound fluid from chronic wound as compared

Table 2. Growth factor super-families and their role in wound healing.

Super-family	Member	Discussion
Platelet-Derived Growth Factor	PDGF	PDGF has three isoforms; PDGF-AA, AB, BB. PDGF affects cells of mesodermal origin. PDGF has its primary effect on these cells and could also be secreted from other polymorph nuclear cell cells, fibroblast, and smooth muscle cells. Its roles include chemotaxis, proliferation, and new gene expression in these cells (34).
	Vascular endothelia growth factor (VEGF)	Its receptors are exclusive to the endothelial cells and it acts as an effective mitogen during angiogenesis. FGF-4, PDGF, Tumor necrosis factor (TNF)-α, Insulin-like growth factor (IGF), and some interleukins stimulate VEGF production and others inhibit it (35).
Epidermal Growth Factor	EGF	EGF is released after degranulation of platelets. Most cells have receptors for EGF but epithelial cells have the largest number of receptors. Other sites for the receptors include endothelial cells, fibroblasts, and smooth muscle cells. EGF is chemotactic in nature, stimulates angiogenesis and collagenase activity and acts as a potent mitogenic stimulant for epithelial cells (36,37).
	TGF-α	TGF-α has 30% structural homology as EGF. It is produced by activation of macrophages, platelets, and keratinocytes. It stimulates mesenchymal, epithelial, and endothelial cells via chemotactic effect (36,37).
Fibroblast Growth Factor	aFGF, bFGF	FGF has 2 different forms; the acidic and basic, both have 50% homology. Both forms stimulate endothelial cell proliferation and motility, thereby contributing to wound angiogenesis. bFGF is 10 times more potent as an angiogenic stimulator. Other stimulatory effects include collagen synthesis, wound contraction, epithelialization, and fibronectin and proteoglycan synthesis (36,38).
	Keratinocyte growth factor (KGF)-1, KGF-2	High quantity of KGF is produced after tissue damage, which is mainly produced by fibroblast. KGF-1 is the most potent mediator of keratinocyte proliferation and motility. KGF-2 has been shown to increase granulation tissue formation by directly stimulating the migration of fibroblasts into wounds. Glutathione peroxidase, a DNA repair enzyme, protects the damaging effect of reactive oxygen species, which are released into the wound by neutrophils (39,40).
Transforming Growth Factor	TGF-β1, β2, β3	TGF-β has been isolated from platelets, macrophages, lymph-cytes, bone, and kidneys. It is also released by platelet after degranulation. It stimulate monocytes to secrete other growth factors (FGF, PDGF, TNF- α, and IL-1). TGF is chemotactic for macrophages and regulates its own production within the macrophages in an autocrine fashion. It also stimulates fibroblast chemotaxis and proliferation. The presence of other growth factors and the variability in concentration of TGF-b could modulate the role of TGF-b. This is either an inhibitory or stimulatory effect on cellular proliferation. TGF-b stimulates fibronectin, proteoglycan synthesis by fibroblast. It organizes extracellular matrix and may be involved in scar remodeling and contracture. It also stimulates epithelial cell proliferation and inhibits endothelial cell proliferation, but with a co-factor it will stimulate angiogenesis (36,41–46).
Insulin Growth Factor	IGF-I, IGF-II	IGF-II is most prominent during fetal development, where-as IGF-I persists throughout life and is synthesized in the liver, heart, lung, kidney, pancreas, brain, and muscle. IGF is stimulated by human growth hormone (especially in the liver) and the two together stimulate skeletal cartilage and bone growth. Degranulation of platelets will also produce IGF-I and the stimulation of fibroblasts. IGF-I is a potent chemotactic agent for endothelial cells, and is also involved in neo-vascularization. It may also act with PDGF to enhance epidermal and dermal growth. The effect of IGF-I on wound healing depends on the amount of available free IGF-I (47,48).

with acute wounds (50). Also, FGF and TGF-β concentrations are down regulated in chronic wounds and are significantly lower when compared with acute wounds (50). It has also been shown that the use of a synthesized single growth factor in treating chronic diabetic wounds has only produced a limited degree of success. With the recent understanding of bio-tissue engineering, autologous derived platelet rich plasma gel can effectively and safely deliver all the needed growth factors to stimulate tissue reparation. The rationale for employing this technique is to mimic the normal physiological wound healing and reparative tissue process.

Autologous derived platelet gel (APG) has a wide and safe application within the post operative surgical field both as a wound sealant and a tissue repair agent. Its application has extended to patients that are prone to higher surgical complications, and this is also true of diabetic patients. The modification of cell saver technology has made it possible to synthesize AGP with structural and functional properties, as close as possible, to those of natural soft tissue and epidermis. Besides, the ability of AGP to deliver multiple growth factors with a synergistic effect to wound sites, the clot formed from AGP serves as a scaffold and a protein reservoir, thereby locally concentrating and magnifying their effect. The platelet plug acts as a barrier to micro-organism invasion of wounds, achieved with the help of highly concentrated leukocyte buffer, present within APG. The availability of concentrated leukocyte with about 5-fold the baseline value makes the graft matrix infection free. It also promotes mitogenesis of mesenchymal stem cells at the wound site. As a three dimensional volumetric soft connective tissue replacement, it provides a matrix medium for cell migration, granulation tissue formation, and epithelia wound contracture in addition to serving as a non-disturbed wound patch. The overall efficacy of AGP in treating wounds is likely to be a function of many variables such as the platelets concentration, the volume of AGP delivered to the wound, the extent and the type of injury and perhaps, the overall medical condition of the patient.

AGP contains autologous-derived living cells, which are able to deliver a program of healing to the wound that may not be achieved with a single growth factor. Another added advantage of autologous preparation technique is the inherent safety and therefore freedom from concerns of transmissible disease, such as, human immunodeficiency virus, hepatitis, and Creutzfeldt-Jakob disease.

Preparation of Platelet Rich Plasma Gel

Platelet rich plasma gel (PRP) can be prepared either through standard blood banking techniques or through a point of care device. The blood bank techniques are more costly, highly controlled logistic systems, to prevent mismatch. However, the point of care device does not require large predonated blood volumes. The tabletop centrifuge system has been used to manufacture smaller volumes of PRP from lesser amounts of whole blood (50–150 mL). With the advent of small compact office devices, 6 mL of platelet-rich plasma could be prepared from 45–60 mL of blood (54). This in a way obviates the need for re-transfusion of the remaining blood products (54). All the different devices available, such as Smart PReP (Harvest Technologies Corp., Norwell, MA), angel (Sorin Group, Arvada, CO), and the Magellan (Medtronic, Minneapolis, MN.), operate on a small volume drawn and on the principle of centrifugation. They all differ widely in their ability to collect and concentrate platelets, but they are able to increase the PRP concentration to about 2-fold to 8-fold of their original platelet concentration (51,54). It is expected that the concentration of the released growth factors would be linearly proportional to the platelet concentration ratio. However, some authors have experienced a little variation in this relationship. Marx (52,54) states that a "working definition" of PRP should be 1,000,000 platelets/μL. Marx states that lesser concentrations of platelets were unable to demonstrate healing properties. However, greater concentrations have not yet been shown to be advantageous. In another study by Anitua et al. (55), they state that the aim is to prepare PRP with a platelet count in the excess of 300,000 platelets/μL. Eppley et al. and Weibrich et al. realized little value in using platelet concentration ratio to predict the resultant platelet-rich plasma secretory protein levels (51,53). Selecting the type of device to be used will depend on the type of surgical procedure, the technical expertise available, and the amount of platelet-rich plasma required.

Preparation Methodology

In the clinical setting, the tabletop device or the cell saver may be used. But for the purposes of this review, we shall concentrate on the tabletop device. Blood is drawn from the anticubital vein carefully and gently into a syringe, using a large bore cannula (>17 gauge). It is important to avoid negative pressure whilst filling the syringe, to avoid damaging the platelets and to avoid activating them before processing. The autologous blood is collected in a sufficient amount of anticoagulation citrate dextrose-A solution (ACD-A). A ratio of 1 mL of ACD-A to 7 mL of whole blood should be maintained. The acid citrate dextrose serves to preserve the integrity of the platelet membrane. The aspirated blood is gently agitated by mixing the anticoagulant with the blood. The whole blood is sequestered into a semiautomatic controlled operation mode by centrifugation, separating the platelet-poor plasma (PPP) from the Buffy coat layer and erythrocytes. The PPP volume is separately collected into another bag. The centrifugation continues to obtain the Buffy coat layer consisting of PRP and leukocytes, which is collected into another separate bag. After this procedure, the erythrocytes are also collected into a separate bag. The PPP and erythrocyte are not re-transfused when a table top processing unit is used but could be re-infused in the case of cell saver. Part of the PPP is used in processing the thrombin.

Platelet Rich Plasma Gel Activation

To initiate the release of the growth factors from APG, the platelets have to be activated. The regenerative potential of platelet-rich plasma depends on the level of secretory proteins that are released during the activation process (51). The protein levels will depend on several factors including: (1) patient factor, the concentration of the protein in the platelet, (2) the platelet handling and processing technique, which determines the quality and quantity produced, and (3) the completeness of platelet activation before measurement (51–53). Thrombin, a potent activator will induce immediate platelet growth factor release when added to the PRP. Human thrombin is generated from a commercial kit Activa (Mirandola, Modena, Italy). It is generated after PPP is mixed with the (Activa) beaded material in a pressurized glass syringe for about 20–25 minutes and the thrombin is squeezed out of the glass syringe following application of pressure on the syringe plunger. Autologous thrombin is preferred in the United Kingdom, so as to avoid the potential complication of pre-prepared bovine thrombin, which has been implicated in the development of antibodies to clotting factor Va, XI, and thrombin. Life-threatening coagulopathies have been previously documented following these reactions. However, it is believed that the antibodies developed against factor Va in bovine thrombin has been essentially eliminated in the manufacturing and processing of bovine thrombin (52).

The harvested cellular Buffy coat is combined with thrombin and 10% calcium chloride ($CaCl_2$) for platelet activation to produce the gelatinous material. The $CaCl_2$ antagonises the effect of anticoagulant in the citrate solution present in the pre-donated blood bag. Once PRP is prepared, it remains stable in the non-coagulated state for about 6–8 hours. The PRP produced can be used to the

desired need of the physician; either to fill three dimensional volumetric wounds or as a spray-on graft for superficial wounds (Figure 1). The tissue graft constructed in this manner has a semisolid physical integrity much like bilaminant skin graft. The graft also serves as an occlusive dressing, which could remain on the wound for 5–10 days (Figure 2). Another additional advantage of PRP is that it has been applied on bone and fracture sites, particularly following amputation or removal of dead bone in osteomyelitis. Mixing autologous platelet gel with sequestered autologuos bone graft materials might create a bioengineered graft. This has the potential to support, promote, and accelerate bone healing (51). The quality and integrity of the formed gel appears central to the delivery of its role. The thrombelastography hemostasis analyzer (TEG) and Sonoclot measures the elasticity of a clot as its forms and subsequently degrades naturally. Cassidy et al., in their attempt to determine the structural integrity of formed gel, concluded that TEG is valid for analyzing platelet gel and Sonoclot appears to be an unreliable tool (66).

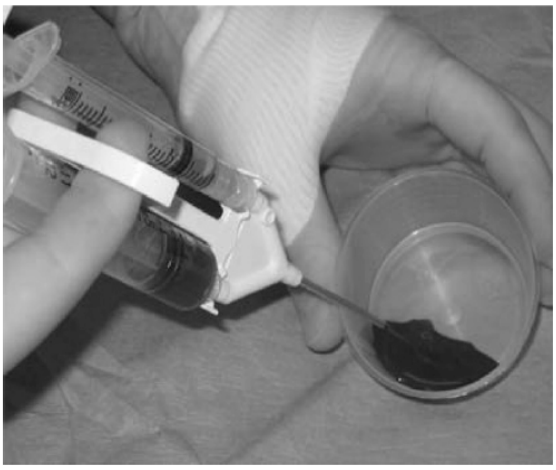

Figure 1. Making of platelet rich plasma gel, a consistent gel that can be used to fill up a three dimensional volumetric wound.

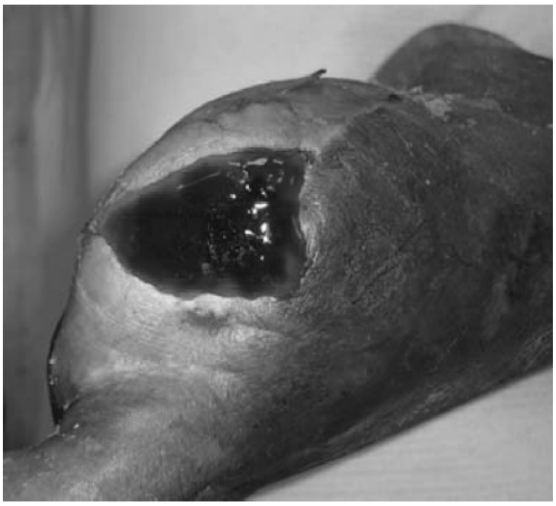

Figure 2. Application of the platelet gel on chronic diabetic foot ulcer after a sharp surgical debridement of a chronic wound.

Pitfalls in Autologous Derived Platelet Rich Plasma Gel Production

Centrifugation forms the basis of the current methods for producing platelet-rich plasma. From the drawn whole blood, platelet fragmentation should be avoided during this process, which could result in the untimely release of high levels of proteins with compromised bioactivity.

A large bore cannula (17G) is recommended for drawing the blood and possibly from a large anticubital vein under no tension. Veno-puncture done under tension could activate the platelet before the centrifugation, which may negate the yield. Low gravity force during centrifugation and minimal platelet activation should be used. Despite all the efforts to prevent activation, bioactive secretory proteins could still be produced properly, but lost during transferring to the surgical bed. This failure is largely due to the delivery technique used. It is important to achieve a good quality gel; the gel serves as a vehicle that delivers the secreted proteins to the wound site. It is imperative to safely transfer the gel to the surgical site and use it within 10 minutes of its production. Otherwise, the gel will retract and lose the originally expressed growth factors. It has been reported that the quality and quantity of the PRP produced is dependent on the quality of the platelet. However, there are other conditions that are relatively contraindicated to the use of PRP, such as pre-existing coagulation defects (thrombocytopenia, hypofibrinogenemia) or potential hypersensitivity to bovine products (64).

Evidence to Support the Clinical Application of Platelet Rich Plasma Gel

Different authors have proposed that platelet-rich plasma technology has relevant application in promoting hard and soft tissue wound healing; and potentially decreasing postoperative wound infections, blood loss, and pain. Platelet-rich plasma has found clinical application in most fields of surgical practice. There are scientific literatures to support its efficacy in periodontal and oral surgery, maxillofacial surgery, aesthetic plastic surgery, heart bypass surgery, orthopedic and spinal fusion surgery, and in the treatment of chronic skin and soft tissue ulcers (51,52,54). Although the various published data appears promising on its efficacy in wound healing, most are case studies or series; hence, most of the evidence is anecdotal. There are very few randomized controlled prospective clinical trials to support the acclaimed potentials. Knighton et al. reported that 17 of 21 patients achieved complete re-epithelization of the

treated chronic lower limb ulcers over an average period of 8.6 weeks, with a course of twice-daily wound treatment with platelet releasate suspended on a collagen base, compared to two of 13 similar wounds treated with placebo (56). After crossover of the placebo group, all the remaining 11 chronic ulcers were treated with the same bio-active agent as the active group. All treated placebo patients achieved 100% re-epithelization in an average of 7.1 weeks (55). Other studies published on the role of platelet-rich plasma in treating diabetic foot ulcers are highlighted in Table 2.

Multidisciplinary Approach to Wound Management and its Future

The complexity and the poor outcome associated with healing chronic diabetic foot ulcers necessitate the need for a multi-disciplinary approach to treating these groups of patients. Most physicians involved with the care of these patients are increasingly advocating for an integrated wound care center. This is aimed at improving the communication amongst the different care givers involved with treating these patients. This center will provide the requisite collaborations for optimal healing, which include in-patient and out-patient care, general medicine, podiatrists, vascular and orthopedic surgeons, diabetologists, nutritionists, radiologists, and neurologists. This center has the potential of galvanizing already available resources, streamlining protocol implementation, reducing cost, and ultimately accelerating wound healing.

Advances in Wound Care

The search for an ideal wound dressing material still remains pivotal to improving the clinical outcome of complex wounds. The ideal dressing needs to ensure that the wound remains moist with exudates but not macerated, and free of infection, excessive slough, toxic chemical, particles, and fibers. It also has to ensure that the wound is at an optimum temperature and pH for healing, and undisturbed by the need for frequent changes (56). The choice of an appropriate local wound dressing material depends on identification of the type of wound that is in question, the phase of the wound healing, the clinical behavior of the wound, such as granulating or epithelializing, the presence of infection, and the conformability of the dressing. The advancement in wound-dressing technology has been directed at solving the problems associated with the non-availability of the ideal dressing material. In the search for the ideal dressing material, many researchers have shown that simulation of the living skin equivalent may hold the key to efficient tissue healing and remodeling (59).

Biological Therapy

Tissue engineering is a biological science that specializes in the development of biological substitutes to restore, maintain, or improve the function of the skin. It seeks to create a readily available tissue replacement with the biologic and pharmacologic properties of the human skin (59). The principle of implementing this method in treating chronic wounds is called biological or cellular therapy. Implementation of cellular therapy is recommended when wound size cannot be decreased by more than 10% within 3 weeks. There are a number of commercially available dermal matrices that have been approved by the United States Food and Drug Administration. This includes Alloderm™ (Life Cell Corp., The Woodlands, TX), altered allograft; Integra™ (Integra Life Science Corp., Plainsboro, NJ), dermal regeneration template; Dermagraft™ (Smith and Nephew, London, UK), synthetic dermal replacement/dermal regeneration template; Apligraf™ (Organogenesis, Inc., Canton, MA), composite skin replacement, and Hyalograft-3D™ (Fidia Advanced Biopolymers, Abano T., Italy).

Biological therapy may be an ideal treatment for diabetic foot ulcers because it adds cells that release growth factors to a growth factor dependent environment, increases cytokines and matrix proteins, and promotes angiogenesis (60). It is also believed that they accelerate healing time and decrease the risk of wound infection. Of all the available cellular therapy techniques, Apligraf™ (organogenesis, previously Graft skin) and Hyalograft-3D™ (esterified hyaluronic acid beneath silicone) have shown efficacy in the treatment of chronic diabetic foot ulcers (61). Falanga et al. in a prospective randomized multicentre clinical trial showed a 55% increased healing rate of chronic neuropathic diabetic foot ulcers.

CONCLUSION

Successful treatment of chronic non-ischemic diabetic ulcers remains challenging for clinicians. Because of the huge financial burden on the health sector and the psychosocial impact on the patients' well being, more clinical trials will be needed to define a definitive treatment protocol and standardization. Sharp wound debridement and a combination of many other discussed treatment modalities hold a promising future. The enhancement of wound healing through the application of PRP is supported by basic scientific and clinical studies. Research has shown that PRP are responsible for actively extruding growth factors, which initiate soft tissue healing and recruitment of stem cell. Although, autologous platelet derived growth factors appear promising, more properly structured clinical randomized controlled trials will be required to confirm these results and to establish under which conditions the application of platelet-rich plasma has merit.

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