Comparative Analysis of Different Activation Methods on Platelet Rich Plasma

Thesis

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Noha M. El-Shishtawy

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List of Abbreviations

Abbrev. Full-term

ADP	**:**	Adenosine diphosphate
ADSC	**:**	Adipose tissue derived stem cells
ANOVA	**:**	Analysis of variance
bFGF	**:**	Basic fibroblast growth factor
BMSC	**:**	Bone marrow stem cells
CBD	**:**	Carboxy-terminal cell binding domain
CIB	**:**	Calcium- and integrin-binding protein
DAG	**:**	Diacylglycerol
DTS	**:**	Dense tubular system
ECM	**:**	Extracellular matrix
EDTA	**:**	Ethylenediaminetetraacetic acid
EGF	**:**	Epidermal growth factor
ELISA	**:**	Enzyme-linked immunosorbent assay
ERK	**:**	Extracellular signal-regulated kinase
Fg	**:**	Fibrinogen
Fn	**:**	Fibronectin
GMP	**:**	Guanosine monophosphate
GP	**:**	Glycoprotein
GPCR	**:**	G protein-coupled receptor
GRB	**:**	Growth factor receptor bound
HGF	**:**	Hepatocyte growth factor
HSC	**:**	Hematopoietic stem cell
IAP	**:**	Integrin-associated protein
ICAM	**:**	Intercellular adhesion molecule
IGF	**:**	Insulin growth factor
IGFBP	**:**	Insulin growth factor binding proteins
IL	**:**	Interleukin
IP_3	**:**	Inositol triphosphate
ITAM	**:**	Immunoreceptor tyrosine-based activatory motif
ITIM	**:**	Immunoreceptor tyrosine-based inhibitory motif

LAMP	**:**	Lysosomal-associated membrane protein
LIBS	**:**	Ligand-induced binding site
LSECs	**:**	Liver sinusoidal endothelial cells
MAP	**:**	Mitogen-activated protein
MAPK	**:**	Mitogen-activated protein kinase
MCP	**:**	Monocyte chemotactic protein
MK	**:**	Megakaryocyte
MPT	**:**	Mitochondrial permeability transition
MSC	**:**	Mesenchymal stem cells
MVBs	**:**	Multivesicular bodies
NBEAL2	**:**	Neurobeachin-like 2
OCS	**:**	open canalicular system
PAC	**:**	Procaspase-activating compound
PADGEM	**:**	Platelet activation dependent granule-external membrane protein
PAF	**:**	Platelet activating factor
PAI-1	**:**	Plasminogen activator inhibitor type I
PAR	**:**	Protease activated receptor
PDGF	**:**	Platelet Derived Growth Factor
PDI	**:**	Protein disulfide isomerase
PECAM	**:**	Platelet endothelial cell adhesion molecule
PF4	**:**	Platelet factor 4
PG	**:**	Prostaglandin
PI3K	**:**	Phosphatidylinositol 3-kinase
PKG	**:**	Protein kinase G
PRGF	**:**	Plasma rich in growth factors
PRP	**:**	Platelet Rich Plasma
PS	**:**	Phosphatidylserine
PtdIns	**:**	phosphatidylinositol
PTKs	**:**	Protein tyrosine kinases
PTPs	**:**	Protein-tyrosine phosphatases
RANKL	**:**	Receptor-activator of nuclear factor kappa beta ligand
ROS	**:**	Reactive oxygen species
S1P	**:**	Sphingosine-1-phosphate

SCF	**:**	Stem cell factor
SD	**:**	Standard deviation
SDF	**:**	Stromal cell-derived factor
SNAP	**:**	Soluble N-ethylmaleimide-sensitive factor attachment protein
SNARE	**:**	Soluble N-ethylmaleimide-sensitive factor attachment receptor
SOCS	**:**	Suppressors of cytokine signalling
STAT	**:**	Signal transducer and activator of transcription
STEM	**:**	Scanning transmission electron microscopy
TFPI	**:**	Tissue factor pathway inhibitor
TGFβ	**:**	Transforming growth factor beta
TIMPs	**:**	Tissue inhibitors of metalloproteases
TLR	**:**	Toll like receptor
TMEM	**:**	Transmembrane protein
TNF	**:**	Tumor necrosis factor
TPO	**:**	Thrombopoietin
TSP	**:**	Thrombospondin
TXA2	**:**	Thromboxane A2
TXA_2	**:**	Thromboxane A_2
TXR	**:**	Thromboxane A_2 receptor
VAMP	**:**	Vesicle- associated membrane proteins
VAMP	**:**	Vesicle-associated membrane protein
VEGF	**:**	Vascular endothelial growth factor
Vn	**:**	Vitronectin
vWF	**:**	Von Willibrand factor

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Introduction

Platelet Rich Plasma (PRP) has been a breakthrough in the stimulation and acceleration of healing for over 20 years, starting by tissue repair in dentistry and expanding its clinical applications to other fields afterwards **(Marx et al., 1998).** It represents a relatively new biotechnology that is part of growing interest for extensive research in tissue engineering and regenerative medicine nowadays **(Niemeyer et al., 2010).**

Platelet-rich plasma is autologous plasma with high platelet concentration used to accelerate healing, due to several different growth factors and cytokines. PRP is easy, cheap, safe, and minimally invasive source for autologous growth factors **(Cavallo et al., 2016).** Platelet rich plasma is prepared by centrifugation with different centrifugal forces, temperature, and time. Accordingly the two-step procedure is most widely used, in which the first step (soft spin) separates plasma buffy coat and cells and a second spin (hard spin) further concentrates platelets with an outcome of platelets exceeding 400 x 10^9/L; unlike platelet poor plasma which gives platelets less than 10 x 10^9/L following vigorous centrifugation **(Alves & Grimalt, 2018).**

Platelets are disk shaped fragments of megakaryocytes originating from stem cells in the bone marrow. Platelets are approximately 2 µm in diameter **(Machlus et al., 2014) (Michelson & Alan, 2013).** The platelets count ranges from

150-400 x 10^9 per litre with a life span of 7-10 days **(Thon et al., 2010).** Although platelets most known function is hemostatic, a process which controls bleeding by forming a clot at the site of a cut or wound by sticking platelets together; other beneficial effects of PRP have been revealed such as cell proliferation, differentiation, improved synthesis of extracellular matrix (ECM), and angiogenesis which results in neovascularization and formation of a new conjunctive tissue necessary for healing **(Marwah et al., 2014) (Cervantes et al., 2018).**

Platelets contain alpha (α) granules, dense granules, and lysosomes; with α granules being most abundant. Each type has specific properties concerning both the structure and function. Alpha granules -80 granules per platelet- contain growth factors (such as PDGF, bFGF, SDF 1α), angiogenic factors (such as VEGF, angiogenin), necrotic factors (such as TNF α, TNF β), hemostatic (such as factor V, VWF, Fibrinogen), and other cytokines **(Blair & Flaumenhaft, 2009) (Whiteheart, 2011).** Alpha-Granules function in adhesion of platelets and healing **(Rendu et al., 2001).** The dense granules contain adenosine diphosphate (ADP), adenosine triphosphate (ATP), ionized calcium, and serotonin **(Orkin et al., 2001).** Dense granules function in recruiting other platelets. Lysosomes contain hydrolases that eliminate platelet aggregates **(Rendu et al., 2001).**

The platelets can be activated in vivo or in vitro. Once activated, platelets show morphological changes from the discoid to round shape, develop pseudopods that spread through the injured tissue, and platelets adhere to one another and to collagen in endothelium forming platelet aggregates. Degranulation of their granules also occur releasing GFs from α-granules by either fusion with the open canalicular system and extrusion of its contents through small channels in the cell membrane,or by exocytosis, that is releasing α granules content outside by fusing the granules with the cell membrane **(Zandim et al., 2012).** Additionally, a platelet gel forms through a clotting process by cleavage of fibrinogen. The factors activate an intracellular signal protein that causes the expression of a gene sequence that directs cell migration, proliferation and differentiation, and promote extracellular matrix accumulation by binding to specific cell surface receptors on target cells (eg. Mesenchymal stem cells, osteoblasts, fibroblast, endothelial cells, and epidermal cells) thus provoking tissue repair and regeneration **(Dhurat and Sukesh, 2014).**

Activation may be initiated in vivo by thrombin, calcium, collagen, and shear stress. In clinical practice, PRP may be pre-activated using in vitro calcium or/and thrombin (bovine or autologous) to induce release of GF from PRP.

However, evidence is lacking for pre-injection activation requirement for therapeutic uses **(Cavallo et al., 2016).**

PRP is prepared for either harvesting platelets for therapeutic purposes or testing platelet function using aggregometer **(Gentile et al., 2010).** Therapeutic clinical applications for PRP have revealed remarkable results in dentistry **(Anitua, 1999),** surgeries **(Patel et al., 2016) (Arnalich et al., 2016),** orthopaedics **(Filardo et al., 2012),** rheumatology **(Cieslik-Bielecka et al., 2009),** chronic wounds **(Rozmam et al., 2007),** and muscle injuries **(Hammond et al., 2009).**

In recent years, platelets were discovered to have a positive influence on the liver thus promoting its regeneration **(Murata et al., 2014) (Takahashi et al., 2013),** improving liver fibrosis **(Takahashi et al., 2013) (Nowatari et al., 2014) (Hesami et al., 2014),** and attenuating liver damage **(Hisakura et al., 2011);** as it accelerates liver regeneration and have anti-fibrosis and anti-apoptotic activity. Platelets have a direct impact on hepatocytes, a supportive effect with liver sinusoidal endothelial cells, and a cooperative effect with Kupffer cells which accelerates liver regeneration. Platelets undergo adenosine-cyclic adenosine 5'-monophosphate signaling pathway which deactivates hepatic stellate cells thus applying anti-fibrotic activity. Platelets activate the Akt pathway and up-regulates Bcl-xL, which in turn suppresses caspase-3 activation thus preventing hepatocyte apoptosis **(Takahashi et al., 2013).**

Aim of the Work

To compare different strategies of PRP activation by evaluating content and release of growth factors for further clinical trials to validate the clinical relevance of best method to help regeneration of liver in hepatic patients.

Chapter 1:
Platelets

Platelets, or thrombocytes, are small (approximately 2-3 µm in diameter), rounded or oval disc-shaped, anucleate cells formed from the fragmentation of long proplatelet extensions, that become interwoven through endothelial pores of the bone marrow sinusoids and are fragmented by shear forces releasing platelets from the megakaryocyte. Megakaryocyte is large multinucleated cell from myeloid lineage of hematopoietic stem cells constituting 0.1% of cellular population of the bone marrow **(Junt et al., 2007, Francone et al., 1990).** A single megakaryocyte can give rise to 1,000–3,000 platelets **(Stenberg and Levin; 1989)** before the residual nuclear material is eliminated by macrophage-mediated phagocytosis **(Radley and Haller; 1983).** Differentiation of stem cells to platelets is predominantly driven by thrombopoietin (TPO) signaling through the cMpl receptor, and is supported by additional growth factors such as interleukin-3 (IL-3), stem cell factor (SCF), IL-6, and IL-11 **(Kaushansky, 2006).**

Thrombopoietin is the principal regulator of thrombo-poiesis and primate platelet counts can be reduced with an inhibitor against TPO without impairment of primary hemostasis **(Kaushansky; 2006, Tucker et al., 2010).** Platelets count ranges from 150,000 to 350.000/ll **(Weibrich**

et al., 2002). The efficiency of platelets is variable, affected by size and age of the platelet, with younger and larger platelets exhibiting better hemostatic function than smaller and older ones **(Karpatkin,; 1978, Hartley; 2007).** Platelets contribute to many aspects of host defense including hemostasis and thrombosis, inflammation, angiogenesis, and wound healing **(Franco et al., 2015).**

Although they lack a nucleus, platelets have an extensive cytoskeleton, organelles, specific granules and membrane features **(Weyrich et al., 2009).**

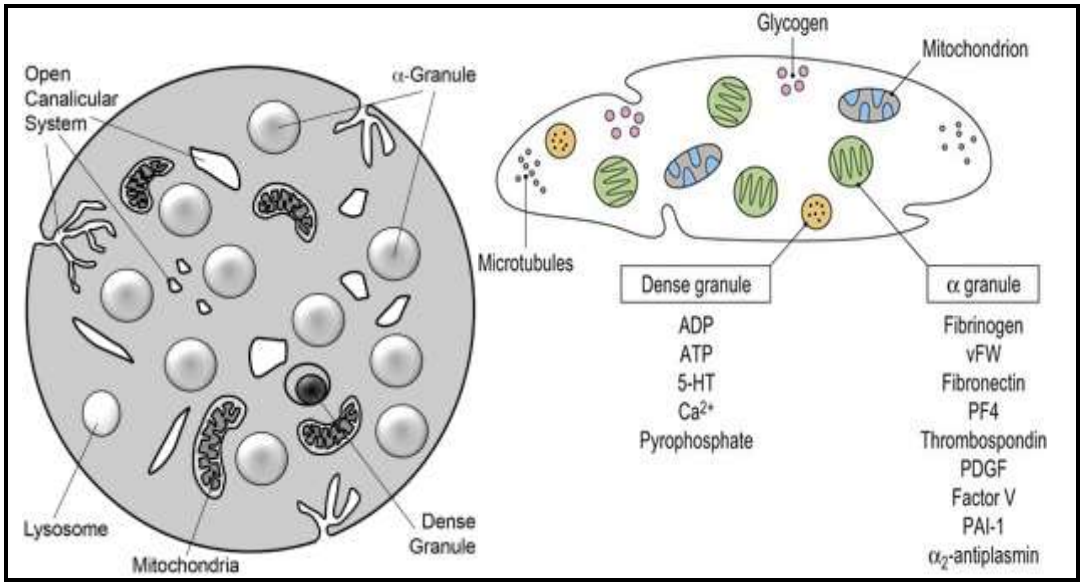

Figure (1): Diagrammatic representation of coronal (left) and longitudinal (right) section of the platelet.