INTRODUCTION

Platelet-rich plasma (PRP) contains a high concentration of thrombocytes and the granules of platelets contain platelet-released growth factors that include molecules such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor, that trigger biological effects including directed cell migration ie, chemotaxis, angiogenesis, and cell proliferation and differentiation, which are key in the processes of tissue repair and regeneration ***(Lubkowska et al., 2012).***

The use of platelet rich plasma to enhance wound healing has increased dramatically over the last decade. However controversies exist in the literature regarding the added benefit of this procedure ***(Alio et al., 2007; Fonder et al., 2008; Alsousou et al., 2009).***

Various studies have been published on the role of platelet concentrates in the treatment of cutaneous wounds compared to control wound care, PRP facilitated wound healing and the ulcers improved significantly in small hard-to-heal acute and chronic wounds ***(Raquel et al., 2013; Sacchidanand et al., 2013).***

Wound healing is a complex process, involving a mechanism of complex cascading regulatory events at both the molecular and cellular levels ***(Rodriguez, 2008; Campos et al.,***

2008). Platelets have a major role in initiation of cutaneous wound healing. They adhere, aggregate, and release numerous growth factors, adhesive molecules, and lipids that regulate the migration, proliferation, and functions of keratinocytes, fibroblasts, and endothelial cells ***(Frykberg et al., 2015)***.

The normal wound healing process is dynamic and complex having three phases: inflammation, tissue formation and tissue remodeling. however, if the normal healing process is interrupted, an ulcer can become chronic in nature due to lack of growth factors and cytokines which delay the healing process ***(Martinez et al., 2012).***

However, there are certain risk factors that commonly affect and contribute to poor wound healing, these include: local causes, such as presence of debris or necrotic tissue, infection in the ulcer, tissue hypoxia, and repeated trauma; systemic diseases, such as diabetes mellitus, immunodeficiency, or malnutrition; and medications, such as corticosteroids ***(Agale et al., 2013).***

Chronic ulcers or non-healing ulcers are defined as spontaneous or traumatic lesions, typically in lower extremities that are unresponsive to initial therapy or that persist despite appropriate care and do not proceed towards healing in a defined time period with an underlying etiology that may be related to systemic disease or local disorders. There are many types of non-healing ulcers that may include venous, arterial, diabetic, pressure and traumatic ulcers ***(Greer et al., 2012; Sebastian et al., 2014).***

The standard available treatment modalities for non healing ulcers are dressings, surgical debridement of necrotic tissue, compression bandage ,provision of a moist wound healing environment, pressure relief in the wound area, infection management using topical antibacterial agents, ischemia management, and medical management of comorbidities, However this conventional therapies cannot provide satisfactory healing since these treatments are not able to provide necessary growth factors that can modulate healing processes ***(Blume et al., 2014).***

Despite treatment, many chronic ulcers fail to heal or persist for months/years and/or recur after healing, requiring additional advanced wound care therapies for adequate healing such as hyperbaric oxygen therapy, skin grafting, vacuum assisted closure (VAC) and angioplasty or reconstructive surgery as needed ***(Suresh et al., 2014; Suryanarayan et al., 2014).***

Platelet rich growth factors products may be allogenic, autologous or recombinant DNA; however there is no standard method for PRP preparation. Lyophilized Platelets Growth Factors are a novel refined form of allogenic PRP preparations that provides an always accessible easy to use source of growth factors for all cases not suitable donors for autologous blood (as anemic or hemodynamically unstable patients). Standardizing the amount of platelets and in vitro activation of platelets to release growth factors might overcome objections to conventional PRP preparations ***(Matthias et al., 2016).***

AIM OF THE WORK

The aim of the study is to evaluate the efficacy and safety of lyophilized human platelets growth factors (L-GFs) as a novel technology for processing and preparation of human platelets in treating chronic cutaneous wounds (non healing ulcers).

Chapter 1
PLATELET-RICH PLASMA & GROWTH FACTORS

Platelet-rich plasma (PRP) has been referred to as platelet-enriched plasma, platelet-rich concentrate, autologous platelet gel, and platelet releasate ***(Mehta and Watson, 2008).*** Platelet releasates have been used to treat wounds since 1985 ***(Driver et al., 2006).***

Platelet rich plasma is an autologous concentration of human platelets above baseline in a small volume of plasma proteins. This concentrate contains clotting factors and trophic GFs that are released once the platelets are activated, both have mitogenic and chemotactic properties ***(Marx, 2004).***

In addition, its use in the treatment of chronic skin and soft tissue ulcerations ***(Pietrzak and Eppley, 2005)***, publications regarding the use of PRP include periodontal and oral surgery ***(Lindeboom et al., 2007),*** maxillofacial surgery, orthopedic and trauma surgery ***(Wrotniak et al., 2007)***, cosmetic and plastic surgery ***(Frechette et al., 2005),*** spinal surgery ***(Eppley et al., 2006),*** heart bypass surgery ***(Pietrzak and Eppley, 2005)*** and burns ***(Henderson et al., 2003).***

Platelets

Platelets are non-nucleated blood cells that originate as megakaryocyte fragments and circulate throughout the body ***(Franz et al., 2007).*** Platelets contain a large number of storage granules, predominantly characterized into three types; lysosomal granules that function as storage for digestive enzymes, dense granules which store and secrete adenosine diphosphate (ADP), which is a potent recruiter and activator of other platelets and alpha granules which store growth factors in an incomplete bioinactive form. Secretion of growth factors is activated by the clotting process. The activation of the clotting process is associated with a structural change in the platelet membrane system, which results in the active secretion of growth factors ***(Marx, 2004).***

Growth Factors:

Growth factors are polypeptides that initiate the growth and proliferation of cells and stimulate protein production. They are named for their tissue of origin, their biological action, or the cell on which they exert their influence ***(Henderson et al., 2003).***

Growth factors may have paracrine or autocrine function whereby they affect not only adjacent cells but also have a self-regulating effect. Some are transported plasma bound to large carrier proteins and thus serve an endocrine function. They are

produced by a variety of cells including platelets, macrophages, epithelial cells, fibroblasts, and endothelial cells ***(Lindeboom et al., 2007).***

Alfa granules of the platelet contain platelet-derived growth factor (PDGF) (AA, BB, and AB),fibroblast growth factor (FGF), transforming Growth Factor (TGF) (b1 and b2), insulin-like growth factor (IGF)-1, vascular endothelial GF (VEGF), epidermal GF (EGF), hepatocyte growth factor and a mixture of the other fundamental GFs (Fig. 1) ***(Anitua et al., 2004).*** Other substances identified in the secretion of alpha granules include osteonectin, von willebrand factor and proaccelerin ***(Lindeboom et al., 2007).***

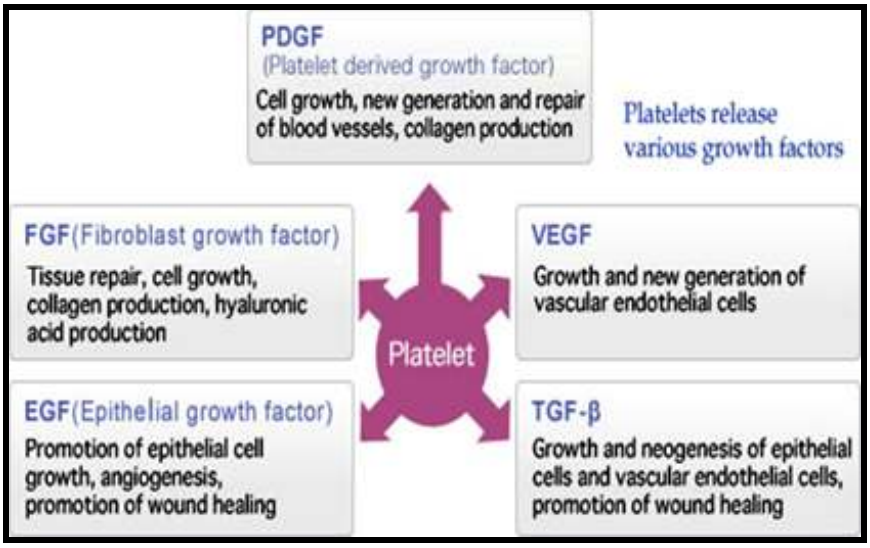

Figure (1): Growth factors by the platelets and their different effects

Role of Growth Factors in Wound Healing

The growth factors involved in wound healing include PDGF, TGF-α & β, EGF, FGF, and insulin-like growth factor (IGF) ***(Mishra et al., 2009).*** These growth factors aid healing by attracting undifferentiated cells in the newly formed matrix and triggering cell division .They interact with macrophages to improve tissue healing and regeneration, promote new capillary growth, and accelerate epithelialization in chronic wounds ***(McAleer et al., 2006).***

Platelet-Derived Growth Factor released from platelets in the wound chemo-attracts fibroblasts and increases collagen production for remodeling of the extracellular matrix during healing ***(Schultz and Wysocki, 2009).*** Another role of PDGF is to stimulate macrophages to synthesize and secrete TGF-b growth factors ***(Dohan et al., 2009)***. It also regulates mesenchymal cell lineages important for cicatrization, PDGF stimulates the production of fibronectin and hyaluronic acid, proteins which are important components of provisional matrix. Collagenase, a protein important in wound remodeling, is also produced in response to PDGF ***(Frechette et al., 2005).***

Transforming Growth Factors are made up of two polypeptide chains; α and β. **TGF-α** has a 30% amino acid homology with epidermal growth factor (EGF). It is named because of its ability to reversibly stimulate the growth of cells ***(Frechette et al., 2005). TGF-b1*** has many important roles in

wound healing, including in inflammation, angiogenesis, re-epithelialization and granulation tissue formation, connective tissue regeneration, chemo-attraction of additional immune cells, and augmentation of macrophage debridement ***(Gurtner and Werner, 2008).***

TGF-b1 stimulates collagen production and synthesis of protease inhibitors to inhibit collagen breakdown. TGF-b1 induces fibrous wound healing leading to scar formation ***(Schultz and Wysocki, 2009).***

Epidermal Growth Factor influences two critical roles in tissue repair: cell proliferation, and cytoprotection. EGF accelerates re-epithelialization and increases tensile strength in wounds .Although EGF does not stimulate collagen production, it increases the number of fibroblasts in the wound ***(Singh and Marwaha, 2009).***

Insulin-Like Growth Factors**,** IGF-1 and IGF-2 are anabolic hormones that can stimulate the synthesis of glycogen and glycosaminoglycans. They increase collagen synthesis by fibroblasts ***(Berlanga and Gavilondo, 2009).***

Vascular Endothelial Growth Factor (***VEGF)*** promotes wound healing by stimulating angiogenesis by regulating endothelial cell proliferation and migration, Other growth factors enhance VEGF-A expression, such as TGF-b1, PDGFBB, bFGF, and EGF ***(Gurtner and Werner, 2008).***

Fibroblast Growth Factor (FGF) is a group of heparin bound growth factors. There are two forms: acidic FGF (a FGF) and basic FGF (b FGF). They are commonly bound to heparin sulfate, which protects them from enzymatic degradation. FGF can be produced by fibroblasts, endothelial cells, smooth muscle cells, and chondrocytes**,** FGFs can stimulate fibroblasts, keratinocytes, chondrocytes, and myoblasts ***(Berlanga and Gavilondo, 2009).***

Keratinocyte Growth Factor is closely related to the FGFs although KGF is found only in fibroblasts, it stimulates keratinocytes, not fibroblasts ***(Singh and Marwaha, 2009).***

Preparation of platelet-rich products:

***Autologous activated PRP*:**

Autologous platelet rich plasma is prepared either ***manually*** or by the use of ***automated devices***. The process must be carried out under strict aseptic conditions and optimum temperature i.e., 20-22°C. Briefly, venous blood is obtained from the patient by venesection of the median cubital forearm vein, shortly before the injection procedure. In order to inhibit platelet aggregation, it is prepared with an anticoagulant, commonly using anticoagulant citrate dextrose solution formula (ACD-A) or sodium citrate. Storage of the blood should be avoided due to loss of platelet activity. The platelets need to be sequestered in high concentrations, enough for achieving

therapeutic benefit and in a viable state at the same time, so that they can actively secrete their GFs ***(Arshdeep and Kumaran, 2014).***

1. ***Manual method*:**

The wide variation in the reported protocols for obtaining manual PRP may lead to samples with different compositions that may induce different biological responses ***(Engebretsen et al., 2010).*** However, the double spin method is preferred over the earlier prevalent single spin method, platelet activation with thrombin and calcium chloride is a crucial step that might influence the availability of bio active molecules and therefore tissue healing ***(Mishra et al., 2009).***

2. ***Automated devices***:

Numerous commercial devices of varying standards are now available for the preparation of PRP, but their application has been confusing because each technique leads to a different product with potentially dissimilar biology and unknown relative efficacy, these adapted kits can be quite expensive as compared to the manual process ***(Arshdeep and Kumaran, 2014).***

Allogeneic platelet concentrates from buffy coats (BC-PC):

In earlier times, therapy with platelet growth factors (GF) was performed with the exclusive use of autologous products,

because of ethical and legal concerns related to the risk of allogeneic blood components as they are antigenic. Homologous platelets such as lyophilised donor platelets may be more practical, a significantly improved level of transfusion safety has contributed to relax allogeneic blood product use limitations ***(Burnouf and Tseng, 2013).***

This novel therapeutic options for patients who cannot easily undergo autologous blood processing **,** for example those who cannot be subjected to repeated bleeding (e.g. infants, patients with septicemia or hematological disorders, elderly subjects affected by comorbidities), or emergency patients who lack the time to undergo autologous preparation. For this kind of patients a convenient PGF source may be represented by allogeneic platelet concentrates procured from a blood transfusion services ***(Matthias and Frank, 2016).***

The preparation of pooled PRP by means of lyophilization may allow physicians to apply a defined amount of growth factors by using a defined amount of PRP powder. Moreover, PRP powder as a dry substance with no need for centrifugation could become ubiquitously available, thus saving time and staff resources in clinical practice. However, before transferring the results of this basic science study to clinical application, regulatory issues have to be cleared ***(Matthias and Frank, 2016).***

Lyophilized Platelets Growth Factors are a novel refined form of platelets growth factors based on the following concepts:

- Use of allogenic pathogen free platelets instead of autologus platelets as a source of growth factors.

- In vitro stimulation of platelets to free growth factors from their site of storage in the alpha granules, and thus avoiding the use of Thrombin or Calcium injections with autologus PRP

- Having a much longer shelf life of the final lyophilized growth factors (12-18 months) when compared to the autologus PRP 8 hours ***(Marx, 2004).***

- Standardizing the amount of growth factors in each vial to the equivalent of the product of 1 million platelets /cmm.

There is a plethora of literature discussing autologous PRP as a source of growth factors and few references discussing how to prepare platelets lysates for clinical use, but no one has developed an integrated global view addressing all issues of viral/pathogen inactivation, ease of use, and eliminating fibrinogen content except LGF**.** Problems with PRP such as absent standardization, lack of consistency among studies, and black box dosage could be solved by using characterized GFs made by separation and lyophilization. The new technique opens up new possibilities for PRP research as well as for the treatment of patients ***(Matthias and Frank, 2016).***

Recombinant DNA Technology and Production of Growth Factors:

Advances in the molecular biology have made it possible to produce highly purified recombinant proteins and recombinant human growth factors to be used as potential therapeutic wound healing agents ***(Kushida et al., 2014).***

The only growth factor that is approved by the US Food and Drug Administration (FDA) is platelet-derived growth factor (PDGF). Platelet-derived growth factor is a dimeric protein composed of 2 disulfide linked polypeptide chains. There are 3 different isoforms: heterodimer PDGF-AB, 2 homo dimers PDGF-AA, and PDGF-BB, Becaplermin is a topical rhPDGF-BB produced by inserting the gene for the B chain of PDGF into the yeast pathogen using recombinant DNA technology**.** Other studies for other recombinant growth factors have been carried out but they haven't got yet the approval of FDA ***(U.S. Food and Drug Administration, 2008).***

Rationale to use platelet-rich plasma products rather than recombinant human platelet-derived growth factors:

The use of PRP represents a greater similarity to the natural healing process, with the application of multiple growth factors in their biologically determined ratios, more closely than the addition of a single growth factor such as PDGF-BB**.** In addition, growth factors may be more effective when directly

delivered via a "depot" platelet plug, allowing a slow release of these factors than when administered in a bolus dose, such as commonly performed with rhPDGF-BB treatment ***(Cullinane et al., 2002)***. The short shelf life of recombinant human growth factors such as rhPDGF-BB is not a concern with the use of PRP, which can be made as needed ***(Nikolidakis and Jansen, 2008).***

Safety of PRP:

Safety of PRP remains to be an issue since increases GF in a local area may be a cancer promoting effects, however the mitogenic effects of PRP are only limited to augmentation of the normal healing process and is theoretically not mutagenic, as the GFs released do not enter the cell or its nucleus, but only bind to the membrane receptors and induce signal transduction mechanisms**.** However, safety concerns with bovine thrombin have been raised about the potential transmission of Cruetzfeld-Jacob disease (mad-cow disease). These have been refuted by some stating that the prion vector has been found only in the neural tissues of cattle, whereas thrombin is solely isolated from the blood and is also further processed by heating ***(Marx, 2004)***. Furthermore, reports of post-operative bleeding due to bovine thrombin-induced factor-V deficiency have made it an unpopular choice. On the other hand, use of $CaCl_2$ as an activator, eliminates the above risks ***(Fufa et al., 2008).***