ANGIOGENIC POTENTIAL OF HUMAN PLATELET RICH CONCENTRATES ON DENTAL PULP STEM CELLS IN AN IN-VITRO INFLAMMATION MODEL

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ABSTRACT

Background: Healing of inflamed dental pulp by stimulating angiogenesis forms the basis of dental pulp regeneration. Dental pulp vitality is paramount for tooth longevity and function. Dental pulp stem cells (DPSCs) are mesenchymal stem cell population present in cell rich zone of dental pulp and demonstrate multilineage differentiation potential in healthy and inflamed microenvironment. Platelet rich concentrates (PRCs), a concentrated suspension of growth factors (GFs) are recognised as promoters of tissue regeneration through their paracrine action. Recently, PRCs has been introduced in the field of oral-maxillofacial and implant dentistry as a treatment modality. However, its effect on DPSCs in inflamed microenvironment is poorly understood. The aim of this study was to gain an insight into the healing and angiogenic potential of two types of PRCs namely human platelet lysate (HPL) and platelet rich plasma (PRP) on DPSCs in an inflamed state. **Objectives**: Firstly, the mesenchymal properties of DPSCs isolated from human adult dental pulp were characterised. Secondly, optimal concentration of bacterial lipopolysaccharide (LPS) to induce inflammation in DPSCs was investigated, followed by determination of two types of PRCs to maintain viability and induce angiogenesis in DPSCs. Finally, we compared the effect of the optimized concentrations of PRCs to induce angiogenesis in DPSCs in inflamed state (iDPSCs). **Methods**: DPSCs were isolated from dental pulp tissue extirpated from healthy premolars of patients indicated for orthodontic extraction and were characterised for their mesenchymal characteristics. Next, LPS was used in different concentrations to induce inflammation in DPSCs. Following this, DPSCs were treated with different concentrations of HPL and PRP to investigate their effect on cell viability and angiogenic effect on DPSCs. Effect of all the treatments given to DPSCs were validated via cell viability assay, gene expression analysis as well as analysis at protein level. Capacity of DPSCs to form tube-like structures after PRCs treatment was investigated by matrigel based functional assay.

Results: Isolated DPSCs demonstrated positive mesenchymal characteristics. LPS for 24 hours considered optimum to induce inflammation in isolated DPSCs as evidenced by expression of pro-inflammatory markers at gene and protein level. 10%, 15%, 20% HPL and 5% PRP treatment on DPSCs maintained the cell viability and morphology, as compared to foetal bovine serum (FBS). It was noted that 20% HPL showed highest expression of angiogenic and cell adhesion surface markers, and capacity of DPSCs to form tube like structures. In iDPSCs, 20% HPL demonstrated significantly high cell viability and angiogenic potential. **Conclusion**: DPSCs could be induced to inflamed state by 1µg/mL LPS treatment for 24 hours. Viability of DPSCs was maintained *in-vitro* by 5% PRP, 10%, 15%, 20% HPL. Potential angiogenic effect on normal and inflamed DPSCs was demonstrated at 20% HPL. **Clinical relevance**: PRCs exhibit potential pro-angiogenic effect on DPSCs in inflamed state. Hence, they can be considered for potential pulp regenerative therapy in inflamed pulp.

ABSTRAK

Latar belakang: Penyembuhan pulpa gigi yang radang menggunakan rangsangan *angiogenesis* adalah asas untuk meregenerasikan pulpa gigi. Gigi yang vital adalah sangat penting untuk mengekalkan fungsinya. Sel stem pulpa gigi adalah populasi sel stem *mesenchymal* yang boleh didapati di kawasan yang kaya dengan sel di pulpa gigi, ianya menunjukkan potensi untuk pembezaan *multilineage* dalam persekitaraan mickro yang normal dan juga radang. Pati kaya platelet (PRC), suatu suspensi pekat dengan faktor tumbesaran telah diketahui sebagai peransang regenerasi tisu melalui tindakan parakrin. Baru-baru ini, PRC telah diperkenalkan dalam bidang oro-maksilofasial dan implant pergigian sebagai satu kaedah rawatan. Namun demikian kesannya ke atas sel stem pulpa gigi dalam persekitaraan mickro yang radang kurang difahami secara mendalam. Kajian ini bertujuan untuk mendapatkan kefahaman tentang penyembuhan dan potensi angiogenik dua jenis pati kaya platelet iaitu *platelet lysate* dari manusia (HPL) dan plasma kaya platelet (PRP) terhadap sel stem pulpa gigi dalam keadaan radang. **Objektif**: Pertama, kajian ini dimulakan dengan mencirikan sifat *mesenchymal* sel stem pulpa gigi yang diperolehi dari pulpa gigi orang dewasa. Kedua, penentuan kepekatan optima 'lipopolysaccharide' bakteria (LPS) untuk mewujudkan keradangan DPSC akan dikaji, dan seterusnya menentukan dan membandingkan dua jenis PRC dalam pemeliharaan kebolehhidupan DPSC dan meransang *angiogenesis*. Akhirnya, kami akan membandingkan kesan PRC yang telah dioptimakan kepekatannya untuk meransang *angiogenesis* bagi DPSCs di dalam keadaan radang (iDPSCs). **Kaedah**: DPSC diasingkan dari tisu pulpa gigi yang diperolehi dari geraham kecil pesakit yang sihat dan dicirikan untuk sifat *mesenchymal*. Gigi geraham kecil ini dicabut kerana pesakit yang menjalani rawatan ortodontik. Kemudiannya, LPS dalam berbagai kepekatan digunakan untuk merangsang keadaan radang dalam DPSCs. Seterusnya, DPSCs akan dirawat dengan HPL dan PRP dalam pelbagai kepekatan untuk mengkaji kesanya terhadap

kebolehhidupan sel dan kesan angiogenic terhadap DPSCs. Kesan rawatan ini dikaji melalui assai kebolehhidupan sel, analisa ekpresi gen dan juga analisa diperingkat protin. Kapasiti DPSCs untuk mendorong pengagregatan sel dalam membentuk struktur seperti kapilari menggunakan *matrigel-based functional assay*. Ini akan menunjukkan *angiogenesis* setelah dirawat dengan PRC. **Keputusan**: DPSCs yang diasingkan menunjukkan ciri-ciri *mesenchymal* yang positif. LPS 24 jam adalah optima untuk meransang keradangan dalam DPSCs berdasarkan ekpresi penanda *pro-inflammatory* pada peringkat gen dan protin. Rawatan menggunakan10%, 15%, 20% HPL and 5% PRP lysate ke atas DPSCs mampu memelihara kebolehhidupan sel dan menunjukkan morfologi yang konsisten berbandingkan serum janin lembu (FBS). Walau bagaimanapun, 20% HPL menunjukkan ekspresi *cell surface marker* angiogenik dan lekatan sel yang tertinggi, serta pengagregatan sel yang menyerupai kapilari. Bagi iDPSC, 20% HPL menunjukkan potesi angiogenik dan kebolehhidupan sel yang tinggi dan signifikan. **Kesimpulan**: DPSC boleh diransang ke situasi keradangan dengan menggunakan 1µg/mL LPS selama 24 jam. Kebolehhidupan DPSCs boleh kekalkan menggunakan 5% PRP, serta 10%, 15% dan 20% HPL. Potensi angiogenik oleh DPSCs normal serta iDPSC boleh dicapai dengan menggunakan 20% HPL. **Kaitan klinikal**: PRC menunjukkan potensi pro-angiogenik ke atas DPSC semasa dalam keadaan keradangan. Maka, sel-sel ini boleh dianggap sesuai untuk digunakan dalam terapi regeneratif pulpa terutamanya bagi pulpa yang mengalami keradangan.

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LIST OF SYMBOLS AND ABBREVIATIONS

Ag	:	Surface antigen
ANG	:	Angiopoietin
ANGPT1	:	Angiopoietin-1
ANOVA	:	Analysis of Variance
BM-MSCs	:	Bone Marrow Mesenchymal Stem Cells
BMP	:	Bone Morphogenic Proteins
CAD	:	Compound Aided Device
CCL11	:	C-C motif chemokine 11
CCL2	:	C-C motif chemokine 2
CD	:	Cluster of Differentiation
CDH5	:	Cadherin 5
cDNA	:	Complementary Deoxyribo Nucleic Acid
CO_2	:	Carbon Dioxide
CXCL	:	Chemokine (C-X-C motif) ligand
DFPCs	:	Dental Follicle Progenitor Cells
DMEM- KO	:	Dulbecco's Modified Minimal Essential Medium - Knock Out
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribo Nucleic Acid
DPBS	:	Dulbecco's phosphate-buffered saline
DPSC	:	Dental Pulp Stem Cells

ECM	:	Extracellular Matrix
ECM	:	Endothelial Cell Media
EDTA	:	Ethylenediaminetetraacetic acid
EGF	:	Endothelial Growth Factor
ErGF	:	Epithelial Growth Factor
ELISA	:	Enzyme Linked Immunosorbent Assay
EREG	:	Epiregulin
ESCs	:	Embryonic stem cells
FACS	:	Fluorescence-activated cell sorting
FBS	:	Fetal Bovine Serum
FGF	:	Fibroblast Growth Factor
FIGF	:	FOS-induced growth factor
FITC	:	Fluorescein Isothiocyanate
FSC	:	Forward Scattered Curve
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
GFs	:	Growth Factors
HA	:	Hydroxyapatite
HDPSCs	:	Human Dental Pulp Stem Cells
HGF	:	Hepatocyte Growth Factor
hMSCs	.:	Human Mesenchymal Stem Cells
HPL	:	Human platelet lysate
iDPSCs	:	Inflammation induced Dental Pulp Stem Cells

IFNA1	:	Interferon A1
IFNB1	:	Interferon B1
IGF	:	Insulin-like growth factor
IL-6	:	Interleukin 6
IL-8	:	Interleukin 8
IL1B	:	Interleukin 1 beta
ITGB3	:	Integrin beta 3
KDa	:	Kilo dalton
L-PRP	:	Leucocyte platelet rich plasma
LPS	:	Lipopolysaccharide
MCP-1	:	Monocyte Chemotactic Protein 1
MEM	:	Minimal Essential Medium
ML	:	Millilitre
mRNA	:	Messenger Ribonucleic Acid
MSCs	:	Mesenchymal stem cell
NFkB	:	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NRP2	:	Neuropilin 2
OCT4	:	Octamer-Binding Transcription Factor 4
P-PRF	:	Pure Platelet Rich Fibrin
PBS	:	Phosphate-Buffered Saline
PCR	:	Polymerase Chain Reaction

PD	:	Population Doubling
PDGF-BB	:	Platelet Derived Growth Factor Beta
PDGFA	:	Platelet Derived Growth Factor Subunit A
PDLSCs	:	Periodontal Ligament Stem Cells
PDT	:	Population Doubling Time
PE	:	Phycoerythrin
PerCP	:	Peridinin-Chlorophyll-Protein Complex
PGA	:	Poly(glycolic) Acid
Ph	:	Potential of hydrogen
PI	:	Pathogen inactivation
PLA	:	Poly Lactic Acid
PLGA	:	Poly lactic-co- Glycolic Acid
PPP	:	Platelet Poor Plasma
PRC	:	Platelet Rich Concentrates
PRF	:	Platelet rich fibrin
PRFM	:	Platelet Rich Fibrin Matrix
PRP	:	Platelet Rich Plasma
PUMA	:	P-53 Upregulated Modulation of Apoptosis
qRT-PCR	:	Quantitative reverse-transcription polymerase chain reaction
RBC	:	Red Blood Cells
RCT	:	Root canal treatment

RIN	:	(RNA) Integrity number
RNA	:	Ribonucleic Acid
RPM	:	Revolutions per minute
RPMI 1640	:	Roswell Park Memorial Institute medium 1640
SCAPs	:	Stem Cells in the apical papilla
SD	:	Standard Deviation
SHEDs	:	Stem cells from human exfoliated deciduous teeth
Sox2- SRY	:	(Sex determining region Y)-box
SSC	:	Side Scattered Curve
STAB1	:	Stabilin 1
TAC 1	:	Tachykinin Precursor 1
TGFB2	:	Transforming Growth Factors beta
TLR 4	:	Toll like Receptor 4
TNF-alpha	:	Tumour necrosis factor alpha
TPA	:	Tissue Plasminogen Activator
UVA	:	Ultra Violet A
V/V	:	Volume by Volume
VEGF	:	Vascular Endothelial Growth Factor A
WBCs	:	White Blood Cells
β TCP	:	Beta Tricalcium Phosphate

CHAPTER 1: INTRODUCTION

1.1 Background and Significance

Dental pulp is a specialized mesenchymal tissue comprising of heterogeneous cell population consisting of odontoblasts (Nör, 2006), fibroblast, and mesenchymal population of stem cells known as dental pulp stem cells (DPSCs). DPSCs, present in the cell-rich part of dental pulp are capable of differentiating into multiple cell lineages like adipocytes, cardiomyocyte (Gandia et al., 2008) myocyte (Yang et al., 2010),corneal cells (Gomes et al., 2010) , hepatocyte (Ishkitiev et al., 2012) neural (Sakai et al., 2012), osteogenic (R d'Aquino et al., 2007) , angiogenic (R d'Aquino et al., 2007) and odontoblasts like cells in-vitro (De Ugarte et al., 2003). Many studies in the past have looked into the application of DPSCs in regenerative medicine (Yamada et al., 2011). DPSCs can be acquired from extracted wisdom tooth or surrounding periapical tissues (Verma et al., 2014). Due to high therapeutic potential and simple, non-invasive attainment of DPSCs, they are the preferred cell of choice in regenerative medicine and dentistry. Studies have investigated the role of DPSCs in regenerative dentistry (Huang et al., 2009a) and oral maxillofacial healing (Riccardo d'Aquino et al., 2009). Damages and injuries to dental pulp by mechanical, chemical, thermal, and microbial irritants activates degenerative, stress related biological events like complex vascular, lymphatic, and local tissue reactions (Johnson, 2003). Unlike other tissue in the body dental pulp lacks the innate immunity and self-renewal potential. The likelihood of pulp tissue regeneration is restricted by several factors, like anatomical location and limited collateral blood supply (G Huang, 2011) (Smith et al., 2008). Dental pulp is housed in hard and firm dental tissues such as enamel and dentine. This makes it difficult for pulp to expand in response to injury as a part of the inflammatory process. During inflammation, ability of dental pulp to self-renew is reduced due to insufficient quantity of blood-derived

factors that are responsible for healing (Huang et al., 2009b; Provenza, 1958). However, their potential in healing and *de novo* pulp regeneration in inflammatory conditions requires further exploration.

Dental pulp injuries often incur by seeping of oral microorganisms and their components through dentinal tubules towards the pulp. When odontoblasts are damaged or destroyed, DPSCs (He et al., 2013) or stem cells from apical papilla (SCAP) (Huang et al., 2009a) are mobilized to accumulate at the injured site, where they subsequently differentiate into odontoblast-like cells that participate in repair of dentine–pulp complex. Process of pulp inflammation starts at the site of bacterial invasion (Berkovitz et al., 2016). Gram-negative bacteria are the most prevalent population in deep caries and pulp inflammation (Gatti et al., 2000). Lipopolysaccharide (LPS) an antigenic constituent of cell wall of gram-negative bacteria is primarily responsible for the inflammatory process in dental pulp (Botero et al., 2010; Vianna et al., 2007). At molecular level, DPSCs have demonstrated an ability to differentiate into intended lineage in response to biochemical cues and specific microenvironment (Arthur et al., 2009; Flavio F Demarco et al., 2010). To understand the desired multipotent properties of dental stem cells in the condition of disease and health, it is vital to comprehend their healing and regenerative potential in inflamed microenvironment (Ennis et al., 2013).

Basic biology behind the therapeutic effects of DPSCs is their paracrine effect that regulates the release of many cytokines and growth factors that control the healing cascades (Mead et al., 2014). Many studies in the past have reported that DPSCs exert inflammation supressing effect in-vitro, confirmed by reduced expression of a potent inflammatory marker TNF alpha and have been reported to stimulate the expression of inflammation supressing cytokines (CD10) (Omi et al., 2015).

Current root canal therapy involves complete removal of dental pulp rendering the tooth non-vital and prone to fracture (Peterson et al., 2010). This calls for a need of a

treatment protocol that conserves and regenerates de novo pulp. A compilation of few clinical cases have shown that in vital pulp therapies, where partial removal of dental pulp is done, regenerative properties of remnant pulp tissue are utilized to stimulate the deposition of reparative dentine that stimulates root end closure (Güven Polat et al., 2014). This indicates that pulp tissue may serve as a source of stem cells in the incident of acute pulp inflammation.

Healing and regeneration requires a finely tuned interaction of cellular processes governing migration, extracellular matrix organization, proliferation, differentiation, and angiogenesis/neovascularization (Gurtner et al., 2008). Platelet rich concentrates (PRCs), a concentrated suspension of growth factors found in platelets has recently gained attention in the field of dentistry. Growth factors released from PRCs are involved in wound healing and are anticipated as promoters of tissue regeneration (Kiran et al., 2011). Besides coagulation factors, platelets store a series of potent bioactive mediators (Semple et al., 2011) and chemokines (Blair & Flaumenhaft, 2009) that control recruitment, proliferation, and activation of fibroblasts, neutrophils, monocytes, MSCs, and other cell types critically involved in wound healing (Gawaz & Vogel, 2013). Their imperative role in wound healing forms a strong basis of their potential in tissue regeneration

There had been a continuous evolution in various fractions PRCs since last decade. Currently, there are three types of PRCs depending on their preparation method and potential use, namely platelet rich plasma (PRP), platelet rich fibrin (PRF) and human platelet lysate (HPL) (Ehrenfest et al., 2014). PRP is an autologous concentrate of human platelets in a small volume of plasma (Bielecki & M Dohan Ehrenfest, 2012). Mechanism of action behind PRP activity is via the degranulation of α granule in platelets, which contain the synthesized and pre-packed growth factors (Battinelli et al., 2011). It has demonstrated anti-inflammatory action by subduing release of cytokine, reducing inflammation and thus, promoting tissue renewal (El-Sharkawy et al., 2007) (Kim et al.,

2014). In clinical set up, PRP has been widely studied for orthopaedic and plastic surgery where effective union of poorly vascularized and damaged tissue is mandatory (Alsousou et al., 2013). It has demonstrated successful clinical outcome for bone, cartilage, tendon and muscle healing (Alsousou et al., 2013). In dentistry, autologous PRP has been reported to be a successful tissue healing modality during bone graft and dental implant placement procedures (Carlson & Roach, 2002). PRF is the latest development in the PRCs fraction. It is widely used in dental clinics for bone graft procedures and implant surgeries (Anitua et al., 2006). Due to simple processing and minimal biochemical handling of blood, PRF is the most clinically translatable fraction of PRCs (Choukroun et al., 2006). However, due to short half-life of this preparation, it is challenging to use this fraction in in-vitro laboratory set-ups. HPL has been used in in-vitro cell culture studies as a replacement for commercially available bovine serum (Suri et al., 2016) for adipose tissue derived stem cell expansion owing to its non-xenogeneic properties (Dessels et al., 2016; Jonsdottir-Buch et al., 2013; Schallmoser et al., 2007). It has demonstrated potential angiogenic promoting effect by stimulating the growth of vascular structures (Barsotti et al., 2013; Oliveira et al., 2015). The potential growth-promoting effect of PRCs is by the virtue of cocktail of growth factors that might augment the regenerative and angiogenic effect of DPSCs.

1.2 Gaps in Literature

Dental pulp regeneration is an anticipated alternative to retain tooth vitality and maintain the longevity of tooth (Kaushik et al., 2016), which is rendered non-vital after complete removal of pulp. For successful pulp regeneration a complete vascularization of target tissue is mandatory. In the past various regenerative modalities have been attempted like scaffolds (Bohl et al., 1998; Hiremath et al., 2008; Kim et al., 2009; Mooney et al., 1996) and cell homing techniques (Nimura et al., 2008) to achieve

formation of pulp like tissue. Among the difficulties encountered to achieve successful pulp regeneration, maintenance of functional blood supply to the regenerated pulp tissue had always been a challenging gap to address.

PRCs comprise of significantly high concentration of bioactive molecules that have imperative role in wound healing and tissue homeostasis (Anitua et al., 2004; Fréchette et al., 2005; Henderson et al., 2003; Lacci & Dardik, 2010). Although, many studies have reported on healing and regenerative effect of PRCs in orthopaedics and plastic surgery, their regenerative role in dental pulp healing and regeneration has not been reported widely.

1.3 Research questions

With the current understanding pertaining to research topic, following research questions are framed for further investigations:

1. Can inflamed microenvironment be induced using bacterial lipopolysaccharide?
2. Could media supplement from autologous PRCs be used for DPSCs expansion in-vitro?
3. Are PRCs capable of inducing angiogenesis in iDPSCs?

Hypothesis of current study is that "Human autologous platelet rich concentrates have healing and angiogenic effect on inflammation induced DPSCs (iDPSCs)"

1.4 Aims and objectives

In line with the proposed hypothesis, current study is divided into four interconnected aims:

Aim 1: To isolate and characterise isolated DPSCs for mesenchymal properties.

Objectives:

1. To isolate DPSCs from human extracted adult dental pulp tissue.
2. To characterise and validate mesenchymal like properties of DPSCs.
3. To determine the population doubling time of DPSCs at various subcultures.

Aim 2: To induce inflammation using bacterial lipopolysaccharide (LPS) and evaluate the maintenance of stemness in DPSCs in inflamed state (iDPSCs)

Objectives:

1. To determine an optimal concentration of LPS through cell viability, gene expression and cytokine release profile.
2. To evaluate the maintenance of MSC characteristics via MSC specific surface marker expression in iDPSCs.

Aim 3: Optimization of concentration of two PRCs (HPL and PRP) for their viability and pro-angiogenic effect on DPSCs.

Objectives:

1. To optimise the suitable concentration of HPL and PRP to maintain viability of DPSCs, comparable to FBS in -vitro.
2. To determine the pro-angiogenic effect of optimised concentrations of HPL and PRP on DPSCs.

Aim 4: Optimization of concentration of HPL and PRP to induce angiogenesis in inflammation induced DPSCs (iDPSCs).

Objectives:

1. To optimise the suitable concentration of HPL and PRP to maintain viability of iDPSCs
2. To determine the pro-angiogenic effect of optimised concentrations of HPL and PRP on iDPSCs.

1.5 Importance of Study

DPSCs have been studied widely in the past for their properties of being highly proliferative and multipotent. However, their behaviour in inflamed microenvironment has not been reported widely. In the present study, we intend to investigate three significant areas. First, to establish a LPS based in-vitro inflammatory microenvironment. The purpose of this part of the study is to explore the viability and pluripotent behaviour of stem cells in the presence of inflamed microenvironment. This will help us to investigate regenerative modalities in DPSCs in an inflamed microenvironment, which is a typical situation in a clinical scenario. Second, we will optimize the most suitable concentration of two types of PRCs that can support viability and exhibit pro- angiogenic effect on DPSCs. Thirdly; the angiogenic effect of optimised concentrations of HPL and PRP will be established on iDPSCs. This study will help us to gain an insight into the angiogenic potential of two autologous PRCs on DPSCs cultured in inflamed microenvironment. The workflow in Figure 1.1 provides an overview of this study.

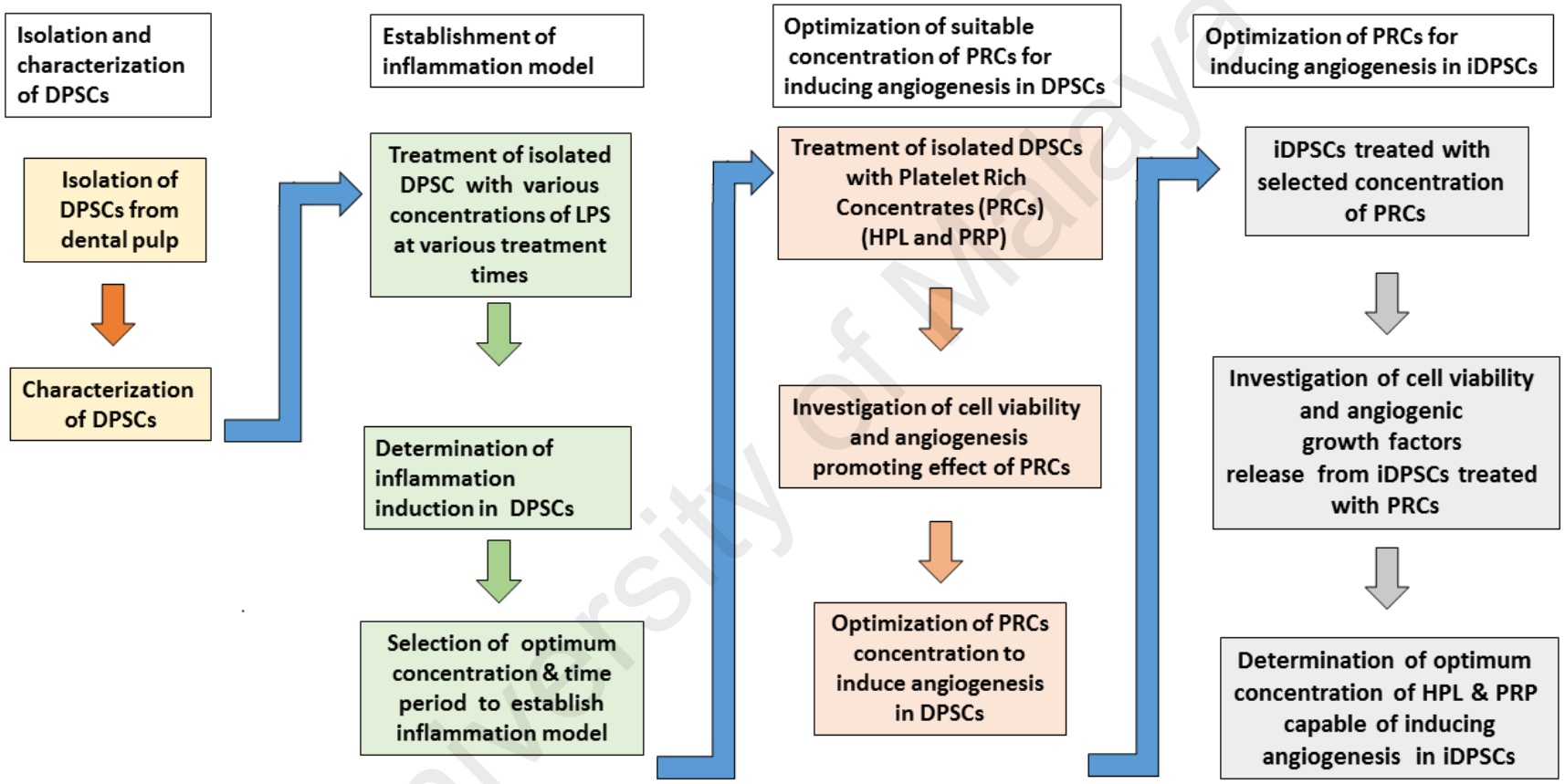

Figure 1.1: Schematic representation of workflow of the study

CHAPTER 2: LITERATURE REVIEW

2.1 Dental Pulp

Dental pulp is a connective tissue of mesenchymal origin located in the centre of a tooth. It consists of specialized cells, odontoblasts, arranged peripherally in direct contact with dentin matrix. This association between odontoblasts and dentin is known as "pulp-dentin complex (Orchardson & Cadden, 2001). Apart from providing support and maintenance to tooth, it also triggers immune response against any incoming harmful invasion. Pulp vitality is imperative for the tooth longevity, since it provides nutrition and detects pathogenic stimuli. The vitality of the dental pulp both during health and after injury, depends on pulp cell activity and the signalling processes that regulate the cells behaviour (Ahmed, 2011).

2.2 Dental Pulp Inflammation

Throughout life, pulp goes through a series of physiological and pathological changes like age changes, mechanical and chemical irritation, trauma and microbial invasion to name a few. Amongst them, microbial infection arising from dental caries is considered to be the most common cause of pulp inflammation and eventually tooth loss (Caufield & Griffen, 2000). Main etiological agent of dental caries is the adipogenic gram- negative oral bacterial. Lipopolysaccharides (LPS), an antigenic component of gram-negative bacterial cell wall, is an important mediator for inflammatory reaction of dental pulp in response to caries (Khabbaz et al., 2000) and is reported to be largely found in peri-apical lesions and root canal of infected tooth (Barthel et al., 1996). When LPS from gram-negative bacteria comes in contact with pulp tissue, it leads to an interplay of T-lymphocytes and other neuropeptides, thus triggering immunological reactions,

causing the release of various interleukins namely IL-6, IL8 and TNF alpha. These factor are the characteristics of a classic immune reaction involved in acute inflammation (Hahn & Liewehr, 2007; Jontell et al., 1998).

Pulp inflammation is characterized by an increase in blood flow in the pulp cavity that has two effects., namely faster spread of infection and excessive pressure on surrounding nerve tissues causing pain (Heyeraas & Kvinnsland, 1991). Since, it is surrounded by intact hard tissues namely enamel and dentine, undissipated vascular pressure in the pulp cavity leads to increased blood flow in pulp canal experienced as pain (K Hargreaves & Goodis, 2002; Heyeraas & Berggreen, 1999) .

Radicular pulp therapy involves removal of infected pulp tissue, and sealing the empty space with synthetic material. However, over time, the root treated tooth became brittle and prone to fracture. In terms of aesthetics, endodontic therapy can often result in discoloration of the tooth crown, mainly due to staining from endodontic filling material (Glickman & Koch, 2000). Thus, an effective treatment strategy is required to regain vital dental pulp to treat dental pulp diseases. The emergence of modern tissue engineering and regenerative medicine has opened possibilities for dental pulp regeneration (M Nakashima & Iohara, 2011).

2.3 Dental Pulp Tissue Engineering

Tissue engineering is an emerging interdisciplinary science, aiming to develop strategies for regeneration of damaged organs and tissues, based on principles of engineering and life sciences. It is interplay of three essential components: scaffolds, responsive cells, and signalling molecules. Reactive cells are generally stem cells, which are undifferentiated cells capable of both self-renewal and multilineage differentiation. Regenerative medicine comes into play, when the body uses its own systems, with the help of foreign

biological material to recreate cells and rebuild tissues and organs (KM Hargreaves et al., 2008; Lanza et al., 2011; Mason & Dunnill, 2008) .

2.3.1 Scaffolds

Scaffold is a 3D framework serving as an extracellular matrix for cells. An ideal scaffold should facilitate attachment, migration, proliferation, and three-dimensional spatial organization of the cell population (Hutmacher, 2000). Alongside, scaffold should enable diffusion of vital cell nutrients and expressed products required for structural and functional replacement of the target tissue (Hutmacher, 2000). For effective dental pulp tissue engineering strategies, the choice of an appropriate scaffold is paramount. Since, there is a direct interaction of stem cells and growth factors with the scaffold; biocompatibility is of utmost importance to avoid adverse tissue reactions. (Galler et al., 2011a). The most desirable scaffold for dental pulp regeneration should allow contamination control in the root canal, vascularization and innervation of a long and narrow space, while incorporating growth factors (Flavio F Demarco et al., 2010; W Zhang et al., 2006).

Biomaterials employed for making scaffold can be synthetic or natural (Sharma et al., 2014). Synthetic biomaterials can be customized to obtain shape, cell differentiation properties, mechanical and chemical characteristics especially the strength, pore characteristics and degradation rate (Basu et al., 2010; Galler et al., 2011b). However, they may lack cell adhesion and sometimes lack appropriate compatibility. Pulp tissue engineering has been attempted by growing pulp cells onto synthetic polymer scaffolds *in-vitro* and *in-vivo* (Rutherford, 1999).

The two categories of materials that are most commonly used in pulp tissue engineering synthetic polymers such as poly(lactic) acid (PLA), poly lactide-co- glycolic acid (PLGA) (Galler et al., 2011a) and poly glycolic acid (PGA) (Marler et al., 1998). Other synthetic materials tested for and dentine pulp complex regeneration include

inorganic calcium phosphate materials like hydroxyapatite (HA) or beta tricalcium phosphate (β TCP) (Zheng et al., 2011).

Natural biomaterials used in pulp regeneration include (a) proteins like collagen, silk (Prescott et al., 2008) and fibrin (Shivashankar et al., 2012), polysaccharides like chitosan (Coimbra et al., 2011), hyaluronic acid (found in synovial fluid and ECM connective tissues), alginate and agarose (made up of 90% of water) (Cheung et al., 2015; Glowacki & Mizuno, 2008). Contrary to synthetic biomaterials, natural biomaterials have the advantage of good cellular compatibility in terms of cell survival and function hence better performance, and biocompatibility. A promising self-assembling peptide hydrogel-based nanofibrous scaffold has been developed with the commercial name Puramatrix™ (Rosa et al., 2011). A combination of SHED stem cells and Puramatrix was administered inside the full-length root canals in order to form a pulp-like tissue (Prescott et al., 2008). The macroporous polymer scaffolds fabricated by polymer casting have also been utilized for pulp tissue engineering (Sakai et al., 2004).

In all the above mentioned studies involving scaffolds, it was found that these scaffolds support cell attachment, proliferation and cell differentiation. However, their high intrinsic stiffness raised clinical concerns in terms of scaffold attachment to dentine walls along the root canal length, a key feature for successful pulp regeneration.

A sufficiently vascularized pulp tissue was noticed with new dentine deposition in pulpotomized pulp-chamber spaces of dog teeth filled with collagen scaffold (Iohara et al., 2009). A human case study reported that using collagen scaffold with blood clot in the root canal resulted in a functional vascularized pulp-like tissue, with positive response to electric pulp testing (Garcia‐Godoy & Murray, 2012). A recent study reported pulp-like tissue formation by utilizing silk fibroin scaffold loaded with basic fibroblast growth factor (bFGF) and seeded with DPSCs (Gao et al., 2015). A combination of stem cells and inductive scaffolds could be employed to enhance the efficacy of dentin-pulp

complex regeneration (Galler et al., 2011a). However, they have limited range of mechanical properties and lack of control over pore size. Recently platelet rich plasma successfully created a scaffold for regenerative endodontic treatment (Galler et al., 2011b). In a study by Zhang et.al it was demonstrated that a reconstituted collagen and PRP scaffold showed promising results in terms of cell adhesion and growth factors release (Bezgin et al., 2015). These studies show that single matrix may not be an ideal scaffold material. Hybrid scaffolds may be best suited keeping in view the variations in tissue composition (Zhang et al., 2016). However, a balanced interplay between scaffold, stem cells and growth factors is essential to initiate regeneration process.

2.3.2 Stem Cells

Stem cells are specific cell population that have the potential of self-renewal and growth. Mesenchymal stem cells (MSCs) are heterogeneous subset of stromal stem cells and are isolated from various adult tissues (Sharma et al., 2014). Human Mesenchymal Stem Cells (hMSCs) are the multipotent stem cells with the capacity to differentiate into mesodermal lineage such as osteocytes, adipocytes, chondrocytes as well as ectodermal (neurocytes) and endodermal lineages (hepatocytes) (Uccelli et al., 2008). Stem cells of dental origin have recently gained attention as they are easy to obtain, available all throughout life and have equivalent properties to that of embryonic stem cells that are considered benchmark of pluripotent stem cells (Horst et al., 2012; Ullah et al., 2015). They have shown to exhibit angiogenic potential: by (1) secreting angiogenic factors that induce angiogenesis from host endothelial cells (Huang et al., 2009a) and (2) by differentiating themselves into endothelial cells and thus actively incorporating into the walls of blood vessels (Gandia et al., 2008). The dental stem cells can be obtained from various dental tissues such as human exfoliated permanent teeth (DPSCs), dental follicle (DFPCs), periodontal ligament (PDLSCs), and from apical papilla (SCAPs) (Sieveking

& Ng, 2009). Sharpe and Young in 2005 pioneered the use of stem cells in dental tissue engineering (Seo et al., 2004; Sonoyama et al., 2006; HH Sun et al., 2011).

DPSCs are present in cell rich zone in dental pulp and usually remain quiescent when they are within the dental pulps, but respond quickly after injury. DPSCs have a high proliferative capacity and immediately differentiate into odontoblasts (Sharpe & Young, 2005), osteoblasts (J Yu et al., 2006) and chondrocytes (R d'Aquino et al., 2007) to produce dentin, bone, and cartilage tissues respectively for this repair process (Y Yamada et al., 2006). They were first introduced, as one of the favourable stem cell source for the practice of regenerative medicine (Potdar & Jethmalani, 2015). They are isolated from dental pulp of deciduous and permanent teeth and have many benefits over other stem cells sources. Unlike controversial embryonic stem cells, dental pulp offers source of stem cells (in post-natal phase) that are, a readily available and can be obtained with minimally invasive procedure.

Various studies have shown that these cells have unique features of stem/progenitor cells having the capacity to differentiate into dentin forming odontoblasts (Gronthos et al., 2000). The roots of the third molar are often incomplete at the age of eighteen, therefore these teeth contains a conspicuous pool of undifferentiated cells, resident within the dental pulp (Loomba et al., 2012). In an *in vitro* model, DPSCs were derived from supernumerary mesiodens' at various stages of tooth development, demonstrated that DPSCs derived at the stage of crown development are more proliferative than at later stages (Gandhi et al., 2011; Loomba et al., 2012).

Previous studies have compared the regenerative potential of DPSCs with the gold standard of MSCs that were obtained from bone marrow (BMSCs). It was reported that DPSCs are mesenchymal in nature due to the properties like adherence to plastic surfaces, expression of stem cell surface markers and trilineage differentiation potential. Recently, stem cells obtained from the dental pulp tissue have been shown to diffrentiate into

adipose and neural cells (Huang et al., 2009b). In addition to their capacity to regenerate dentine, regeneration of periodontal tissues and skeletal articular tissues of craniofacial region, DPSCs have also been reported to be effective in the treatment of neurotrauma, autoimmune diseases, myocardial infarction, muscular dystrophy and connective tissue damages *in-vitro* (Chang et al., 2014).

2.3.3 Growth factors

Growth factors (GFs) are the protein based signalling molecules that bind to specific cell membrane receptors and control the activity of stem cells e.g. by regulating the rate of proliferation, inducing differentiation into another cell type, or by stimulating cells to synthesize mineralized matrices (X Yang et al., 2010). A cocktail of GFs like VEGF, FGF, BMP and TGF beta are involved in regenerative process. These growth factors, in combination with other transcription factors, then activate a set angiogenesis promoting genes. The growth factors also induce specific changes at the cellular level. All of these effects are controlled by feedback mechanisms involving binding proteins and other growth factors (Stevens et al., 2005). These molecules play key role in dentine pulp complex repair by initiating signalling cascade (Carlson & Roach, 2002). The growth factors of BMP family play an important role in the biology of pulp cells. Studies have shown that the expression of BMP-2 is increased during reparative dentine formation (Tziafas, 2003). In a study on pulp of mature teeth displayed evident formation of new capillary formation, directly underneath the odontoblastic layer, through the regulatory effect of growth factors. Examples of growth factors involved in pulp biology are several members of the transforming growth factors (TGF-b), the bone morphogenic proteins (BMP-2, 4, 7, 11), fibroblast growth factor (FGF), insulin growth factor (IGF), vascular endothelial growth factor (VEGF) (Misako Nakashima & Reddi, 2003). Growth

factors embedded in scaffold with bioactive molecules such as growth factors guide the tissue formation or network vessel development (Wei et al., 2006).

It is well known that successful tissue engineering relies on the establishment of an effective vascular network to sustain the high metabolic activity of cells that are engaged in tissue regeneration (Sloan et al., 2000). To address this we need strategies such as embedding angiogenic factors into the scaffold to promote ingrowth of micro vessels have been proposed (Flávio Fernando Demarco et al., 2011). Numerous growth factors and environmental conditions contribute to vascular network formation. Studies have shown that vascular endothelial growth factor (VEGF), a pro-angiogenic factor induces stem cell differentiation into endothelial cells (Chrobak et al., 2006). VEGF induced DPSCs to acquire endothelial cell-like features when cultured in a 3-d fibrin mesh, displaying capillary-like structures (Sakai et al., 2010). When SHED cells were cultured in collagen matrices, they organized themselves into capillary structures that resemble micro vessels. Also, VEGF enhanced the differentiation of SHED into vascular endothelial cells (Marchionni et al., 2009). Possible sources of these GFs are mainly extra cellular matrix (ECM) mimicking polymer matrices and spatiotemporal regulation of single or multiple externally injected growth factors (Sakai et al., 2010). In majority of the tissue engineering techniques involving growth factors, allogeneic and commercially produced growth factors are utilized. Serum-free culture media are formulated commercially by trial and error to suit specific cell lines (Lee et al., 2011). Serum-free blends offer the possibility to eliminate most of the problems like xenogeneic contamination and others. Growth factors and other substances supplied to serum-free media need to be thoroughly purified and scanned.

One of the most promising natural source of sustained and autologous growth factors release is human blood platelets, that are known to release a cocktail of growth factors during wound healing process (Tekkatte et al., 2011).

2.3.4 Human Platelets: Biological Role

Platelets, or thrombocytes, are enucleated products of large multi-nucleated cells in the bone marrow called megakaryocytes. Each megakaryocyte can produce thousands of platelets during thrombopoieses by the formation of long pseudopods. Platelets contain numerous growth factors that are known to aid in tissue repair and angiogenesis (Blair & Flaumenhaft, 2009).

Platelets contain α-granules and dense granules. The α-granules are rich in various soluble factors such as coagulation factors, thrombolytic molecules, growth factors and chemokines. Alpha granules affect wound healing through several types of growth factors including the following: platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), endothelial growth factor, fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor beta (TGFβ), and insulin-like growth factor (IGF). In general, these factors chemotactically recruit and activate stem cells as well as induce their mitogenesis and differentiation. In contrast, dense granules promote tissue regeneration by secreting mediators such as serotonin and histamine, which increase vessels permeability and tissue perfusion (Liao et al., 2013).

Products enriched in platelet products also support cellular proliferation of osteogenic and tendon cells among others (Blair & Flaumenhaft, 2009). These factors initiated the use of platelet rich concentrates (PRCs) in the treatment of orthopaedic defects and further research is focused on the potential clinical role of platelets apart from haemostasis (Feltsan et al., 2010). Recently, platelet rich concentrates have gained attraction due to release of cocktail of GFs from platelets that promote tissue repair, angiogenesis, inflammation and immune response. These GFs create chemotactic gradients favouring the recruitment of the stem cells, stimulating cell migration, differentiation, and promoting repair. Thus, the use of autologous platelet concentrates is a promising application in the field of tissue regeneration and can be used in clinical

situations requiring rapid healing. The composition of platelets derived serum include variable amounts of growth factors and cytokines, like epidermal growth factor (ErGF), endothelial growth factor (EGF). bone morphogenetic proteins (BMPs), platelet derived growth factor (PDGF) just to name a few and they are mostly uncharacterised with distinct lot-to-lot variation which may influence their reproducibility (do Amaral & Balduino, 2015; Herrera & Inman, 2009).

2.3.5 Platelet rich concentrates (PRCs)

Being autologous in nature and rich in growth factors, PRCs are the most sought after natural non-immunogenic and economical source of growth factors (Salvade et al., 2009). Use of platelet concentrates as a therapeutic treatment to enhance healing and control inflammation has gained attention in sports medicine and orthopaedics (Mehta & Watson, 2008). By facilitating recruitment, proliferation, and maturation of cells participating in regeneration of tendon, ligaments, bone, and cartilage, platelet concentrates may prove beneficial for tissues with restricted blood supply, slow cell turnover, and limited extracellular matrix restoration (Ehrenfest et al., 2014).

The advantages of PRCs are their autologous nature, simple collection, easy chair side preparation, and clinical application without the risks associated with allogeneic products. Currently, various preparations of PRCs are being utilized in orthopaedics and sports medicine (Boswell et al., 2012), dental maxillofacial surgery (Lopez-Vidriero et al., 2010), wound healing (Del Fabbro et al., 2011) and hair regrowth (Frykberg et al., 2010).

A study on MSCs cultured in PRP, reported that PRP enhance MSCs proliferation without compromising differentiation capacity or immunophenotype. Suggesting PRP to be a possible animal-free medium supplement for MSCs culture (Valente Duarte de Sousa & Tosti, 2013). However, PRP requires a complicated manufacturing process, while HPL, in contrast, can be produced by simple freeze thaw cycles of platelet units, being rich in growth factors released from the platelet fraction (PDGF, IGF-1, bFGF and TGF-

b1) (Bieback et al., 2009; Kocaoemer et al., 2007). Contrary to PRP that requires pre-activation of platelets to release growth factors, human platelet lysate (HPL) does not require activation and contains cocktail of growth factors in active state with cell proliferative effect. HPL has proven as an efficient substitute of FBS for MSCs expansion, with cells showing higher proliferation (Bieback et al., 2009; Lohmann et al., 2012). Azouna and colleagues demonstrated in their study that HPL-containing media not only allowed cells to preserve their phenotype, as well as their differentiation capacity but also shorten culture time by increasing their growth rate (Azouna et al., 2012). HPL remains one of the most efficient FBS replacement for large-scale expansion when compared with other currently available human blood-derived alternatives (Azouna et al., 2012). HPL is a derived from human blood plasma derived and is readily available in most hospital and transfusion centres. Since, HPL is prepared from PRP obtained from donors of different age, gender and ethnicity , there are possibilities of batch to batch variation In order to minimize these batch-to-batch variation, HPL obtained from different donors are pooled together (Tekkatte et al., 2011). Recently, Lohmann and colleagues demonstrated that there is a significant effect of HPLs donors age on stem cell expansion rate (Lohmann et al., 2012).

There is also a possibility of contamination from adventitious agents that might be present in these blood-derived substitutes. However, this can be minimized by following the blood bank quality standards.

Ideally, for a growth medium intended for MSCs culture in the clinical arena, serum should be substituted with human-derived supplements including human growth factors, hormones and proteins. In the search for an adaptable solution for serum replacement, human blood-derived elements have been suggested, such platelet rich plasma (PRP) and platelet lysates (HPL) (Lohmann et al., 2012). These are viable choices since it would both prevent cellular contact to FBS, hence limiting immune reactions

(Mannello & Tonti, 2007). During a state of disease, clinically relevant numbers of cells is required, which is an unlikely scenario by just using serum and plasma for cell expansion and is beyond donor capabilities (Pérez‐Ilzarbe et al., 2009). Pooling plasma or serum to obtain a heterogeneous solution has been attempted but the results are contradicting, with some studies claiming successful isolation and expansion of MSCs while others describe poor proliferation rates and growth arrest (Pérez‐Ilzarbe et al., 2009). Even though serum and plasma don't fully support the growth of MSCs, adding platelets or platelet secretory products has been shown to greatly improve the expansion while maintaining pluripotency characteristics (Mannello & Tonti, 2007; Pérez‐Ilzarbe et al., 2009).

2.3.6 Types of platelet rich concentrates

PRCs namely the platelet-rich plasma, platelet rich fibrin and human platelet lysate were categorized depending on their preparation technique and leucocyte-fibrin content.

i) Platelet-rich plasma (PRP)

Platelet-rich plasma (PRP) is a fraction of plasma that can be produced by centrifugal separation of whole blood. Since PRP contains multiple growth factors in high concentration, autologous PRP has been applied clinically in orthopaedic and sports injuries to facilitate bone and tissue healing (Sankaranarayanan et al., 2011; Schallmoser et al., 2007). In an animal study conducted on an injured cartilage of mice it was reported that PRP stimulates cell proliferation and extracellular matrix metabolism by articular chondrocytes, being a simple and safe new approach to the engineering of cartilage constructs (Kanno et al., 2005). PRP is high in the concentration of platelets within a relatively low volume of plasma. Due to high growth factor concentration and potential benefits in tissue healing, PRP has been successfully used to treat orthopedic disorders (Akeda et al., 2006). Reportedly, PRP also displays antimicrobial activity against E. coli

and S. aureus, evidently beneficial in the clinical environment (Redler et al., 2011). As a supplement for MSCs growth media, PRP has proven to enhance cellular proliferation and retain and even improved chondrogenic and osteogenic differentiation (Redler et al., 2011). PRP requires activation to release growth factors that is accomplished by adding calcium or thrombin to PRP, as shown in Fig 2.1 adapted from (Mishra et al., 2009).

For preparation of pure platelet rich plasma (PRP), 350mL of blood is collected into tubes with anticoagulants. Collected blood is then subjected to differential ultracentrifugation (3000g). The different blood components, such as platelets, leucocytes and RBCs, are first separated from the platelet poor plasma and the first buffy elements in the serum, are collected into a separate bag as the platelet concentrate (PRP). This PRP requires activation for triggering the release of growth factors. Activation of PRP is done by either calcium chloride or thrombin. Second type of PRC is leucocyte platelet rich plasma (L-PRP). This fraction of platelet concentrate contains a high quantity of leucocytes compared to conventional PRP. In the preparation of L-PRP after first centrifugation, blood components are separated into three layers ; RBCs, buffy coat and platelet poor plasma (PPP). The combined layer of buffy coat and PPP makes L-PRP. This is also activated by bovine thrombin and calcium chloride similar to PRP. Both these fractions of PRP dissolve easily and make a weak matrix. PRPs were shown to strongly stimulate proliferation of MSCs but inhibited their differentiation. This method for tissue engineering with MSCs and PRP might be relevant in reconstructive craniofacial surgery or in orthopaedics.

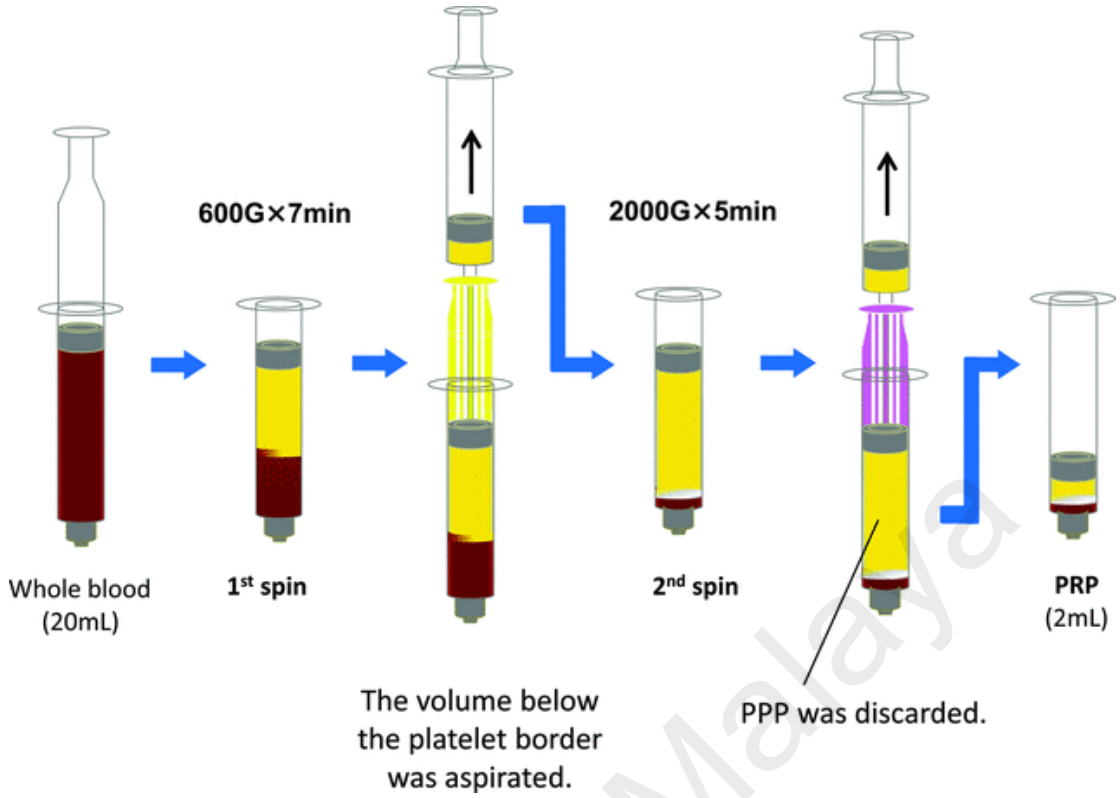

Figure 2.1: Step by step procedure for preparation of platelet rich plasma.

adapted from (Mishra et al., 2009)

ii) Platelet rich fibrin (PRF)

Another type of PRC is platelet rich fibrin (PRF). This is again sub classified into pure platelet rich fibrin (P-PRF) and leucocyte platelet rich fibrin (L-PRF). For preparing PPRF, small amount of blood (10mL) is drawn into collection tube containing anticoagulant and centrifuged at high speed. Out of three, typical layers RBCs, buffy coat and PPP, buffy coat and PPP are transferred into another tube with calcium chloride. The tube is then immediately centrifuged for 15 min with clotting process triggered. After centrifugation, platelet rich fibrin matrix (PRFM) clot is obtained.. LPRF also called Choukron's PRF is a second-generation platelet concentrate produced without any anticoagulants or jellifying agents (Kushida et al., 2015). Due to the absence of anticoagulants and platelet activation, fibrin polymerization is triggered immediately. After first centrifugation three layers of RBC, acellular plasma top layer and a PRF clot is formed in the middle. This PRF clot forms a strong fibrin matrix with complex three-

dimensional architecture, rich in platelets and leucocytes. PRF clot becomes a strong ready to use membrane, when pressed between two gauges (Liao et al., 2013). Its application include oral maxillofacial and plastic surgery (Dohan et al., 2007). Unlike the PRP, Choukroun's PRF does not dissolve quickly after application; instead, the strong fibrin matrix is slowly remodelled in a similar way to a natural blood clot. Other advantages of PRF over PRP include low cost and the great ease of the procedure allowing the production of many concentrates quickly and by natural means, without the use of chemicals or unnatural conditions. Therefore, this method seems to be most suitable for widespread use in clinics (Braccini & Dohan, 2006).

iii) **Human platelet lysate (HPL)**

Lysing the platelets in PRP, and thereby releasing the growth factors into the plasma, is one possibility that is gradually gaining more and more attention. Platelet lysates can be prepared from PRP by mechanically lysing of platelets, in which PRP units are frozen and subsequently thawed again at 37°C, resulting in platelet rupture and growth factor release (Ehrenfest et al., 2009b). Human platelet lysates (HPL) is a promising supplement for MSCs. Since, it allows MSCs to retain their basic characteristics and immunomodulatory abilities to the same extent as MSCs cultured in FBS (Tekkatte et al., 2011). MSCs have been reported to proliferate at a higher rate when cultured in HPL compared to FBS, without showing chromosomal abnormalities (Schallmoser et al., 2007). Similar results were exhibited with other cells such as renal epithelial cells and myocardial fibroblasts (Sankaranarayanan et al., 2011). Many growth factors found in HPL are known to enhance osteogenic differentiation of MSCs on osteogenic inducing environment (Rauch et al., 2011). The discovery of the potential use of platelets as growth medium supplement is of major interest and an important step towards the development of serum-free growth medium for MSCs. Since HPL supplemented growth media seems

to have positive effects on other cell types as well, it could be option for serum-free, culture media that is autologous in nature and is not restricted to a special cell line (Verrier et al., 2010).

For preparing HPL, PRP prepared from 8-10 donors are pooled together and subjected to freeze thaw cycles, leading to lysis of platelet, releasing their contents. A mix of bioactive trophic factors is separated from cellular debris through subsequent centrifugation as shown in Fig 2.2, *adapted from (Tekkatte et al., 2011).*

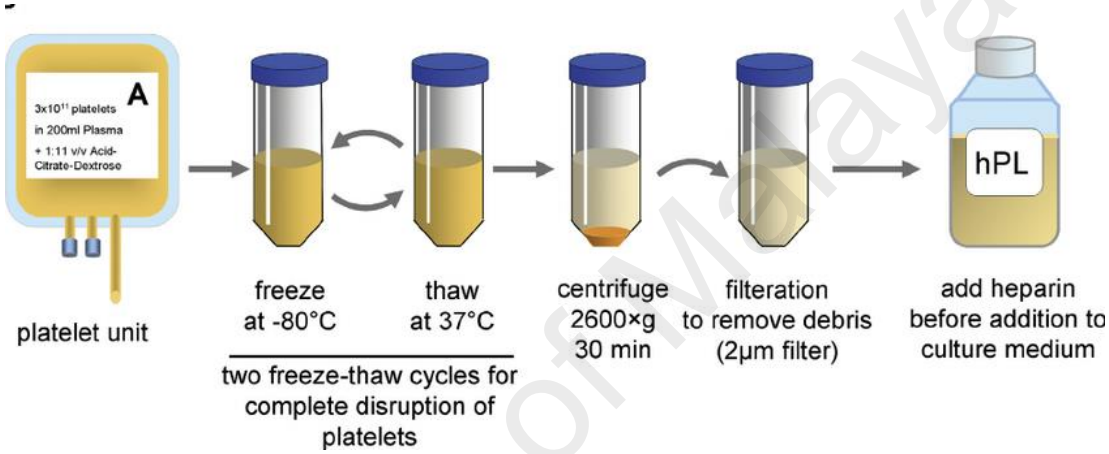

Figure 2.2: Step by step procedure for preparation of human platelet lysate.

Mechanical lysis of platelets to retrieve platelet growth factors is faster, easy and more economical method than chemical activation of platelets by through extrinsic agents like bovine thrombin and calcium chloride. In addition, it reduces the risk side effects that may occur due to the use of bovine thrombin (Kushida et al., 2015). To maintain the stability of growth factors, HPL is stored at −20 °C for a long time spans (Bieback, 2013). Previous studies have shown that HPL supports the isolation and expansion of MSCs. In addition, it is also favourable for maintaining the differentiation capacity of MSCs especially to osteogenic and chondrogenic lineages (Rauch et al., 2011).

Table 2-1: Comparison of three platelet rich concentrates

HPL	PRP	PRF
Applicable for variable cell types. Used in particular for human MSCs and fibroblasts	Used for expansion of human MSCs at higher concentrations.	Used in clinical set up as surgical adjunct to seal the open wounds.
Enriched in growth factors released after the lysis of platelets	Trophic factors are secreted from alpha granules of platelets, after activation with calcium chloride or thrombin	High concentration of growth factors compared to other preparations
Proliferation of MSCs—particularly of MSCs derived from adipose tissue—is significantly faster than in FBS.	Proliferation of MSCs at higher concentration (20%) is comparable to that of FBS	Not widely used in stem cell culture experiments
HPL is easily generated by freeze-thaw procedures (prepared from expired platelet units) followed by centrifugation to remove platelet debris.	Prepared by gradient centrifugation of fresh blood	Prepared by gradient centrifugation of fresh blood
Has been used in clinical trials—no critical side effects were reported.	Already in use in clinical set up for cosmetic surgery, periodontal surgery and wound repair	Used chair side as fibrin glue preparation for sealing open wounds
No threat of xenogeneic induced immune response or transmission of prions from bovine source. Can be used in autologous settings to reduce risks of contamination or immune reactions.	No threat of xenogeneic induced immune response or transmission of prions from bovine source. Can be used in autologous settings to reduce risks of contamination and immune reactions.	No threat of xenogeneic induced immune response or transmission of prions from bovine source. Can be used in autologous settings to reduce risks of contamination or immune reactions.
Not precisely defined; yet, platelet units might be more standardized than bovine foetal blood.	Composed of platelet derived growth factors, leucocytes, clotting factors	Composed of concentrated platelet derived growth factors, leucocytes
Variation exists between individual HPLs, which can be reduced by pooling.	No lot to lot variation	No lot to lot variation

HPL is initiated by some culture companies for commercial distribution	Prepared chair side by commercially available machines	Prepared chair side by commercially available machines
Less probability of endotoxin contamination	No endotoxins due to autologous preparation	No endotoxins due to autologous preparation
Danger of transmission of human diseases by known or unknown viruses such as human immunodeficiency virus and human t-lymphotropic virus in allogeneic setups (quarantine storage cannot completely exclude this risk). Contamination with mycoplasma should be excluded.	No chances of cross contamination due to autologous preparation	No chances of cross contamination due to autologous preparation

Partially adapted from (Doucet et al., 2005).

2.3.7 Platelet rich concentrates in dentistry

In dentistry, effect of PRP on bone regeneration has been investigated extensively. It has been used in different clinical procedures (i.e. sinus floor elevation, alveolar ridge augmentation, mandibular reconstruction, maxillary cleft repair, treatment of periodontal defects and treatment of extraction sockets), where it has been applied alone or in addition to autogenous bone, inorganic bone mineral and organic bone substitutes (Hemeda et al., 2014). Rationale of PRP addition to bone grafts is that high concentrations of platelets in a bony wound will increase the local concentration of secreted growth factors and subsequently enhance the initial bone-healing response. Later on, the direct influence of PRP fades away and physiological mechanisms of bone repair will continue to work on an accelerated level (Camargo et al., 2002; Whitman et al., 1997). PRP has also been investigated for its potential in dental pulp regeneration. Studies have shown that that PRP is able to stimulate root thickening in immature root in an animal model (Jakse et al., 2003). However, formation of pulp like connective tissue was not evident. In similar studies done on beagle dogs did not report any hard or soft tissue formation on treatment with PRP (W Zhu et al., 2013; X Zhu et al., 2012). PRF consists of a matrix of autologous fibrin and has several advantages over PRP, including easier preparation and not requiring chemical manipulation of the blood, which makes it strictly an autologous preparation (Dohan et al., 2006). PRF is a material with a great potential for bone and soft tissue regeneration. It may be used alone or in combination with bone grafts, promoting haemostasis, bone growth, and maturation. This autologous matrix demonstrated a great potential to increase cell attachment (Kiran et al., 2011) and a stimulation to proliferate and differentiate osteoblasts (Wu et al., 2012). PRF has immunological and antibacterial properties, may lead to leukocyte degranulation, and has some cytokines that may induce angiogenesis and pro/anti-inflammatory reactions (Ehrenfest et al., 2009a). A clinical case study done on a traumatised central incisor with incomplete immature root and

necrotic pulp showed that, on using PRF in a sterile root canal, it showed an evident dentine tissue formation, resulting in root end closure (Dohan Ehrenfest et al., 2010).

HPL is another fraction of PRCs, that has been reported to be an efficient culture media supplement compared to commonly used foetal bovine serum (FBS) and has demonstrated efficient scaling up of MSCs (Shivashankar et al., 2012). DPSCs cultured in presence of HPL exhibited a higher healing rate after injury and are less susceptible to toxicity mediated than those cultured with FBS (Govindasamy et al., 2011). However, HPL has not been investigated widely for its role in tissue regeneration.

This study will evaluate the biologic effect of platelet rich concentrates on the proliferation and the differentiation of human dental stem cells, and find the key cytokines inducing these effects to estimate the clinical feasibility of PRC for dental tissue engineering.

CHAPTER 3: ISOLATION AND CHARACTERIZATION OF DENTAL PULP STEM CELLS

3.1 Introduction

Mesenchymal stem cells (MSCs) are pluripotent cells possessing two remarkable features: multi-potency and self-renewal (Nombela-Arrieta et al., 2011). Growing evidences demonstrated that dental pulp is a highly vascularized tissue and contains several niches of stem cells known as dental pulp stem cells (DPSCs) (Sumita et al., 2009) It is considered a rich and most accessible source of MSCs that can be easily isolated non-invasively from primary and permanent teeth (Huang et al., 2009a)

Dental pulp stem cells from adult teeth are known as DPSCs and stem cells obtained from human exfoliated deciduous teeth are known as stem cells from human deciduous teeth (SHED) (Miura et al., 2003). DPSCs are highly proliferative and have the potential to differentiate into several cell types, including odontoblasts, neural progenitor cells, osteoblasts, chondrocytes, and adipocytes (Dupin & Coelho‐Aguiar, 2013; Volponi et al., 2010). A study done on a tooth root model filled with DPSCs and further implanted into subcutaneous tissue of mice, demonstrated formation of pulp like tissue into the canal with adequate vascularization (Huang et al., 2009b). In another study by Gronthos et al. suggested that when ex-vivo expanded DPSCs were implanted along with hydroxyapatite, subcutaneously into mice, it revealed the formation of dentine pulp complex like structure with well-formed collagen network (Gronthos et al., 2000). These characteristics facilitate ex vivo expansion and enhance the translational potential of these cells (Casagrande et al., 2011).

According to the International Society for Cellular Therapy (ISCT), plastic adherence, expression of specific surface antigen (Ag) and multi-differentiation potential

are the minimal criteria for MSCs characteristics (Dominici et al., 2006). The aim of this study was to isolate and characterise DPSCs from primary source. Specific objectives were:

a. To isolate DPSCs from dental pulp tissue obtained from human extracted permanent teeth.
b. To characterise and validate the MSCs like properties of DPSCs.
c. To determine the population doubling time of DPSCs at various subcultures.

3.2 Material and Methods

3.2.1 Sample collection

Ethics approval for the sample collection and processing was obtained from the Medical Ethics Committee, Faculty of Dentistry, University of Malaya {DFDP 1304/002 (P)}. All samples were obtained after well-informed written consent from the patients. Freshly extracted sound premolars from five healthy donors (n=5) were collected at different instances from Department of Oral Surgery, Faculty of Dentistry; University of Malaya, from the patients, who were indicated orthodontic treatment. Exclusion criteria for the teeth selection involved patients with evident periodontitis, fractured tooth, patients with any history of pain and trauma. Extracted teeth were thoroughly cleaned using povidone-iodine solution in a 100mm petri dish. Debris and periodontal ligament on the root was cleaned using scalpel blade & washed with 1:1 solution of povidone iodine. A high-speed sterilized diamond disk was used to cut the tooth at the cemento-enamel junction. The pulp tissue was then gently extirpated using a small size barbed broach. A blunt non-cutting forceps was used to transfer the pulp tissue into a small centrifugation tube (BD Falcon) containing transport media and transported to the laboratory immediately.

3.2.2 Isolation and expansion of DPSCs

Culture media used in the experiment was prepared with Dulbecco's Modified Eagle's Media, knockout media (DMEM-KO) (Gibco, Invitrogen, USA), that was supplemented with 10% FBS (HyClone USA), 50units/mL Penicillin-Streptomycin (Gibco,Invitrogen, USA) and 2mM L-glutamax (Gibco, Invitrogen, USA). The enriched media was filter sterilized through a 0.2 μm syringe filter (Millipore, Thermoscientific, USA) and stored at 4°C until further use.

DPSCs isolation procedure was conducted under biohazard laminar flow hood. The extirpated pulp tissue was transferred into sterile centrifugation tubes containing transport media and washed twice using Dulbecco's phosphate-buffered saline solution (DPBS) supplemented with 1% antibiotic-antimycotic solution. Pulp tissue was then minced using sterile surgical scissors into smaller pieces and digested with 3-mg/mL collagenase type IV (Gibco, USA) enzyme for 30 minutes at 37^{o}C. The minced pulp tissue was then re-suspended into a 15 mL centrifugation tube (BD Falcon, USA) containing 6mL of complete media and centrifuged at 1250 rpm for 6 minutes. The supernatant was discarded and the cell palette was seeded in a T25 culture flask and incubated in a humidified incubator maintained at 37°C and 5% CO2 for cells to adhere. Cell outgrowth was observed from pulp tissue after 6 days. On reaching 70% confluence, DPSCs were sub-cultured at ratio of 1:4 (passage 1). In all experiments, the results represent average of three culture replicates.

3.2.3 Mesenchymal characterization of DPSCs

According to the International Society for Cellular Therapy (ISCT), MSCs should demonstrate plastic adherence, expression of specific surface antigen (Ag) and multi-differentiation potential (Dominici et al., 2006). In this study, mesenchymal

characteristics of DPSCs isolated from dental pulp were characterised by following analysis.

3.2.3.1 Plastic adherence of stem cells from human extracted permanent teeth

Plastic adherence of DPSCs was confirmed by observing the culture flasks under an inverted microscope (Leica Microsystems, Germany).

3.2.3.2 Expression of surface antigens on DPSCs using FACS Analysis

Flow cytometry was used to detect the expression of MSC specific surface antigen on DPSCs (at passage 3). The cells were stained with MSC phenotyping kit and measured using MACSQuant® analyser 10 (Miltenyl Biotech, Germany). This specialized type of flow cytometer provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time using FACS (fluorescence-activated cell sorting) technique. This technique is based upon the specific light scattering and fluorescent characteristics of each cell. Briefly, DPSCs yielded at passage 3 from five healthy donors (n=5) were first dissociated using 0.25% trypsin (Gibco, USA). Number of nucleated cells were counted using tryptan blue (Gibco) and aliquoted ($1x10^6$ cells each) into seven pre-labelled (i.e. 1, 2, PerCP, PE, APC, FITC and blank) in micro centrifuge tubes. Cells were centrifuged at 1250rpm for 10 minutes and supernatants were completely discarded. Cells in the tube labelled 'blank' were re-suspended into 500 µL of buffer (2% FBS containing DPBS) and kept in a container containing ice. Cells in the other six tubes were re-suspended into 100 µL buffer. 10 µL of MSC phenotyping cocktail containing the combination of (anti-human antibodies; CD14-PerCP, CD20- PerCP, CD34- PerCP, CD45- PerCP , CD73-APC, CD90-FITC, CD105-PE). An aliquot of 10 µL of isotype control cocktail (anti-human: mouse IgG1-FITC, mouse IgG1-PE, mouse IgG-APC, IgG1-PerCP, mouse IgG2a-PerCP), 10 µL of anti-human CD73-Biotin, 10 µL

of anti-human CD90-FITC were added into tubes labelled 1, 2, PerCP, PE, APC and FITC respectively. Cells were mixed gently and incubated for 10 minutes in the dark at 4ºC. After 10 minutes, Cells were washed by adding 1mL of buffer and centrifuged at 1250 rpm for 10 minutes. Supernatants were discarded completely. Except, for the tube labelled PerCP, cell palettes in all other tubes were re-suspended in 500 µL buffer. Tubes labelled 1 and 2 were used to analyse the specific antigen on DPSCs. Other tubes were used to compensate (standardise) the instrument.

After standardising the instrument, gating was performed to exclude cell debris and unwanted aggregates (FSC/SSC dot plot). Cell surface markers expression of Cluster of Differentiation (CD) factors CD73 (APC), CD90 (FITC) and CD105 (PE) were analysed.

3.2.3.3 Trilineage differentiation

To further elucidate the mesenchymal characteristics of isolated DPSCs, multi-lineage differentiation potential of DPSCs into osteogenic, adipogenic and chondrogenic lineages was investigated, using the standard induction media (StemPro differentiation kit, Invitrogen USA).

DPSCs yielded at passage three (n=3) were seeded into two six well plates at 5,000 cell/well and incubated with complete media. At 60% cell confluence, complete media was aspirated and washed thoroughly with DPBS. Set of three wells (three replicate wells for each test group) were washed thoroughly with DPBS. Following washing, the test wells were incubated with relevant differentiation (osteogenic, chondrogenic and adipogenic) media respectively as provided in the Stem Pro differentiation kit (Invitrogen). Three replicates for each differentiation marker were carried out. Media was changed every 3 days for 20 days for osteogenic differentiation, 25 days for chondrogenic differentiation and 14 days for adipogenic differentiation.

Osteogenic differentiation was analysed by Alizarin Red staining that was performed by washing the cells twice with PBS followed by treatment with 70% ice cold ethanol for 10 min, DPSCs were then rinsed with distilled water and incubated with Alizarin Red stain for 10 min at room temperature. Stained cells were then rinsed five times with distilled water and PBS to the cells for further observations. Observations were recorded under a phase contrast microscope (Leica Microsystems, Germany).

Chondrogenic differentiation was analysed by Safranin O staining. Media from culture wells was discarded and DPSCs were washed once with DPBS. DPSCs were then fixed with 4% formaldehyde solution for 30 minutes at room temperature. Following fixation, cells were washed with DPBS and cells were stained with 1% Safranin O solution prepared in 0.1 N HCL for 30 minutes. DPSCs were washed three times with 0.1 N HCL. Following washing, distilled water was added to the DPSCs to neutralize the acidity. After neutralization observations were recorded under a phase contrast microscope (Leica Microsystems, Germany).

Adipogenic differentiation was analysed by oil-red O staining. DPSCs were rinsed with DPBS twice and then fixed with 10% (v/v) formalin for half an hour at room temperature. Oil red O working solution was freshly prepared by mixing three parts Oil red O stock solution with two parts of deionised water. Solution was allowed to rest for 10 minutes and then filtered through glass fibre filter paper. After 30 minutes formalin solution was removed and DPSCs were rinsed twice with DPBS to wash out formalin. After removing DPBS, 60% (v/v) isopropanol was added to the culture wells and incubated for 5 minutes. After 5 minutes, isopropanol was removed and 2 mL of Oil red O working solution was added to the wells and incubated at room temperature for 10 minutes. After 10 minutes of incubation Oil red O was removed and 2mL of 60% isopropanol was added to the wells to rinse off the excess Oil red O. Finally, the cells

were washed with PBS three times and observations were recorded under a phase contrast microscope (Leica Microsystems, Germany).

3.2.3.4 Population Doubling Time

The growth rate of DPSCs was monitored by plating 5000 cells per cm2 into three separate T25 culture flasks (BD Bioscience, USA). Each donor had three replicates. After reaching 90% confluence DPSCs were yielded using 0.25% trypsin. Tryptan blue dye was used to count the cells before the next sub-culture. Replating of cells was conducted for consequent sub-culture, and five sub-cultures were analysed in this trial. Growth kinetics was studied by calculating cell population doubling (PD) time. The PD time at each passage was calculated using the following formula:

PDT=tplg2/(lgNH-lgNI)

PDT: Population doubling time; NI: the inoculums cell number; NH is the cell harvest number and t is the time of the culture (in hours). tp: culture period

3.3 Results

3.3.1 Isolation of DPSCs from human extracted permanent teeth

Six days after cell seeding, spindle shaped cells were observed under an inverted microscope (Leica Microsystems, Germany). Distinct colonies that exhibited spindle shaped morphology and uniform size distribution were observed from day 6 to 18. Typically, on day- 18, it reached confluency and the first subculture could be established. Figure 3.1 shows DPSCs outgrown from pulp tissue on day 6, 11, 15 & 18.

3.3.2 Mesenchymal characterization of DPSCs

Mesenchymal properties of freshly isolated DPSCs were confirmed by investigating following characteristics.

3.3.2.1 Plastic adherence of stem cells

Homogenous monolayer of adherent spindle shaped DPSCs were observed after 18 days of culture, under an inverted microscope (Leica Microsystems, Germany) (Figure 3.1) confirming plastic adherence capacity of DPSCs.

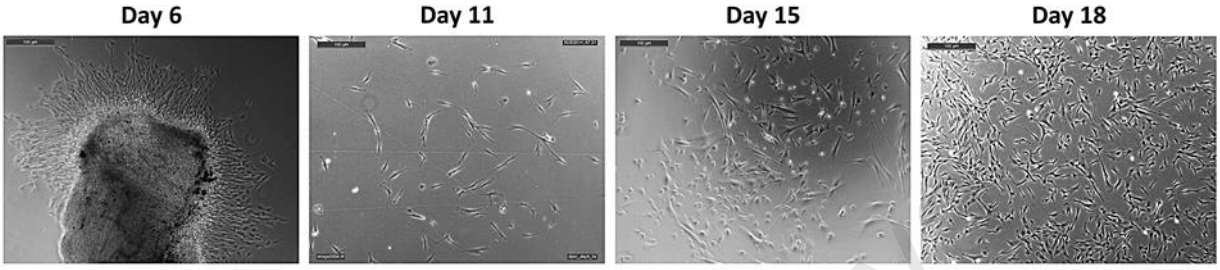

Figure 3.1: Plastic adherence of DPSCs at 6, 11, 15 and 18 days. Micrographs were captured using inverted microscope. Scale bar =100µm.

3.3.2.2 Mesenchymal cell surface marker expression

MSC phenotyping results showed that 98% of DPSCs derived from human dental pulp showed positive expression of the MSCs-related surface markers for CD73 (APC), CD90 (FITC) and CD105 (PE) (Figure 3.2).

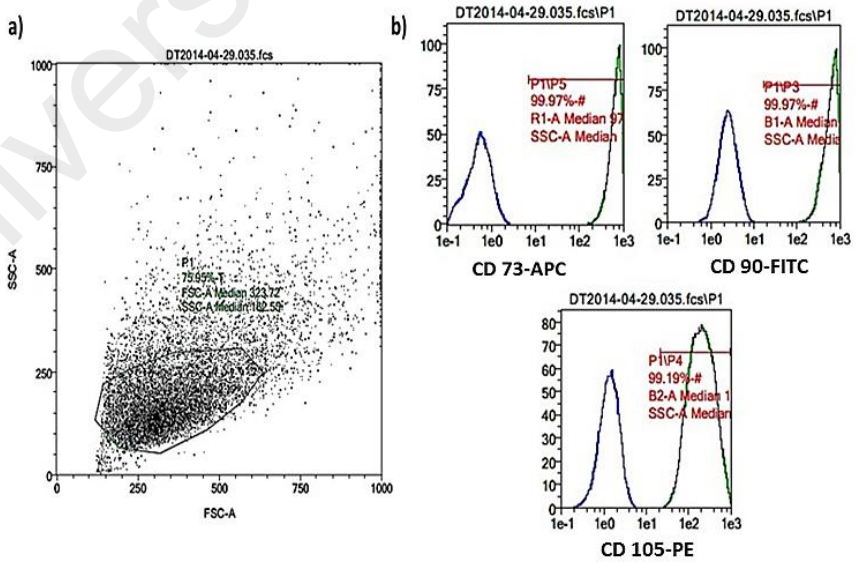

Figure 3.2: MSC Phenotyping a) Gated DPSCs population**, b)** Mesenchymal surface marker expression of CD 73 (APC), CD 90 (FITC) and CD105 (PE) in gated DPSCs population

3.3.2.3 Trilineage differentiation

Figure 3.3 shows the trilineage differentiation expression. Osteogenesis was confirmed by mineralized matrix deposition stained with Alizarin Red. The DPSCs demonstrated cuboidal shaped morphology, similar to osteoblast-like cells.

Adipogenesis was detected by oil droplet formation stained with Oil red O. Results of adipogenic differentiation showed that morphology of DPSCs changed from elongated to round. Formation of lipid droplets was seen as red fluorescence under the microscope after staining with Oil red O. Chondrogenic differentiation was demonstrated as change in appearance of DPSCs from spindle shaped cells to flat and multi-angled cells. Chondrogenesis was detected by the presence of proteoglycans stained with Safranin O.

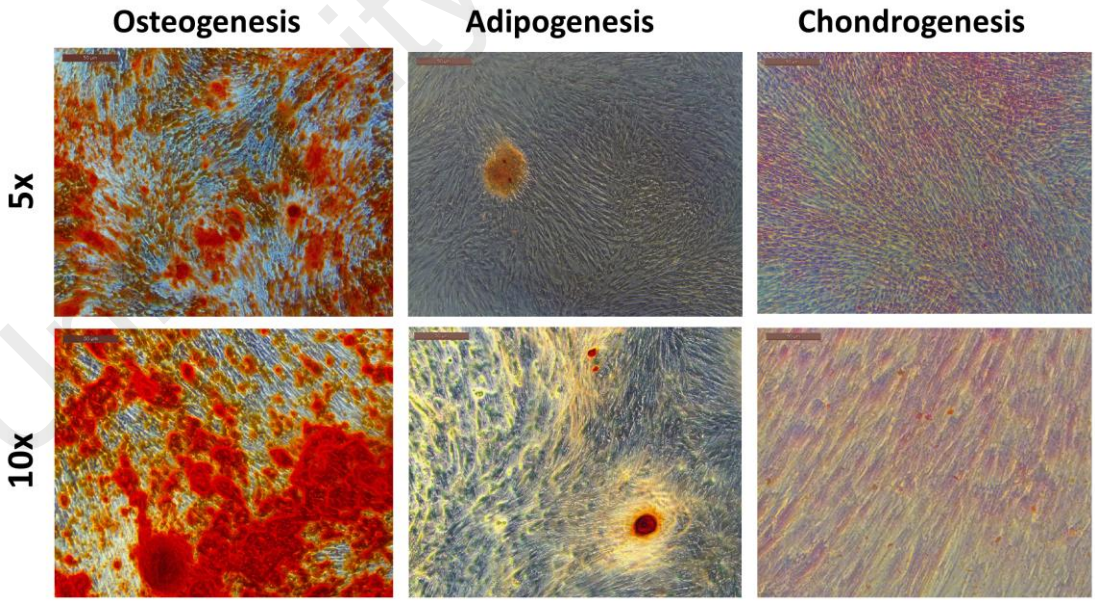

Figure 3.3: Trilineage differentiation of isolated DPSCs into osteogenic indicated by formation of mineralized matrix stained with Alizarin Red, adipogenic lineage indicated by oil droplets stained with Oil red O and chondrogenic lineage indicated by peptidoglycan formation stained with Safranin O. Scale bar = 200µm for 5x, 100 µm for 10x.

3.3.3 Growth Kinetics of DPSCs

A significantly ($p<0.05$) high cell yield (~280000 cells) was evident at SC3 compared to other SCs (Figure 3.4 A). For further experiments, SC3 was chosen due to their highly proliferative performance. Population doubling time of freshly isolated DPSCs (n=3, SC1-SC5) revealed, that time in hours at subculture 1 (SC1) and subculture 5 (SC5) was 45.45 ± 4.03 hours and 51.34 ± 2.9 hours respectively (Figure 3.4 B).

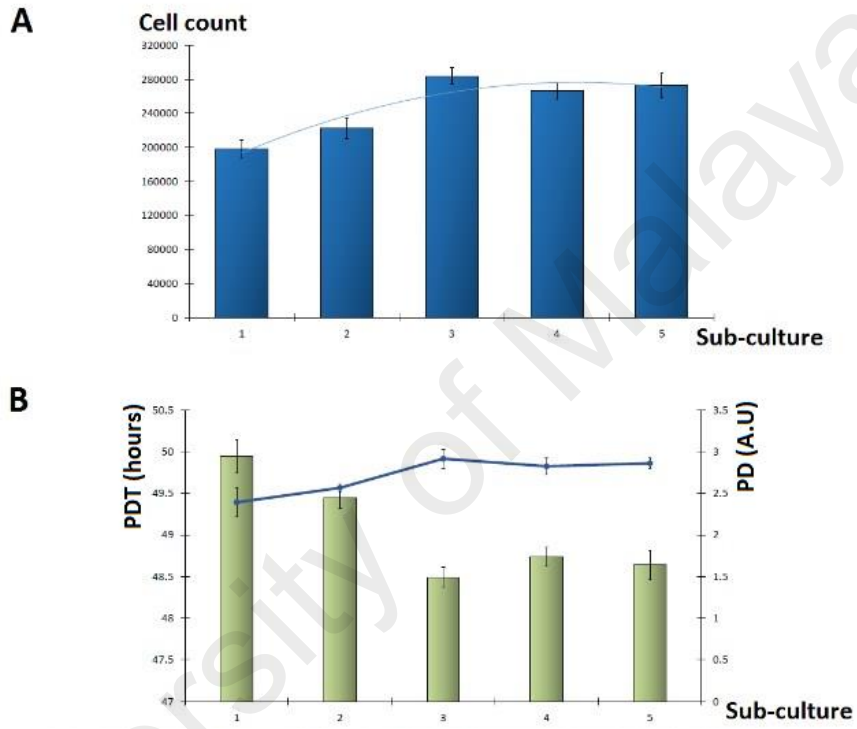

Figure 3.4: Population doubling time of DPSCs at 5 subcultures: (A) Cell count **(B)** Population Doubling Time (PDT) in hours and population doubling (PD) in A.U (Australian units) of DPSCs at five consecutive cultures. Cell counts were represented in bar graphs while the line graphs represented PDT. Data was presented as mean ± SD.

3.4 Discussion

There are evidences of MSCs been present in almost all post-natal organs and tissues (da Silva Meirelles et al., 2006). Amongst all known post-natal stem cells sources, DPSCs have been widely explored due to their neural crest origin and their native

propensity towards multi lineage (Abe et al., 2012; Dupin & Coelho‐Aguiar, 2013). In this part of the study, DPSCs were isolated from dental pulp using enzymatic dissociation, and direct outgrowth of stem cells from pulp tissue explants was observed. In order to reduce the statistical variation, all the experiments were carried on samples from three donors. All experiments were carried out in triplicates on all the samples.

The DPSCs were characterised in-vitro by their plastic adherence capacity, differentiation into three lineages (osteogenic, adipogenic and chondrogenic) and expressions of mesenchymal specific surface markers. It was shown that the cells isolated from adult dental pulp maintained the trilineage differentiation properties in specific induction culture media. The findings in this study were in line with a similar study that reported that stem cells derived from permanent teeth exhibit similar differentiation behaviours under the same conditions as that in bone marrow stem cells (BMSCs) (Shi & Gronthos, 2003).

MSC phenotyping of cells using a panel of mesenchymal cell surface antigens revealed a similar expression pattern for a variety of markers for both DPSCs and periodontal stem cells (used as isotype cell population). In the present study, the cells were strongly positive for lymphocyte differentiation marker CD73, neurogenic surface marker CD90 and angiogenic surface marker CD105. Our findings were partially similar to that of a study performed by Uccelli et al. where for MSCs, typical markers were reported, i.e. CD44, CD73, CD90, CD105, CD271 and STRO-1 (Uccelli et al., 2008)

In this study, the CD73 marker, which indicates a cell adhesion molecule that mediates lymphocyte binding to endothelial cells (Airas et al., 1995) , was found positive in our study. This is in agreement with other studies (Pivoriūnas et al., 2009; Y Yamada et al., 2010). The CD90 (Thy-1), a marker for cell–cell and cell–matrix interactions of leukocytes (Rege & Hagood, 2006), has been commonly reported in bone marrow-derived mesenchymal stem cells (Dennis et al., 2007). This marker has also been

expressed significantly high in DPSCs samples. This finding was in accordance with other studies (Agha‐Hosseini et al., 2010). CD105, also known as endoglin, is a homodimeric membrane glycoprotein primarily associated with the human vascular endothelium (Ríus et al., 1998).

Endoglin is a component of the transforming growth factor-beta (TGFβ) receptor complex and binds TGFβ-1, and is reported to be expressed on DPSCs (Marchionni et al., 2009). DPSCs samples in this study showed >90% expression of CD105, that is in agreement with another study that reported that CD105 positive dental pulp cells exhibit high proliferation and migration activities, along with multi-lineage differentiation potential (Misako Nakashima et al., 2009). A study reported that of DPSCs and bone marrow stem cells (BMSCs) show the similar marker expression of mesenchymal surface markers (Gronthos et al., 2000; Marrazzo et al., 2016).Some studies have also reported otherwise, suggesting marked difference in MSC marker expression profile of BMSCs and DPSCs (Tamaki et al., 2013). However, the significant diversity in surface marker expression in different studies might be due to either cultivation conditions such as passage number, composition of media or other inter individual variations between different donors. Results of our study suggests that isolated population of DPSCs as in this study can be confirmed as mesenchymal stem cells.

Furthermore, in this study it was shown that at SC3 there was significantly high cell count with at low PDT. These findings were in line with a similar study done on DPSCs (Govindasamy et al., 2010), suggesting the presence of relatively high cell count, with low PDT render DPSCs as preferred cells of choice for cell replacement therapies. Based on this, dental pulp tissue may represent a promising reservoir of stem cells for tissue engineering and clinical applications.

3.5 Conclusion

Isolated DPSCs in our study demonstrated MSCs like properties as evidenced by expression of mesenchymal surface markers namely CD73, CD90 and CD105, typical differentiation markers for MSCs. Mesenchymal properties of isolated DPSCs were supported by trilineage differentiation i.e. osteogenic, chondrogenic and adipogenic. DPSCs at passage 3 were considered highly proliferative at shortest time span. Hence, suggesting adult dental pulp could be potential donor pool for DPSCs, potential stem cells for cell based regenerative therapies.

CHAPTER 4: OPTIMIZATION OF LIPOPOLYSACCHARIDE (LPS) TO INDUCE INFLAMMATION IN DPSCs

4.1 Introduction

Dental pulp does not own a specific innate immunity due to its anatomical location and organization of blood vessels within the tooth. It is confined within rigid structures namely enamel and dentine (Berkovitz et al., 2016). Blood supply to the dental pulp is accomplished by small blood vessels entering the pulp chamber through a small opening at the tooth apex, known as apical foramen (Provenza, 1958). Due to the absence of collateral blood supply, dental pulp lacks a strong defence mechanism to fight against bacterial infection (Heyeraas & Berggreen, 1999).

Majority of oral diseases are caused by a mixed population of gram-negative bacteria found in the oral biofilm (Takahashi et al., 2011). Dental caries is one such gram-negative microbial infection that is primarily responsible for pulpal inflammation. When active caries reaches the pulp chamber (Haapasalo et al., 2005), it provides an activating stimulus through bacterial lipopolysaccharides (antigenic cell wall component of negative bacteria) leading to integrated actions of inflammatory mediators in the pulp (Botero et al., 2010). Dental pulp inflammation manifests as increase in microvascular density. This eventually activates the stem cells residing within the cell rich zone of the pulp to release chemokines such as substance P and somatostatin (Avila et al., 2014). A study reported that the dental pulp utilizes cell surface receptors to recognize molecular patterns involving cytokines and chemokines, which are common to microbes, to initiate defence against bacterial invasion (Hahn & Liewehr, 2007).

DPSCs play a significant role in homeostasis, angiogenesis and health of dental pulp by responding to injury and bacterial invasion (Rombouts et al., 2016). Recent studies revealed that the multipotency and angiogenic capacity of DPSCs in response to

injury could be employed as a therapeutic strategy for dental pulp tissue regeneration (Cordeiro et al., 2008; Mangano et al., 2011). However, before DPSCs could be used for clinical applications, it is imperative to understand their biological properties in response to an injury. Studies have demonstrated that the differentiation capacity of stem cells is largely dependent on the niche in which they are thriving (Moore & Lemischka, 2006). To comprehend the potential of DPSCs in healing and regenerative applications, it is important to understand their behaviour in an inflamed microenvironment that has not been explored widely.

In this present study, we aimed to induce inflammation of DPSCs using LPS in an *in vitro* condition. The specific objectives are as follows:

1. To determine the optimal concentration of LPS and treatment time to induce inflammation in DPSCs *in vitro* through cell viability, gene expression and cytokine release profile.

2. To evaluate the maintenance of MSC characteristics via morphological change and MSC specific surface marker expression in iDPSCs.

4.2 Material and Methods

4.2.1 Sample Collection

Ethics approval for the sample collection and processing was obtained from Medical Ethics Committee, Faculty of Dentistry, and University of Malaya {DFDP 1304/002 (P)}. Freshly extracted sound premolars (n=5) were collected following an informed written consent obtained from the donors undergoing orthodontic treatment. Extractions were carried out at the Department of Oral Surgery, Faculty of Dentistry, University of Malaya. Exclusion criteria for the teeth selection involved patients with evident periodontitis, fractured tooth, patients with any history of pain and trauma. Under aseptic condition following surface sterilization, teeth were sectioned at cemento-enamel

junction using high-speed diamond disc. The pulp tissue was then gently extirpated using a small sized endodontic barbed broach. A blunt non-cutting forceps was used to transfer the pulp tissue into a micro-centrifuge tube (BD Falcon, USA) containing transportation media and was transported to the laboratory immediately.

4.2.2 Isolation of DPSCs from extirpated pulp

The extirpated pulp tissue (n=5) was transferred into sterile centrifugation tubes and washed thrice using phosphate-buffered saline (DPBS) (Gibco,USA) solution supplemented with 1% antibiotic-antimycotic solution (Gibco, USA). Then, the tissue was minced using sterile surgical scissors into smaller pieces prior to enzymatic digestion with 3mg/mL collagenase type IV (Gibco,USA) enzyme for 30 minutes. After incubation with collagenase, the digested pulp was transferred to a 15 mL centrifuge tube (BD Falcon, USA) containing 6 mL of complete media and centrifuged at 1250 rpm for 6 minutes. The supernatant was discarded; the cell pellet was re-suspended in the complete media and seeded in a T25 culture flask. The culture flask was incubated for 3 days in a humidified incubator maintained at 37°C, 5% CO^2. At day 6 outgrowth of the cells were detected under phase contrast microscope (Leica Microsystems, Germany).

Culture media used in subsequent experiments was prepared with standard cell culture media, knock out- Dulbecco's modified eagle's media (DMEM-KO) (Gibco, Invitrogen, USA), that was supplemented with 10% FBS (HyClone, USA), 50 units/mL Penicillin-Streptomycin (Gibco, USA) and 2mM L-glutamax (Gibco, USA). The complete media was filter sterilized through a 0.2 µm syringe filter (Millipore, Thermoscientific, USA) and stored at 4°C until further use.

4.2.3 Expansion and Sub-Culturing of DPSCs

Once the cell monolayer reached 80% confluence, DPSCs from five donors (n=5) were further expanded in T75 flasks with complete media for further experiments. Culture

media from T25 flask was discarded. Adherent DPSCs were washed twice with DPBS to re-move any residual culture media. Pre-warmed 0.25% trypsin-EDTA (Invitrogen, USA) was then added as dissociation reagent to the flask and incubated for three minutes. Cells were observed under inverted microscope (Leica Microsystems, Germany) to confirm complete dissociation. After that, complete culture media was added to neutralize the trypsin action and centrifugation was carried out at 1,250 rpm for 5 minutes to obtain a cell pellet. Supernatant was discarded and pellet was re-suspended in 10 mL of complete culture media. DPSCs were counted using tryptan blue dye and were seeded at a seeding density of $2x10^3$ mL in T75 flask containing 10mL of complete culture media and incubated at 37°C, 5% CO_2 for 24 hours. Culture media was changed every 3 days.

4.2.4 LPS media preparation

Five milligram of commercially available E. coli LPS powder (Sigma Aldrich, USA) was dissolved in 5 mL of DMEM-KO medium to prepare the LPS stock solution at the con-centration of 1mg/mL. Specific amount of the stock solution was added to 10 mL DMEM-KO resulting in five different LPS based culture media with varying concentrations of LPS as illustrated in Table 4.1.

Table 4-1: Composition of LPS-based culture media with various LPS concentrations.

LPS media composition	LPS concentration

2.5µl LPS+10mL DMEM-KO	0.25µg/mL
5 µl LPS +10mL DMEM-KO	0.5 µg/mL
10 µl LPS +10mL DMEM-KO	1 µg/mL
20 µl LPS +10mL DMEM-KO	2 µg/mL
40 µl LPS + 10mL DMEM-KO	4 µg/mL

4.2.5 Treatment of DPSCs with various concentrations of LPS

DPSCs from five healthy donors (n=5) yielded at passage 3 were seeded into 6-well plates at a seeding density of 2×10^5 cell per well in 2mL of culture media. After 24 hours of incubation at 37°C and 5% CO^2 in a humidified incubator, culture media was discarded and cells were gently washed with DPBS (Gibco, USA). DPSCs were then incubated in culture media containing various concentrations of LPS (0.25µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL and 4 µg/mL). DPSCs incubated in DMEM-KO constitute the positive control. All concentrations of LPS and control were tested in triplicates.

Any morphological changes of DPSCs treated with various concentrations of LPS were observed under an inverted microscope (Leica Microsystems, Germany) and recorded at three different time points 24, 48 and 72 hours post-incubation.

4.2.6 Effect of various concentrations of LPS on viability of DPSCs

DPSCs (n=5) at passage 3 were seeded into 96-well plates in triplicates at a density of 5×10^3 cell per well in 200 µl of complete culture media. After 24 hours of incubation at 37°C and 5% CO2 in a humidified incubator, culture media was discarded and DPSCs were washed thoroughly with DPBS. All cells were then incubated in various concentrations of LPS media (0.25µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL and 4 µg/mL) prepared previously for 12, 24, 36, 48, 60 and 72 hours. After each time point 100µl of DMEM-KO (Gibco, USA) along with 10% Alamar Blue reagent (Thermo fisher scientific, USA) v/v was added to each well and plates were further incubated for 3 hours at 37°C and 5% CO_2 in a humidified incubator. Absorbance was measured at 450nm with reference wavelength set at 590nm by Tecan micro plate reader (Infinite 200 PRO Tecan, Life sciences, Switzerland) to determine effect of LPS on viability of DPSCs. Cell viability was calculated as percentage reduction from the reduction in recorded absorbance values.

4.2.7 Effect of various concentrations of LPS on expression of pro-inflammatory genes in DPSCs

DNA and mRNA from the cultured DPSCs, were extracted using DNEasy Blood & Tissue Kit (Qiagen, GmBH Germany) and RNeasy micro kit (Qiagen), respectively, according to the manufacturer's instructions. The quality (A260/A280, A260/230) and concentration of the gDNA (ng/µL) was determined using the spectrophotometer ND-2000 (NanoDrop Technologies, Wilmington, DE, USA). The integrity of RNA was assessed using the Agilent Bioanalyzer-2100 (Agilent, Palo Alto, CA, USA) and samples with RNA integrity number (RIN) more than six were used for cDNA synthesis and subsequently used for qRT-PCR analysis. The expression of the housekeeping gene

GAPDH was used as reference for measurements. The ΔCt calculation method was used to evaluate the relative quantity of gene expression. Experiment was carried out on samples from three different donors in triplicates. Expression of pro-inflammatory cytokines was investigated using qRT-PCR analysis. The pro-inflammatory genes and primers used for the qPCR analysis is tabulated in Table 4.3.

Table 4-3: List of pro-inflammatory genes and cytokines with their functions

Primer	Primer ID	Function
IL-6 (Interleukin 6)	Hs00985639_mL	➢ Contributes to the progression of inflammatory diseases in lungs inflammation through NF-κB cascade (Govindasamy et al., 2010). ➢ High marker expression in acute periodontal and periapical inflammation (Rincon, 2012).
IL-8 (Interleukin 8)	Hs00174103_mL	➢ Detected in large quantities in inflamed dental pulp (Barkhordar et al., 1999). ➢ Contributes to immune response for inflammation onset by increasing chemotaxis (Park et al., 2004). ➢ Responsible for macrophage mediated angiogenesis in inflammatory conditions (Clark-Lewis et al., 1994).
TAC-1 (Tachykinin Precursor 1)	Hs00243225_mL	➢ Initiates phagocytosis in leukocytes during acute inflammatory process (Koch et al., 1992). ➢ Detected in large quantities in bacteria induced inflammation (Bar-Shavit et al., 1980).
TPA (Tissue Plasminogen Activator)	Hs00263492_mL	➢ Responsible for tissue remodelling post-injury (Swain et al., 1992). ➢ Regulates coordinated adhesion of cells during inflammation(Coussens & Werb, 2002).
TNFα (Tumour Necrotising Factor alpha)	Hs00275871_mL	➢ Regulates innate immune response of host cells during inflammation through NF-κB cascade (Lo et al., 2002). ➢ Primary marker for inflammation induced angiogenesis (Pasparakis et al., 1996).

IL-1α (Interleukin1alpha)	Cat.336161	➢ Responsible for inflammatory process involving, pain, inflammation and autoimmune conditions(Coussens & Werb, 2002). ➢ A prominent mediator of neuro inflammation (Ren & Torres, 2009).
IL1β (Interlukin1beta)	Cat.336161	➢ A potential pro-inflammatory marker for pathogenesis of acute phase reaction (Shaftel et al., 2008). ➢ Mediates collagen degradation in inflamed pulp when coordinates with IL-6 and TNFα

4.2.8 Effect of various concentrations of LPS on release of pro-inflammatory cytokines

DPSCs (n=5) at passage 3 were seeded in six T75 cell culture flasks at the density of $2.1x10^6$ cells/well and expanded till 70% confluence. Cells were then thoroughly washed with DPBS to remove any residual culture media. Washed DPSCs were then incubated with 10mL of LPS media in various concentrations for 24 hours. The supernatant from the culture flask was collected for ELISA assay after 24 hours.

DPSCs cell lysate was prepared by scrapping the adherent cells at the bottom of the T75 flask mentioned above, using a plastic cell scrapper. Cells were transferred into 15mL centrifugation tube and centrifuged at 1500rpm at 4ºC for 5 minutes. After discarding the supernatant, cells were re-suspended into 5mL of ice cold PBS and again centrifuged at 1500rpm at 4ºC for 5 minutes. Supernatant was removed without disturbing the cells and cell lysis buffer was added (Qiagen, USA). Cell suspension was pipetted up and down 8-10 times and incubated on ice for 5 minutes. The entire content was then transferred to 1.5mL micro centrifugation tube and centrifuged at 20000rpm for 10 min at 4ºC. Lysate obtained after centrifugation in the form of supernatant was used to run the ELISA assay.

Reagent preparation was carried out as per the manufacturer's protocol (Qiagen, USA). Six hundred fifty microliters of each sample was used to carry out the assay that was performed using a standard antigen cocktail for standard curve preparation.

Cytokines in the supernatant were detected using detection antibodies. Presence of cytokines was detected using Tecan micro plate reader (Infinite 200 PRO Tecan, Life sciences, Switzerland) with test wavelength adjusted at 450nm and reference wavelength at 650nm, compared against the control. Pro-inflammatory cytokines release in DPSCs lysates and media supernatants after treatment with various concentrations of LPS was analysed using ELISA (Enzyme Linked Immunosorbent Assay) (Qiagen, USA). Pro-inflammatory cytokines evaluated in the study were IL-6, IL-8, IL-1α and TNF α.

Selection of pro-inflammatory cytokines namely investigated in this experiment was based on their release profile as described by some previous studies that investigated on tissues with acute inflammation (Cavalcanti, Rode, França, & Marques, 2011; Cooper et al., 2010; Engels-Deutsch et al., 2003; W He et al., 2013; Patel, Park, Lin, Chiappelli, & Huang, 2003; Yamaguchi et al., 2004).

4.2.9 Effect of various concentrations of LPS on mesenchymal cell surface markers expression in iDPSCs

Expression of specific surface antigens indicating mesenchymal properties was determined by staining DPSCs with human MSC phenotyping kit. Sample preparation for FACS analysis was done as mentioned in Chapter 3 section 3.2.3.2 . The following antibodies were used to mark the cell surface epitopes- CD105 (PE), CD90 (FITC) and CD73 (APC). All analyses were standardized against the negative control DPSCs incubated with isotype-specific CD14-PerCP, CD20- PerCP, CD34- PerCP, CD45-PerCP purchased from MACS Miltenyl Biotech, Germany. Cells acquisition was performed using FACSCalibur flow cytometer and was analysed using MACSQuant® Analyser 10 (Miltenyl Biotech, Germany).

Seeding density of any given experiment was chosen depending on the area of the well plates selected for the specific experiment. E.g: Experiment for cell viability was carried out in a 96-well plate with a seeding density at $1.5x10^4$.

Similarly, experiment for gene expression required higher number cells in range of millions. Hence, the experiment was carried in 75mm^2 flask with a cell density of 3.7x10^6.

4.2.10 Statistical Analysis

Data was presented as mean ± standard deviation. Two-way ANOVA with post-hoc analysis was performed to determine the LPS concentration and time points that could significantly induce inflammation of DPSCs.

4.3 Results

4.3.1 Morphology of DPSCs after treatment with various concentrations of LPS

Upon exposure to LPS at various concentrations, DPSCs showed elongated spindle shaped morphology, similar to that of control group (Figure. 4.1). This morphology was maintained throughout at all concentrations and time points.

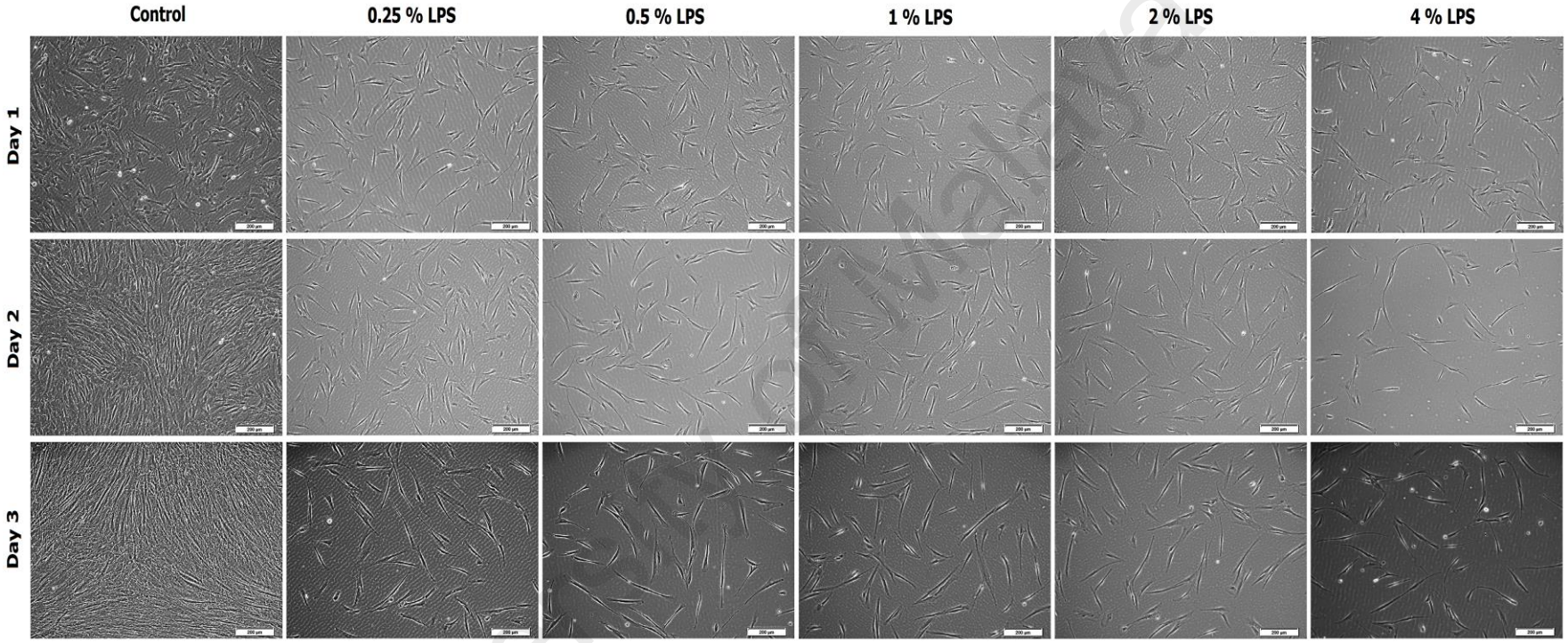

Figure 4.1: Morphology of DPSCs upon exposure to various concentrations of LPS at three different treatment times. Scale bar = 100µm**.** Upon exposure of DPSCs to various LPS concentrations (0.25, 0.5, 1, 2 and 4) µg/mL at 24h, 48h and 72h, it was observed that DPSCs morphology remained elongated spindle shaped at all concentration and time points.

4.3.2 Percentage viability of DPSCs after treatment with various concentrations of LPS

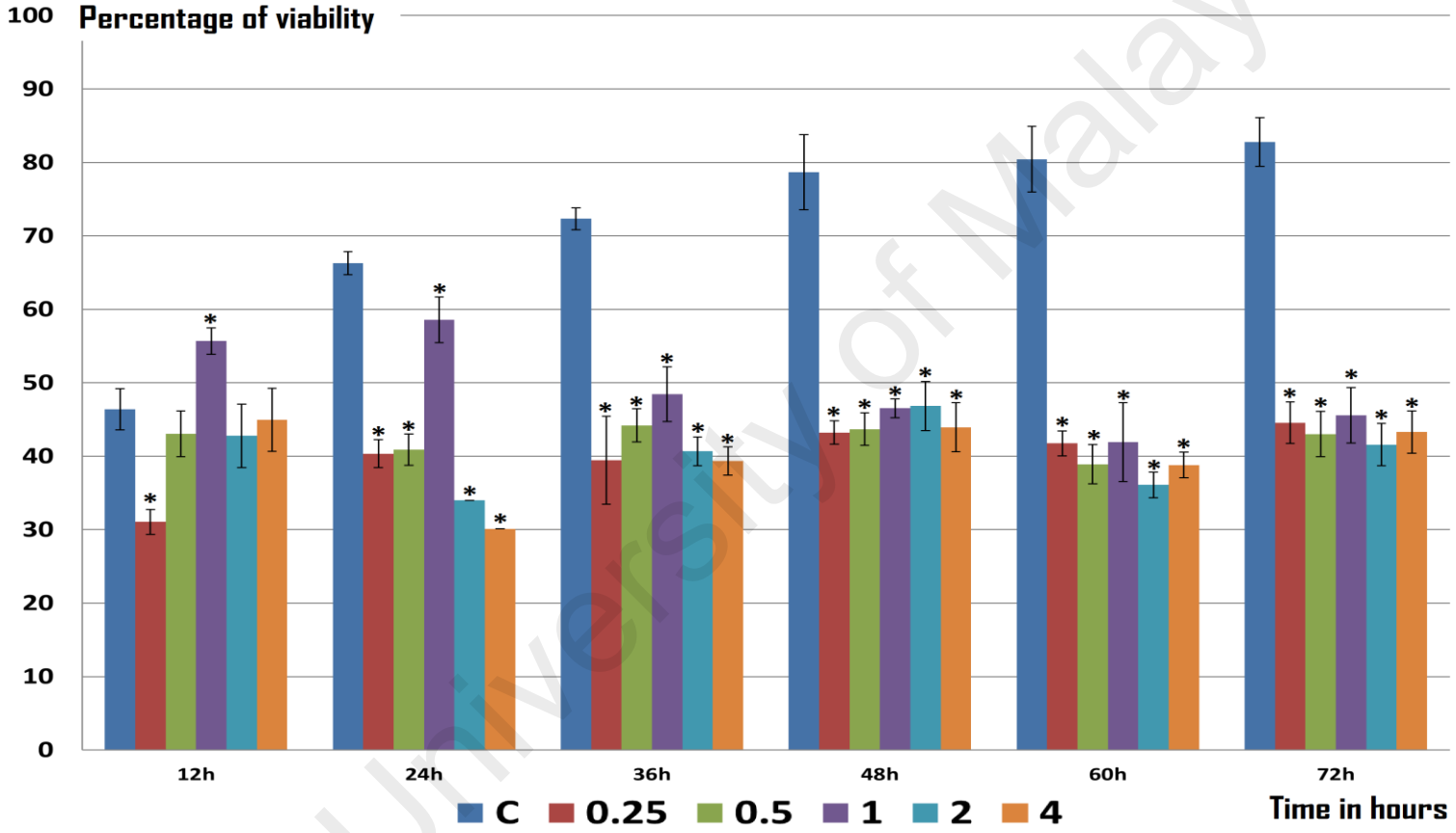

Figure 4.2: Percentage viability of DPSCs upon exposure to various concentrations of LPS at six different time points. Upon exposure of DPSCs to various LPS concentrations (0.25, 0.5, 1, 2 and 4) µg/mL for 12h, 24h, 36h, 48h, 60h, and 72h showed significantly ($p<0.05$) high viability at 1 µg/mL LPS concentrations at 12 and 24h.

4.3.3 Expression of pro-inflammatory cytokines and genes in DPSCs after treatment with various concentrations of LPS

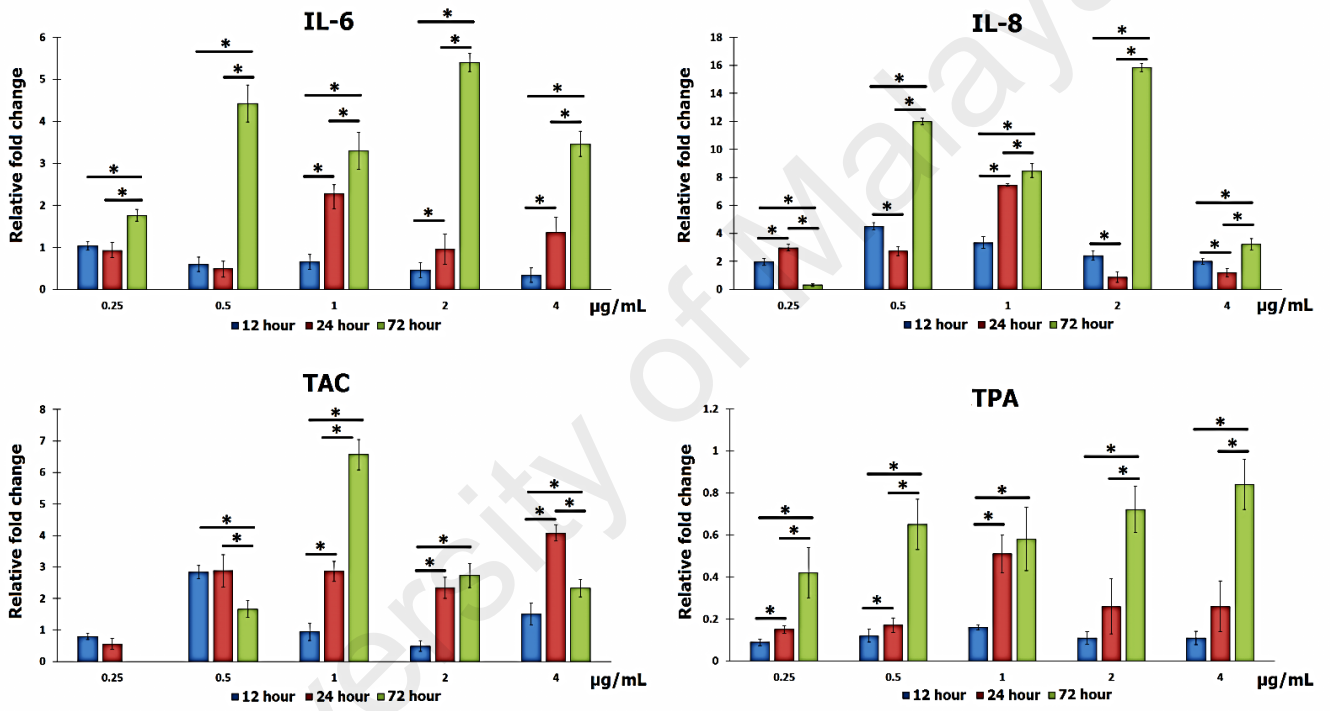

Figure 4.3: Relative fold change in pro-inflammatory genes expression in DPSCs upon exposure to various LPS concentration at 12h, 24h, and 72h Exposure of DPSCs to various concentrations of LPS (0.25, 0.5, 1, 2 and 4) µg/mL at 24h, 48h and 72h demonstrated significantly high fold change in gene expression with increasing time at all concentrations.

Effect of various LPS concentrations on cell viability of DPSCs is presented as percentage viability, as shown in Figure 4.2. In general, it was shown that LPS treatment significantly reduced cell viability when compared to control group at all tested concentrations, except at 12h treatment ($p<0.05$). A significant decrease in DPSCs viability was observed with increasing LPS concentration, from 12h to 72h. Statistical analysis showed that viability was significantly ($p<0.05$) high at 1 µg/mL LPS concentration at 12 and 24h compared to all LPS concentrations and higher than the control group at 12 hours treatment. DPSCs viability started to decrease after 36h and remained constant till 72h.

The extent of inflammation progression stimulated by various LPS concentrations was determined by expression of key inflammatory markers; Interleukin 6 (IL-6), Interlukin-8 (IL-8), Tachykinin precursor (TAC 1) and Tissue Plasminogen Activator (TPA) was assessed determined at 12th, 24th and 72nd hour. As shown in Figure 4.4, it was observed that expression of IL-6 at all concentrations except 1µg/mL, showed a sudden increase from 24 hours to 72 hours. IL-6 expression showed five-fold increase in expression at 0.5µg/mL LPS when treatment time was increased from 24h to 72h. Similarly, exposure to 2µg/mL and 4 µg/mL revealed comparable pattern whereby the expression was increased by five-fold from 0.4 to 5.2 at 72 hours. However, for 1µg/mL LPS concentration, there was a gradual increase in IL-6 expression with increasing treatment time, indicating a gradual onset of inflammatory process.

A decrease in expression of all cytokines was observed at 24 hours, in samples exposed to 0.25 µg/mL, 2 µg/mL and 4 µg/mL LPS. Interestingly, cells exposed to 1µg/mL LPS showed increased expression from 12 hours to 72 hours. The expression pattern of TAC1 revealed mixed results but at concentration of 1ug/mL LPS, an incremental pattern was observed in which the expression of TAC1 increased from 0.8 at 12 hours to 6.35 at 72 hours, respectively. At 24 hours there was no significant difference

in the expression of TAC1 for concentrations 0.5µg/mL, 1µg/mL and 2µg/mL. Expression pattern of TPA was similar to that of IL-6 and IL-8. A sudden increase in expression was evident from 12 hours to 72 hours treatment. However, at 1µg/mL concentration there was gradual increase in expression with increasing treatment time. Interestingly, there was an insignificant difference in TPA expression at 24 hours and 72 hours. Expression of TNF α was not present at any concentration or time point.

The folds change of pro-inflammatory cytokines (IL-6, IL-8) as well as pain signalling. TAC1 showed a significantly higher expression pattern in DPSCs upon exposure on to increasing concentration from 0.25µg/mL to 4µg/mL of LPS.

4.3.4 Release of pro- inflammatory Cytokines from DPSCs after treatment with various concentrations of LPS

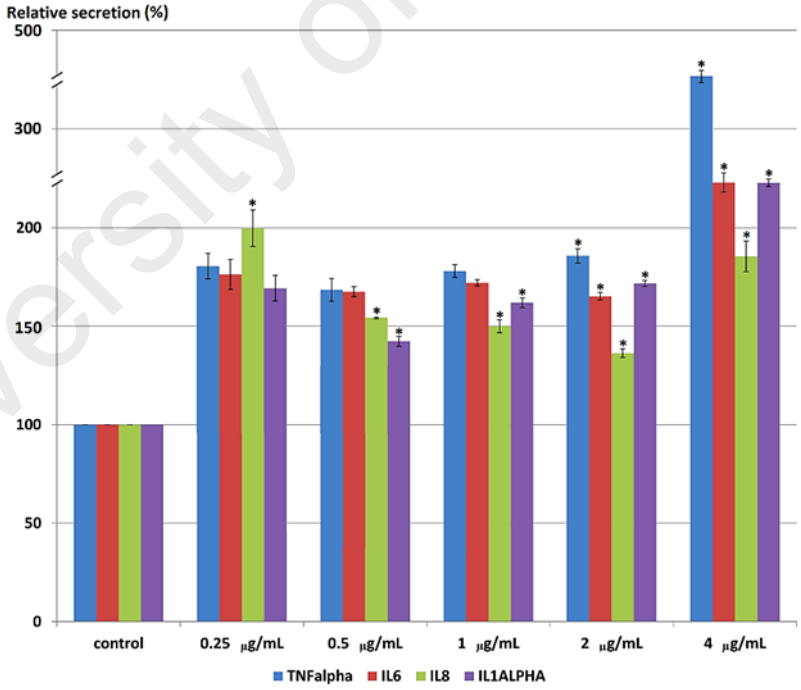

Figure 4.4: Relative secretion of pro-inflammatory cytokines upon exposure of DPSCs to various LPS concentrations for 24h. Exposure of DPSCs to various concentrations of LPS (0.25, 0.5, 1, 2 and 4) µg/mL at 24h demonstrated significantly high release of cytokines at 4µg/mL LPS concentration compared to control group. No significant change in the release pattern was observed between 0.25, 0.5, 1and 2µg/mL LPS.

Levels of pro-inflammatory cytokine released from DPSCs exposed to various LPS concentrations were also evaluated at protein level using ELISA. As shown in Figure 4.4, the secretion level of each protein had generally increased with increasing LPS concentration. Cytokines showed decreased release especially at 0.5 µg/mL LPS concentration. IL8 release was decreased distinctly from 200 ± 4.5% at 0.25µg/mL to 153 ± 3.2% at 0.5µg/mL and showed significant increase in release 185 ± 3.3, except at 2µg/mL. Similarly, release of IL-1α also presented a decrease from 172 ± 4.1% at 0.25µg/mL; to 148.3 ± 2.1% ($p < 0.05$). Higher LPS concentrations demonstrated increased cytokines release. It was observed that at 4µg/mL LPS concentration significantly high release of cytokines was evident compared to other LPS concentrations. The secretion profile of all cytokines at LPS concentrations 0.25µg/mL to 2µg/mL was noted to be within reasonably consistent level. They did not show significant change in the release pattern above 1µg/mL LPS, with the exception of TNF alpha, that demonstrated a remarkably ($p < 0.05$) high release at 4µg/mL LPS. It was noted that release of all inflammatory cytokines was significantly elevated at all LPS concentrations, compared to control.

4.3.5 MSC specific surface antigen expression in DPSCs after treatment with various concentrations of LPS

DPSCs treated with various concentrations of LPS demonstrated, MSC specific cell surface markers expression, similar to those of untreated DPSCs. All three mesenchymal markers namely CD73, CD105, CD90 were highly expressed (>90%) in all the samples, representing MSC like properties in iDPSCs.

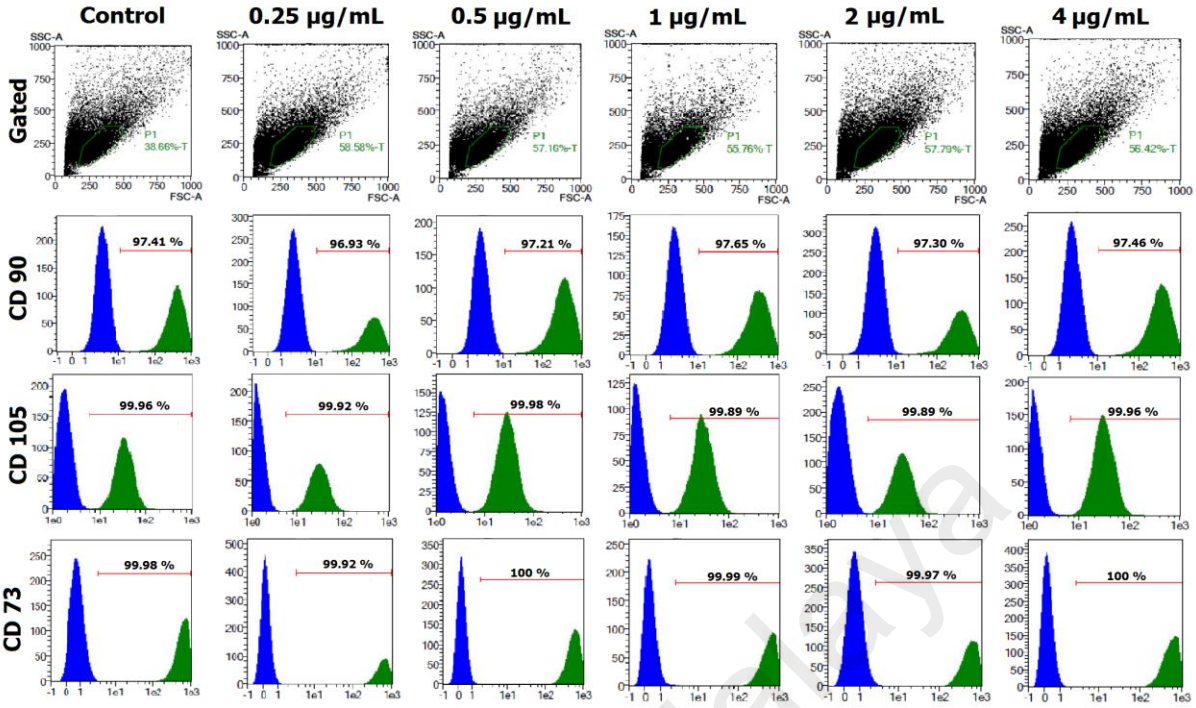

Figure 4.5: MSC phenotyping of DPSCs upon exposure to various LPS concentrations for 24h. FACS analysis of DPSCs exposed to various concentrations of LPS (0.25, 0.5, 1, 2 and 4) µg/mL at 24h demonstrated remarkably high > 90% expression of MSC specific cell surface antigens (CD73, CD105 and CD90), similar to that of control group.

4.4 Discussion

Inflammatory process induced by microbial infection, progresses in dental pulp through lipopolysaccharide (LPS) component present in the cell wall of gram-negative bacteria. This process has huge implication on the functionality of affected tooth and various cell populations residing in the pulp tissue (C-B Zhu et al., 2006). DPSCs are a pluripotent stem cell population present in cell rich zone of dental pulp. Studies have shown that DPSCs hold a great potential to initiate healing at the inflamed site (Ennis et al., 2013). Efficiency of DPSCs to initiate healing and regeneration is determined by their performance in an inflamed microenvironment (Gronthos et al., 2000). Dental pulp is commonly exposed to bacterial microenvironment, in incidences of acute pulp inflammation (Ma et al., 2014). Properties of DPSCs in terms of morphology, viability and inflammatory gene expression both at mRNA and protein level, during inflamed state, are yet to be explored extensively.

Our intention in this part of the study was to develop an *in-vitro* LPS-based inflammatory microenvironment for DPSCs isolated from adult human tooth pulp to various concentrations of LPS. The selection of pro-inflammatory cytokines and genes tested in the study was done based on their participation in regulating the acute inflammatory process (Abd-Elmeguid et al., 2013; Guo et al., 2000). To investigate the inflammatory microenvironment created by treating DPSCs with LPS, various analytical tests were undertaken. Seeding density for any given experiment was chosen depending on the available culture surface area recommended for the specific experiment, such as cell viability was carried out in a 96-well plate with a seeding density at $1.5x10^4$. Similarly, experiment for gene expression required higher number cells in range of millions, hence, the experiment was carried in $75mm^2$ flask with a cell density of $3.7x10^6$.

In a study done on human umbilical cord mesenchymal stem cells (hUCMSCs) showed that on exposing hUCMSCs to various concentrations of *E.coli* LPS (0.01, 0.1, 1, 10, 20, 30, 40 or 50) μg/mL, a remarkable reduction in cell viability was evident at LPS concentrations > 10μg/mL. At concentrations < 1μg/mL no significant increase in viability was evident. At 1 μg/mL a significantly high viability was observed compared to other concentrations tested. However, it was found that a pre-treatment of hUCMSCs with 1μg/mL showed significant reduction in cell apoptosis (Loesche, 2007). A similar study done on periodontal ligament mesenchymal stem cells (PDLMSCs) reported that when PDLMSCs were treated with a range of LPS concentrations (0.1, 1 and 10) μg/mL, significantly high cell viability was recorded at 1μg/mL LPS concentration. Osteogenic differentiation potential and pro-inflammatory cytokine expression was recorded to be significant high at 1μg/mL LPS concentration. However, 10μg/mL LPS demonstrated a significant expression of few but not all pro-inflammatory cytokine (Hou et al., 2015). Based on the findings from studies mentioned above, ranges of LPS concentrations (0.25, 0.5, 1, 2 and 4) μg/mL were selected to induce inflammation in DPSCs.

In acute inflammatory process, several pro-inflammatory cytokines like IL-1β, IL-6 and IL-1 are expressed at gene and protein level by activated immune cells (Albiero et al., 2015). Lipopolysaccharides (LPS), induce MSCs to release pro-inflammatory cytokines, including interleukin-1-beta (IL-1β), IL-6, IL-8, IL-12, type I interferon (IFNs), and tumour necrosis factor-alpha (TNF-α) (J-M Zhang & An, 2007) in cases pulpitis and periodontal diseases (Ha et al., 2008). Pro-inflammatory cytokines investigated in the study were IL-6, IL-8, TAC1 and TPA. The selection of cytokines and genes for the study was done based on their participation in regulating an acute inflammatory process, as described in Table 4.1 and as reported in previous references (Sledziewski & Glińska, 2014)

IL-6 has been shown to play a key role in acute phase of inflammation. It is known to have a dual effect, pro-inflammatory and anti-inflammatory. However, its pro-inflammatory action is found to be dominant in acute inflammatory conditions (Abd-Elmeguid et al., 2013; Guo et al., 2000; He et al., 2013; Kamio et al., 2007; Leal et al., 2015; Silva et al., 2009; Tamura et al., 1996; Ueda & Matsushima, 2001). It has also been demonstrated by previous studies that IL-6 is not only responsible for inflammation progression, it is also involved in metabolic and regenerative process regulation (Scheller et al., 2011). In our study, it was observed that at gene and protein level IL-6 release was significantly high at 1µg/mL at 24 hours treatment compared to control. However, highest expression of IL-6 was recorded at 4 µg/mL at 72 hours (Figure. 4.4) This finding in our study was in line with a similar study that demonstrated increased release of IL-6 in a co-culture comprising of monocytes and MSCs in an inflammation induced rat model (Scheller et al., 2011). Furthermore, previous studies have also shown that LPS from E. Coli induces the expression of cytokines such as IL-6 in periodontal ligament fibroblasts (Gabay, 2006).

IL-8 is a potent pro-inflammatory indicator, which is up regulated in response to bacterial lipopolysaccharide (Botero et al., 2010; Crisostomo et al., 2008; Morsczeck et al., 2012). Studies have shown that IL-8 expression is stimulated by gram negative bacteria found in inflamed dental pulp and is responsible for recruitment of neutrophils at the inflamed site (Nagaoka et al., 1996) (J Chang et al., 2005; He et al., 2013; Y Sun et al., 2010) It is known to be involved in innate response like inducing phagocytosis and activation of neutrophils at the site of inflammation (Jiang et al., 1998). As demonstrated in our study, expression of IL-8 at gene and protein level was significantly high at 1µg/mL LPS concentration and increased with increasing treatment time (Figure. 4.4). These findings were in line with those found in a study done on stem cells from apical papilla (J-M Zhang & An, 2007). Interestingly, IL-8 was not induced at low concentrations (0.25

and 0.5 µg/mL) of LPS in the tested DPSCs population. This finding was similar to that found in study done on dental follicle cells (J Zhang et al., 2013). This indicates that inflammation induction has started to set in after 1µg/mL LPS and was not that evident at lower LPS concentrations.

Other key inflammatory genes investigated in the study were TPA (Tissue Plasminogen Activator) and TAC1 (Tachykinin1). TPA gene encodes a protein called plasmin that is responsible for breakdown of fibrin network in event of acute inflammatory process (Morsczeck et al., 2012). TPA expression has been shown in early stages of inflammation (DeMers et al., 2012) and its expression was significantly higher in grossly carious painful specimens than in grossly carious asymptomatic specimens. These data would suggest that the expression of TPA within pulpal nerves undergoes dynamic changes following caries, which may have an important clinical significance in terms of inflammation and pain experience (Huang et al., 2005). In our study it was shown that at 24 hours TPA expression was significantly high in DPSCs treated with 1µg/mL LPS among all tested groups. However, at 72 hours, there was a sudden increase in TPA expression, which indicates an initial fibrin breakdown that occurs with inflammation advancement. Hence, considering 24 hours to be an appropriate treatment time for inflammation induction in DPSCs. There is not much evidence in literature showing expression of TPA in response to LPS. However, a study performed exclusively on TPA , reported remarkably high expression of TPA gene in inflamed pulp tissue, compared to normal pulp tissue after qPCR analysis (Rodd & Boissonade, 2000).

TAC1 gene is associated with pain and acute inflammation of neurogenic origin (Tsai et al., 2005). However, expression of TAC1 has been found to be significantly high in patients suffering from diabetic periodontitis (Peroutka, 2005). TAC1 is also found to play a regulatory role in immune response of dental pulp tissue to pathogenic bacteria (Andriankaja et al., 2012; Gronthos et al., 2000). Results of our study have demonstrated

highest expression of TAC1 at LPS concentration higher than 0.5µg/mL. TAC1 expression was found to be consistently high at 72 hours at all LPS concentrations (Figure. 4.4), indicating that acute inflammatory phase starts to set in above 0.5µg/mL LPS concentration at 24h treatment time. From this viewpoint, it was noted that LPS microbial toxin has distinct influence on induction of inflammation in DPSCs, which was reflected through molecular expression of IL-6, IL8, TAC1 and TPA.

IL-1α is a pro-inflammatory cytokine found in large concentrations in exudates obtained from infected root canals (Tokuda et al., 2004). In our study, it was shown that IL-1α release showed an incremental pattern with increasing LPS concentrations (Figure. 4.5). Thus, suggesting a progressive onset on inflammatory process with increasing bacterial LPS exposure. These results were similar to a study done on LPS induced pulpitis in a mice model IL-1α was observed to be released in high concentrations from pulp fibroblasts (Matsuo et al., 1994).

Results of cytokine release demonstrated a typical phenomenon with regards to TNFα. At protein level its release was significantly high, but at gene level expression of TNFα was not evident. This could be because LPS from *E.coli* initiates inflammation through dual pathways TLR4 and TLR2. All the pro-inflammatory cytokine expression at gene level and release at protein level are regulated by TLR4 pathway. However, TLR2 pathway causes impairment of TNF α expression visibility at gene level, due to cross-signalling and camouflage (Coil et al., 2004). Sudden increment of TNF-alpha in the cytokine profiling might suggest that TNF-alpha pathway could have been activated as well due to prolonged exposure with LPS, as also reported by a study done on BMSCs (Bradley, 2008).

In our study it was found that release of pro-inflammatory cytokines such as IL-1α, IL-6, IL-8, and TNF-alpha presented increment with respect to time and concentration. This pattern was in accordance to earlier studies indicating that these cells

were preparing themselves to initiate healing mechanism in which such cytokines are necessary to trigger NF-κB (Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells) pathway that is a key molecular mechanism involved in inflammation (Stanley et al., 2014). On the whole, the expression of these genes indicated that series of inflammatory signals were turned on, that might have contributed to behaviours associated with inflammatory process.

An increasing trend in gene expression with increasing LPS concentration was observed in this study. However, the expressions of both gene and protein especially upon exposure with 0.25µg/mL and 0.5µg/mL have revealed a decreasing pattern instead. This would be suggestive of negative feedback mechanism (Lawrence, 2009) that might be responsible to trigger proliferation and initiate healing mechanism. As reported by Farges et al, balance between the inflammatory response and the repair process is of paramount importance, as they dictate the rate of pulpal healing (Farges et al., 2015).

It is noteworthy that the morphology of LPS treated DPSCs in our study did not show obvious differences to that of control group throughout the culture period. In addition, it was also observed that the DPSCs had maintained their mesenchymal characteristics, despite being exposed to various LPS concentrations evidenced by mesenchymal specific markers expression (CD73, CD90, and CD105). In our study, we did not investigate the multilineage potential of iDPSCs. However, in an *in-vitro* study, it was observed, increase in osteogenic differentiation pattern occurs when mesenchymal stem cells isolated from the bone marrow of healthy individuals were exposed to the LPS from *E.coli* (Bradley, 2008). However, another study demonstrated contrasting results showing that DPSCs isolated from inflamed pulp did not exhibit mesenchymal properties compared to DPSCs isolated from healthy pulp (Picanço-Castro et al., 2014). This also indicated that this microbial toxin has least effect on their mesenchymal phenotype. This would perhaps indicate that although, DPSCs were responding to bacterial toxin in terms

of expression of pro-inflammatory cytokines, they were capable to differentiate into intended lineages. This feature is very beneficial especially when the DPSCs are to differentiate into certain cell lineages such as odontoblasts to facilitate repair on damages done by LPS (Yazid et al., 2014).

Cell viability is physiologically and pathologically controlled by alteration in cellular microenvironment especially when they are exposed to a microbial toxin, like *E.coli*. In this study, the change in DPSCs viability was observed every 12 hours for all concentrations, to closely observe any effect of increasing time and LPS concentration on DPSCs viability. It was observed that the DPSCs viability was significantly reduced in LPS treated cells as compared to non-treated cells at all time points and concentrations. In particular, it was observed that LPS concentration less than 2µg/mL showed higher DPSCs viability, on increasing the concentration, above 2µg/mL the viability dropped significantly. Similar findings were observed in study conducted by Liu et al (2014) which reported that higher concentrations of LPS from *Streptococcus mutans* were able to inhibit the proliferation of DPSCs *in vitro* (He et al., 2015). However, there are studies reporting otherwise whereby LPS from *P. gingivalis* promoted cell proliferation in periodontal ligament stem cells (PDLSCs). In another study by Dongmei Li et.al, cell adhesion was more strongly promoted by 1 µg/mL LPS and not at higher LPS concentrations (D Li et al., 2014). This finding indicated that there may be a threshold concentration of bacterial toxin for DPSCs to stimulate a protective molecular cascade to support cell survival. However, molecular mechanism underlying the regulation of cytoprotection stimulated by LPS remains unclear. In a similar study on epithelial cells and gingival fibroblasts, Basso et.al showed that fibroblast viability was significantly reduced at 48 and 72h on exposure to LPS (Basso et al., 2015). In a separate study by Gonçalves et al. on cancer cell lines it was demonstrated that LPS, at higher concentrations (>1 µg/mL) showed significantly reduced cell viability when compared to

control, for all the times tested (24, 48 and 72 h) (Gonçalves et al., 2007). Morsczeck et al in their study showed that after 24 h of incubation with LPS the cell proliferation of dental follicle cells was significantly decreased (Morsczeck et al., 2012). From our results it can be reflected that concentration of less than 2µg/mL for time period of 24h – 48h are the most favourable conditions to induce inflammation in DPSCs. In a separate study TLR4 receptor stimulation in BMSCs regulates proliferation and apoptosis via PUMA (p-53 up regulated modulation of apoptosis) (Neal et al., 2012), that might be one of the reason behind reduced viability post 2µg/mL LPS concentration and 48th hour. The threshold stimulation of TLR4 receptor might have shot up too high resulting in reduced cell viability, which might not be that prominent at lower concentration and earlier time points. These heterogeneous effects of bacteria on the induction or inhibition of cell viability across studies could imply the complexity of the underlying mechanisms that rule the interactions between host cells and bacteria. These inconsistencies could be due to perhaps different cell type or even due to activation of different pathway by LPS itself (Y Liu et al., 2014).

This varied pattern of increased cytokine expression with increased concentration and treatment time was evident throughout the study . The findings in our study were in line with a similar study done on umbilical cord stem cells, that displayed up-regulated expression of pro-inflammatory cytokines such as interleukin IL-1α, IL-6, IL-8 and tumour necrosis factor after infection with *E. coli* (Kato et al., 2014). However, taking into account the cell viability results, LPS treatment with 1µg/mL concentration at 24 hours is considered to be optimum to induce inflammation in DPSCs without compromising their proliferative and mesenchymal characteristics. This phenomenon still requires further elucidation especially by targeting the possible pathway where genes and the proteins would execute their functions.

Cell response to bacterial stimuli seems to be associated with the cell type, bacterial strain and specific bacterial components used in each experimental setting (Yilmaz, 2008). This indicates that DPSCs cultured in an inflamed microenvironment (iDPSCs) is suitable to simulate inflammation-like condition for *in-vitro* testing of regenerative protocols.

4.5 Conclusion

Within the limitations of the study, it can be concluded that amongst the selected LPS concentrations, treatment with 1 µg/mL LPS for 24 hours is chosen to be most appropriate condition to induce inflammation in DPSCs. However, for further elucidation, LPS induced molecular pathway involved in the inflammation induction in DPSCs would be required.

CHAPTER 5: PLATELET RICH CONCENTRATES IN INDUCTION OF ANGIOGENESIS IN DENTAL PULP STEM CELLS

5.1 Introduction

The fundamental of tissue regeneration is based on the concept of restoring the physical integrity of cells, tissues and organs by means of the organisms' own repair mechanisms. A successful regenerative therapy involves sufficient population of stem cells and plethora of enzymes, signal proteins, ligands and a functional vasculature (Kleinheinz et al., 2005). Mesenchymal Stem Cells (MSCs) are the most popular and easily available source of stem cells for regenerative therapy. Regenerative studies in the past have reported MSCs to be safe for therapeutic use in orthopaedics and sports injuries (Hare et al., 2012). Dental pulp stem cells (DPSCs) are one such type of MSCs that due to their easy availability and non-invasive mode of isolation hold great potential to be used in regenerative protocols.

DPSCs have higher angiogenic, neurogenic, and regenerative potential, presenting a versatile stem cell source for cellular therapies(Iohara et al., 2013). In dental field, DPSCs have demonstrated the ability to differentiate into odontoblasts under stimulation of bioactive materials that makes them a potential source of stem cells for dentine and pulp regeneration (Prescott et al., 2008). Apart from the deciduous teeth, they can be obtained from permanent teeth and harvested in a minimally invasive and safe manner.

In order for stem cell therapy to be effective, cells should be in sufficient numbers, usually in the order of millions (Horn et al., 2010).Culture expansion and maintenance is of considerable importance for advancement of both allogeneic and autologous cell therapies. In the majority of clinical trials, commercially available foetal bovine serum

(FBS) is used for stem cells isolation and expansion. It provides rich composite of growth factors to yield sufficient cell proliferation and expansion during cell culture experiments(Bettiol et al., 2007). However, it does pose a risk of immunogenic reactions in response to xenogeneic proteins (Mirabet et al., 2008). During culture, these prions could be transmitted to stem cells from FBS. It has been observed, that cells cultured in the presence of FBS, when injected into the human body, exhibited an immune reaction due to the animal nature of the sera used for cultures(Mirabet et al., 2008). Threat of unknown infectious agents in FBS is of significant concern. Hence, it is of paramount importance to explore the alternatives that are cost effective, autologous, prion free in nature and can effectively improve the proliferation and differentiation of the stem cells *in-vitro*.

A recent strategy in many regenerative fields including regenerative dentistry is the use of platelet rich concentrates (PRCs). In general, PRCs are concentrated source of autologous platelets or their extracts in a small amount of plasma (DMD Ehrenfest et al., 2014). They are blood-derived products used for the prevention and treatment of haemorrhages(Barsotti et al., 2013). Use of PRCs in wound healing dates back to 1990's demonstrating their role in accelerating healing process at inflamed site (Kiran et al., 2011). Other clinical uses of PRCs have been reported in the fields of dermatology, orthopaedics, dentistry, and ophthalmology (De Pascale et al., 2015). The cocktail of growth factors (GFs) released from PRCs is responsible for activation of many cellular processes including mitosis/cell cycle, production of collagen (Schär et al., 2015), recruitment of other cells to the site of injury, initiation of angiogenesis, and cell differentiation (Freymiller & Aghaloo, 2004).There are approximately 30 bioactive proteins known to date, that play fundamental role in tissue haemostasis and repair (Schliephake, 2002), namely, platelet-derived growth factor (PDGF), epithelial growth factor (EGF), vascular endothelial growth factor (VEGF), endothelial cell growth factor

(ECGF), fibroblast growth factor (FGF), transforming growth factor beta (TGFβ), and insulin-like growth factor (IGF), hepatocyte growth factor (HGF) and many others (Sell et al., 2011). This molecular pool initiates the interaction of molecules such as collagen, thrombin, platelet-activating factor, serotonin, calcium, magnesium, thromboxane A2, and adenosine di-phosphate with platelet receptors (Kiran et al., 2011) that constitute an integral part of cell regenerative process. Recent proteomic studies have shown the complexity of platelet secretome, and indexed not only GFs but also a vast array of molecules including cytokines and chemokines, adhesive proteins, enzymes, fibrinolytic and anti-fibrinolytic proteins (Del Corso et al., 2010).

A number of studies have shown that stem cells expanded in two-dimensional cultures using PRCs uphold their multipotency and therapeutic properties (Crespo-Diaz et al., 2011; Mishra et al., 2009; Sánchez-González et al., 2012). It has also been shown that cells expanded in PRCs showed no signs of immunogenic reactions when used in clinical trials (Garcia-Godoy & Murray, 2012; Ramos-Torrecillas et al., 2014).

This approach has paved the way for improvements in stem cell properties including cell growth, viability, proliferation and differentiation, leading to an effective regenerative potential (Anitua et al., 2004). Moreover, translation of the combination of stem cell and PRCs in clinical set up is being powered by their easy availability, cost-effectiveness, extensive range of applications, autologous nature, simple collection, easy chair side preparation, and clinical application without the risks associated with allogeneic products (Schär et al., 2015). PRCs may prove beneficial for tissues with restricted blood supply, slow cell turnover, and limited extracellular matrix restoration, owing to their nature of sustained release of growth factors (Bielecki & M Dohan Ehrenfest, 2012).

Angiogenesis, which is a key factor for making any regenerative therapy successful, involves a complex cascade of biological processes like cell adhesion,

proliferation, vascular activation and stabilization (Carmeliet, 2005). It is a pivotal factor in many areas of tissue maintenance and regeneration because insufficient blood supply at the intended site may lead to insufficient oxygen and nutrition supply to implanted tissue, resulting in necrosis (Laschke et al., 2006). In regenerative therapies, initiation of angiogenesis in parallel with tissue regeneration would be an integral part that leads to successful treatment strategies (Jung & Kleinheinz, 2013). DPSCs have been reported to have the ability to induce angiogenesis in a paracrine fashion under the stimulatory effect of pro-angiogenic growth factors (Bronckaers et al., 2013). Tissue regeneration involving PRCs has been attempted in various clinical and pre-clinical settings. It offers numerous advantages since it is neither toxic nor immune-reactive, and can be readily obtained, inexpensively, from patient's own blood. In a study it was been proven that growth factors chemotactically recruit and activate stem cells as well as induce their mitogenesis and differentiation (Liao et al., 2013). Animal studies in the past have suggested a positive influence of PRCs on angiogenesis (Anitua et al., 2015; Choukroun et al., 2006; Pietramaggiori et al., 2008). In a study by Mammoto et al in 2013 it was observed that PRCs promoted vascular growth and stimulated endothelial progenitor cells to form vessel like structures (Mammoto et al., 2013). Anitua et al. recently studied the effect of PRCs that forms a fibrin matrix system resulting in improved perfusion and tissue remodelling in ischaemic tissue of mouse hind limb (Anitua et al., 2013). Hence, use of PRCs, is potential a way to accelerate and enhance the body's natural healing and regeneration mechanisms (Kiran et al., 2011).

All these processes require an intricate interplay of various signalling mechanism, which can be achieved by a diversity of GFs, secreted by PRCs (Peterson et al., 2010). The use of PRCs have been continuously advanced since last few years, depending primarily on their method of preparation and intended site of application. They are classified as platelet rich plasma (PRP), platelet rich fibrin (PRF) and human platelet

lysate (HPL). PRP has been actively employed in clinical practice for treating infra-bony defects, jaw reconstruction and gingival grafts and have demonstrated promising results (Arora et al., 2009). Previous studies have reported promising effect of PRP on the proliferation and differentiation potential of MSCs (Kocaoemer et al., 2007; Niemeyer et al., 2010). There are concerns over the use of bovine thrombin for platelet activation in PRP, that may pose a risk of xenogenic contamination transmitted by commercial preparations.

In dentistry, PRP has been actively employed in clinical practice for periodontal regenerative protocols and implant surgeries (Preeja & Arun, 2014). Dentists have attempted the use of PRP in teeth with immature apex and could get positive results in terms of elimination of inflammation and root apex closure (Güven Polat et al., 2014). Alongside, another study demonstrated that interaction of exogenously implanted stem cells and PRP demonstrated formation of vital pulp-like tissue within the root canals of infected immature teeth of a 9 year old boy who reported to the clinic with a fractured central incisor post trauma (W Zhu et al., 2013).

Another type of PRC preparation is PRF, also known as second-generation platelet concentrate prepared by centrifugation of blood sample without anticoagulant typically at a speed of 3000 rpm for 10 minutes. Due to absence of anticoagulant, the platelets in this concentrate are already in an activated state. This fraction of PRC is most suitable for immediate chair side procedures like sinus lift procedure, treatment of perio-endo furcation defects and as interposition biomaterial (Choukroun et al., 2006; Trombelli et al., 1996).

Another, development of PRC is lysate, also known as human platelet lysate (HPL). It is prepared from PRP, by lysing the platelets and stimulating the release of GFs (Horn et al., 2010; Rauch et al., 2011; Schallmoser & Strunk, 2013). HPL has been explored for bone regenerative studies using stem cells. However, its clinical translation

for therapeutics is still under trial (Altaie et al., 2016). On the same note, HPL has been shown as an effective cell culture additive that is concurrently used in many laboratories and clinical trials (Chen et al., 2012; Doucet et al., 2005; Kim et al., 2004; Tarte et al., 2010; Von Bonin et al., 2009). However, the biological effects and optimal concentrations of HPL for the proliferation and differentiation of human DPSCs has not been explored extensively.

HPL can be produced from PRP units by a simple freeze-thawing procedure. This is a very simple, fast and effective procedure. Pooling of platelet units can reduce variation between individual donor's platelets. Protocols have been developed to generate large pools of HPL to balance the lot-to-lot variation (Schallmoser et al., 2007; Schallmoser & Strunk, 2013). HPL minimizes the risk of immunological reaction (Horn et al., 2010) and can be produced according to good manufacturing practice protocols to scale-up of MSCs production for clinical applications (C Lange et al., 2007; Müller et al., 2006).

These findings indicate that interplay of stem cells and PRCs may be considered as a prospective combination for regenerative protocols *in-vitro*. Therefore, in this study we aimed to compare the effect of two types of PRCs namely HPL and PRP on the maintenance of cell viability, expression of angiogenic surface marker, release of growth factors and the ability to form capillary-like structures indicating the process of angiogenesis. The specific objectives of this study are:

a) To optimize the suitable concentration of two types of PRCs supporting the viability of DPSCs.
b) To determine the pro-angiogenic effect of optimized concentration on DPSCs.

5.2 Materials and Methods

5.2.1 Ethics approval for collection of human extracted permanent teeth

Ethics approval for the sample collection and processing was obtained from Medical Ethics Committee, Faculty of Dentistry, and University of Malaya {DFDP 1304/002 (P)}. Figure 5.1 depicts the schematic representation of the experiment process.

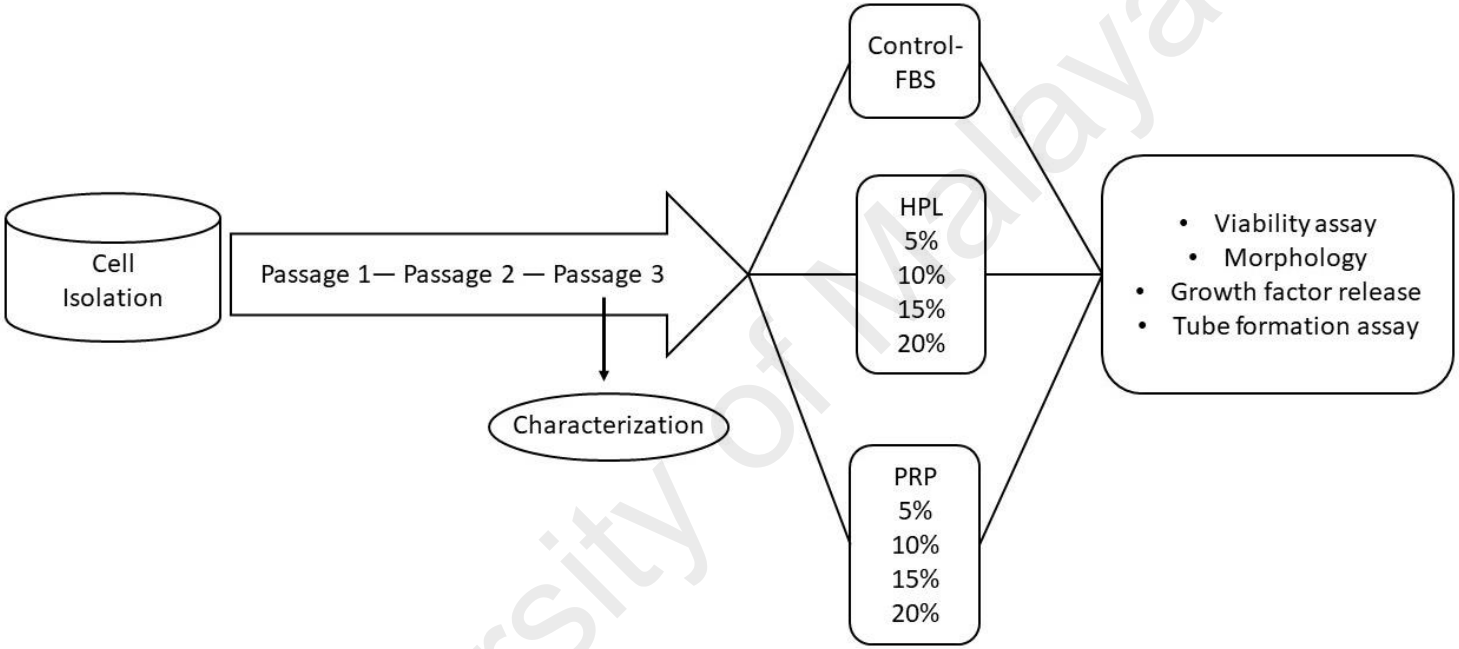

Figure 5.1: **Schematic representation of experiment process.** The study was commenced with isolation of DPSCs from pulp tissue (n=5) cultured up to passage 3. DPSCs were then treated with various concentrations of HPL and PRP and were investigated for their effect on morphology, cell viability and tube formation capacity. Cells cultured with complete media were considered as control.

5.2.2 Preparation of two types of platelet rich concentrates (PRCs)

5.2.2.1 Platelet Rich Plasma (PRP)

PRP samples (n=8) were obtained from Blood Bank University of Malaya Medical Centre, Kuala Lumpur, Malaysia after ethics approval from the Medical Ethics Committee, Faculty of Dentistry; University of Malaya {DFDP 1304/002 (P)}.

Whole blood from eight healthy donors kept in triple bags and was stored at room temperature (20^0C). Exclusion criteria of donors included those consuming any blood thinning drug, undergone major surgery in last one-year smoking, alcohol consumption, narcotics addiction, recently diagnosed with acute or chronic systemic disease. Blood samples were then centrifuged at 3600 rpm for 15 min at 22^0 C in a centrifuge (Heraeus™ Megafuge™ 16 Centrifuge Series, Thermofisher Scientific, USA). After centrifugation, PRP was taken out in a triple bag and hanged on semi-automated separator for automated processing. PRP was extracted completely and transferred into an empty bag. 100mL of PRP from each donor bag was collected in blood collection tubes for research purpose. PRP from all eight donors was pooled (800mL). Bags and collection tubes containing PRP were then placed in the blast freezer for rapid freezing for 15 minutes. After 15 minutes, PRP was then stored in the -70^0C freezer until further use.

5.2.2.2 Human Platelet Lysate (HPL)

HPL was prepared from frozen pooled PRP from eight different donors.. Frozen PRP from the donors was thawed at 37°C in a sterile water bath and were subjected to four freeze thaw cycles to lyse platelets. The lysate obtained was centrifuged three times at 3000 rpm for 30 min to remove the ruptured platelets cell walls and debris. Final products were then stored at -80°C until further use.

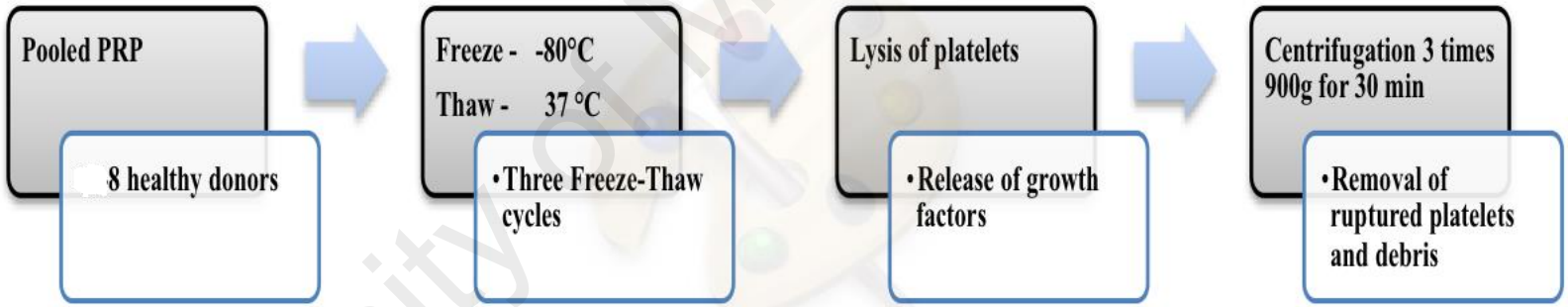

Figure 5.2: Step by step procedure for preparation of human platelet lysate.

5.2.3 Preparation of media with various concentrations of HPL and PRP

HPL and PRP concentrations (Table 5.1) used in this study were selected based on the results obtained in previous studies (Barsotti et al., 2013; Castegnaro et al., 2011; Hayon et al., 2013; Tavassoli-Hojjati et al., 2016). Culture media with various concentrations of HPL and PRP were prepared based on the composition stated in Table 5.1.

Table 5-1: Various culture media groups used in the experiment

Culture media groups	Composition	
Control	DMEM-KO+10% FBS (v/v)	2mM L-glutamax + 50 units/mL Penicillin-Streptomycin
HPL	DMEM-KO + (5%), (10%), (15%), (20%) (v/v) HPL + 5000 IU Heparin	
PRP	DMEM-KO + (5%), (10%), (15%), (20%) (v/v) PRP + 5000 IU Heparin	

5.2.4 *In-vitro* expansion and maintenance of DPSCs in HPL and PRP

DPSCs from five healthy donors (n=5) yielded at passage 3 were seeded into 6-well plates at a seeding density of 2× 105 cell per well in 2mL of culture media. After 24 hours of incubation at 37°C and 5% CO2 in a humidified incubator, culture media was discarded and cells were thoroughly washed with DPBS (Gibco, USA). DPSCs were then incubated with various concentrations of previously prepared culture media (Table 5.1) at 37 °C at 5% CO2 in a humidified incubator. Respective culture medias were changed every three days. All concentrations of PRCs and control were tested in triplicates. Any morphological changes of DPSCs treated with various concentrations of PRCs were observed and recorded under a fluorescence microscope (Leica Microsystems, Germany) at three various time points; Day 0, Day 2 and Day 8 post-incubation.

5.2.5 Effect of various concentrations of HPL and PRP on viability of DPSCs

DPSCs (n=5) at passage three were seeded into 96-well plates in triplicates at a density of 5×10^3 cell per well in 200 µl of complete culture media. After 24 hours of incubation at 37°C and 5% CO2 in a humidified incubator, culture media was discarded and DPSCs were washed thoroughly with DPBS. All cells were then incubated in various concentrations of PRCs media (5%, 10%, 15% and 20%) and complete culture media for 2,4,6,8 and 10 days. After each time point 100µl of DMEM-KO (Gibco USA) along with 10% Alamar Blue reagent (Thermo fisher scientific, USA) v/v was added to each well and plates were further incubated for 3 hours at 37°C and 5% CO2 in a humidified incubator. Absorbance was measured at 450nm with reference wavelength set at 590nm by Tecan (Infinite 200 PRO, Life sciences, Switzerland) microplate reader. Viability of DPSCs after treatment with HPL and PRP was determined using Alamar Blue assay (Thermo fisher scientific). Cell viability was calculated as percentage from the reduction in the absorbance recorded. DPSCs cultured in media supplemented with FBS are considered as a control and reference (normalized to 100%).

5.2.6 Effect of various concentrations of HPL and PRP on pro-angiogenic growth factors release from DPSCs

Release of pro-angiogenic GFs, fibroblast growth factor (FGF), vascular endothelial growth factor-A (VEGF-A), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF-BB), and monocyte chemotactic protein-1 (MCP-1) using multiplex immunoassay (Procarta, Multiplex immunoassay, Affymetrix, ebioscience, USA). GFs used in the study were selected based on their involvement in cell proliferation, migration and angiogenic effect as reported by few studies (Table 5.2). Experiment was conducted using commercially available multiplex immunoassays. Reagents and detection antibodies were supplied by Affymetrix, ebioscience, USA.

Table 5-2: List of GFs with functions

Name of Growth Factor (ID)	Function (References)
Fibroblast growth factor (FGF-2)	➢ Stimulates self-renewal and differentiation in ESC (Govindasamy et al., 2011) ➢ Stimulates *in-vitro* expansion of BMSCs (Kunath et al., 2007) ➢ Regulates migration and adhesion of ESC (Ahn & Brown, 2009)
Platelet derived growth factor BB (PDGF-BB	➢ Accelerates microvascular formation in endothelial cells (Eiselleova et al., 2009) ➢ Regulates proliferation, migration and differentiation of MSCs ➢ Stimulates angiogenesis in MSCs (Battegay et al., 1994)
Vascular endothelial growth factor (VEGF)	➢ Stimulates the expansion of neural stem cells (Carmeliet, 2005) ➢ Enhances the recruitment of BMSCs into blood vessels during angiogenesis(Schänzer et al., 2004) ➢ Enhances proliferation of MSCs in culture(Beckermann et al., 2008)
Hepatocyte growth factor (HGF)	➢ Enhances the motility of endothelial cells to promote angiogenesis (Pons et al., 2008) ➢ Promotes the angiogenic effect of VEGF (Bussolino et al., 1992) ➢ Promotes migration, proliferation, and differentiation of BMSCs(Bussolino et al., 1992)
Monocyte chemotactic protein-1 (MCP-1)	➢ Increases chemotaxis of monocytes at inflamed site (G Forte et al., 2006) ➢ Regulates VEGF mediated angiogenesis (Deshmane et al., 2009) ➢ Enhances migration of macrophages at inflamed site (Hong et al., 2005)

DPSCs (n=5) at passage 3 were seeded in T75 cell culture flasks at the seeding density of 2.1 x 10^6 and incubated with various concentrations of HPL and PRP till 70% confluence for 12h, 24h and 48h respectively in triplicates.

5.2.6.1 Sample preparation for multiplex immunoassay

Aspirated culture media supernatant and cell lysate from all the culture flasks were used for multiplex immunoassay. HPL and PRP supplemented media supernatant was aspirated from all T75 culture flasks and stored at -80ºC for multiplex immunoassay. DPSCs lysate was prepared by scrapping the adherent cells from the flask bottom using a plastic cell scrapper. Cells were then transferred to 15mL centrifugation tube and centrifuged at 1500 rpm at 4ºC for 5 minutes. After discarding the supernatant, cells were re-suspended into 5mL of ice cold PBS and centrifuged again at 1500 rpm at 4ºC for 5 minutes. Supernatant was then removed without disturbing the cells and cell lysis buffer was added (Affymetrix, ebioscience, USA). Cell suspension is then pipetted up and down 8-10 times and incubated on ice for 5 minutes. Cell suspension was then transferred to 1.5mL micro centrifugation tube and centrifuged at 20000 rpm at bench top centrifuge for 10min. Cell lysate obtained after centrifugation was used to run the ELISA assay. 650µl of each sample was used to carry out the assay that was performed using a standard antigen cocktail for standard curve preparation. GFs released in the supernatant and lysate were detected using detection antibodies provided by the supplier. Quantification of GFs was done using Tecan Infinite 200 PRO, Life sciences, Switzerland at absorbance 450nm, compared against the control. GFs were detected in cell supernatant medium and cell lysates in triplicates of at least three donors of DPSCs.

5.2.7 Effect of various concentrations of HPL and PRP on tube formation capacity of DPSCs

To evaluate the tube formation capacity of DPSCs, matrigel based functional angiogenesis assay was performed.

5.2.7.1 Preparation of matrigel matrix

Matrigel matrix (Catalogue no. BIC354234, BD Biosciences, USA) was thawed overnight at 4°C. Pre-cooled pipettes, tips, and tubes were used throughout to avoid any temperature raise around matrigel matrix. A precooled 24-well plate kept on ice bath was then coated with 300µl of 10 mg/mL matrigel matrix (10mg/mL), using pre-cooled 100µl micropipette from the base of the well to avoid inclusion of air bubbles. After the matrigel was evenly dispersed in, all the wells, culture plate was then incubated at 37°C and 5% CO_2 in a humidified incubator for 60 minutes.

5.2.7.2 DPSCs culture on coated matrix

DPSCs yielded at passage 3 (n=3) were seeded into the matrigel coated plates at a seeding density of 1×10^5 cell per well in 2mL of HPL and PRP culture medias in respective concentrations (Table 5.1), culture media was supplemented with FBS and endothelial cell media (ECM) (Science cell, USA). Culture plates were then incubated in a humidified incubator at 37°C and 5% CO_2. Cells incubated with ECM were considered as positive control. Culture plates were observed for any network formation and micrographs and readings were recorded every six hours using a fluorescence microscope (Leica Microsystems, Germany) for 24 hours. Experimental and control groups were tested in triplicates.

5.2.8 Statistical Analysis

All experiments were conducted on samples from three separate donors. Data was analysed using SPSS 22.0 software. Results were expressed as mean and standard deviation of three replicates wells for all samples and controls. Statistical analysis was done using independent student's t-test. $p<0.05$ were considered significant.

5.3 Results

5.3.1 Morphology of DPSCs after treatment with various concentrations of HPL

Morphology of DPSCs treated with various concentrations of HPL and PRP were observed at three time points, Day 0, Day 2 and Day 8. It was observed that morphology of DPSCs remained elongated and spindle shaped, resembling typical MSC morphology, under all culture conditions, throughout the culture period of eight days. Except at 20% HPL on Day 8, where DPSCs demonstrated flat shaped morphology, showing mild resemblance to endothelial cell (Figure 5.3).

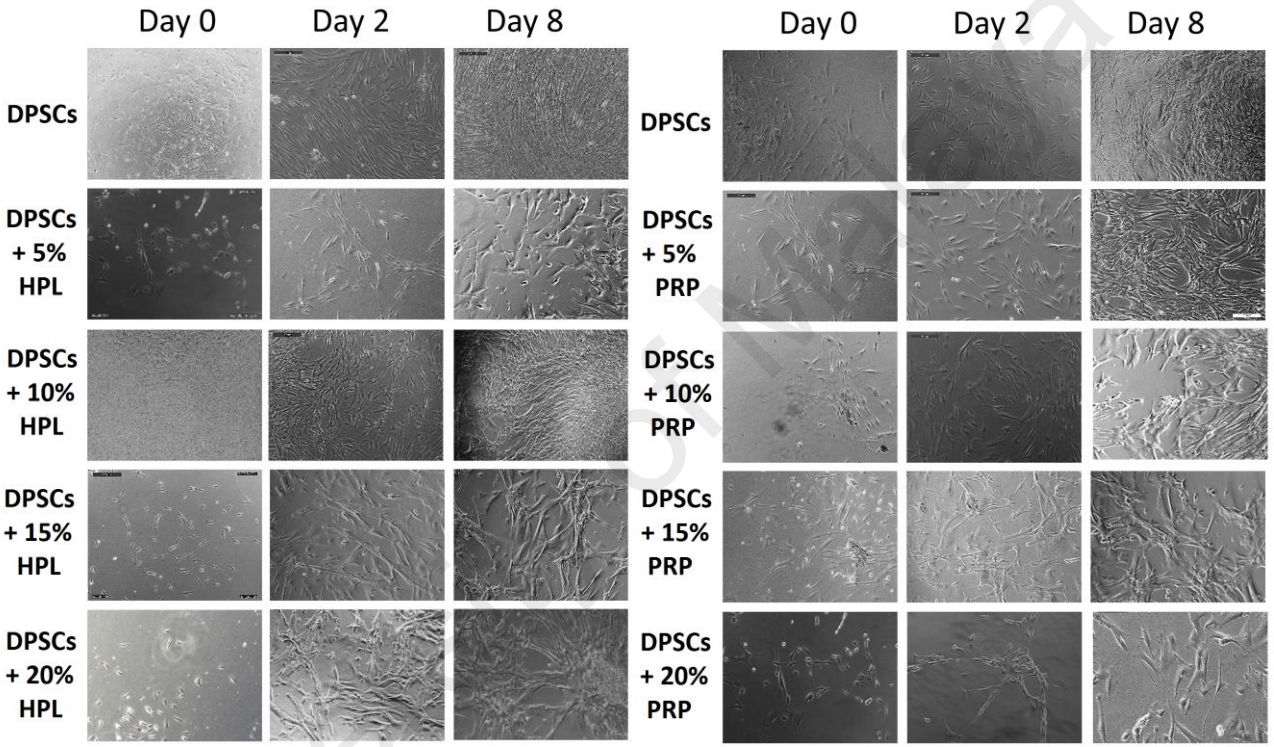

Figure 5.3: Morphology of DPSCs upon exposure to various concentrations of HPL and PRP at three different treatment times. Scale bar = 100µm. Upon exposure of DPSCs to various HPL and PRP concentrations (10% and 20%) at Day 0, Day2 and Day 8, it was observed that DPSCs morphology remained elongated spindle shaped at all concentration and time points. At 20% HPL on Day 8, DPSCs demonstrated flat shaped morphology, showing mild resemblance to endothelial cells.

5.3.2 Viability of DPSCs after treatment with various concentrations of HPL and PRP

Experiment for cell viability was carried for 10 days and observations were recorded after every two days (Figure 5.4). Significantly ($p<0.05$) higher viability was observed in DPSCs treated with10, 15 and 20 percent of HPL compared to that of PRP. However, no significant difference in viability was observed among the DPSCs treated with 5% HPL, 5% PRP and 10% PRP. Viability of DPSCs in both the groups was significantly lower at Day 6 ($p<0.05$) compared to the viability at Day 2, 4, 8 and 10 at all concentrations. In addition, an interesting pattern was observed in all tested concentrations whereby the viability decreased from Day 2 until Day 6 of culture before an increase until Day 10. A significantly higher ($p<0.05$) viability was observed with HPL 10%, 15% and 20% at Day 2 (97%) compared to Day 6 (64%) (Figure 5.5). Moreover, the viability of DPSCs treated with HPL was significantly increased ($p<0.05$) at Day10 compared to that at Day 6. Except at 5%, HPL (~70%) viability of DPSCs in other concentration was about 80%. Whereas, in PRP irrespective of the concentration and time, viability of DPSCs was equal to 80% or below. Viability varied the most amongst various concentrations of PRP. In PRP treated cells especially, it was noted that increasing its concentration more than 5% did not alter the final viability of cells.

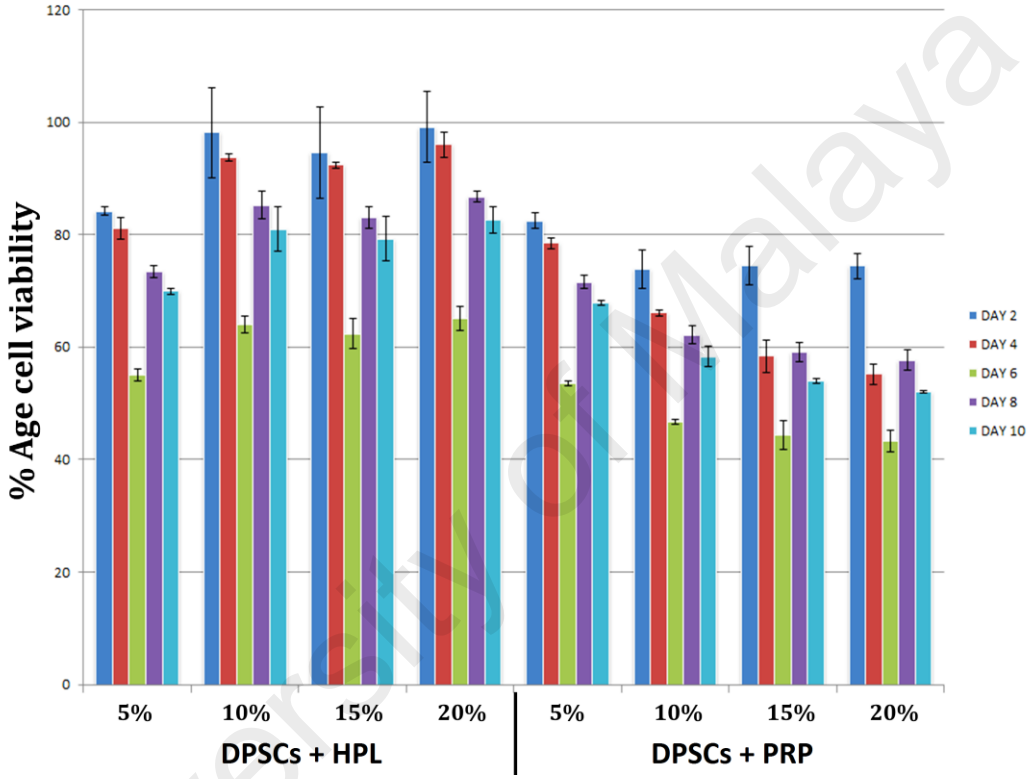

Figure 5.4: **Percentage cell viability upon exposure to various concentrations of HPL and PRP at five different time points.** Exposure of DPSCs to various concentrations of HPL and PRP (10% and 20%) at Day 2,4,6,8 and Day 10 showed significantly ($p<0.05$) high viability at Day 2 and Day 4 for 10%, 15% and 20% HPL media groups.

5.3.3 Pro-angiogenic GFs release from DPSCs after treatment with various concentrations of HPL and PRP

Pro-angiogenic GFs release showed significant difference in release profile of HPL and PRP. Figure 5.5 shows that HPL treated group showed significantly ($p<0.05$) high release of all GFs compared to other media groups. All media groups demonstrated initial high release at 12 hours that declined after 24h. Both 10 & 20% HPL demonstrated significantly ($p<0.05$) high GFs release at 12h and 24h except for FGF-2 at 12h, where control group has shown remarkably ($p<0.05$) high FGF -2 release compared to all media groups. HGF release was observed to be significantly ($p<0.05$) high at 24h in HPL 20% group, when compared to other media groups. VEGF-A release profile shows that all media groups showed significantly higher ($p<0.05$) amount of VEGF-A at 12 hours compared to control group. While, the highest concentration was recorded for HPL 20% followed by HPL 10%. However, at 24 hours both HPL 10 % and 20% group demonstrated similar release, although higher than all other media groups. Results of MCP-1 showed that at 12h, amongst all media groups, a significantly high MCP-1 release was noticed at 10% and 20% HPL group. However, at 24h, all media groups except control showed significantly high ($p<0.05$) release of MCP-1, highest being demonstrated in HPL 10% and 20% group. MCP-1 release dropped remarkably at 48h. PDGB-BB showed similar release profile of being highest in 10% and 20% HPL. However, it was noticed that PRP 20% showed remarkable high PDGF-BB release at 12h.

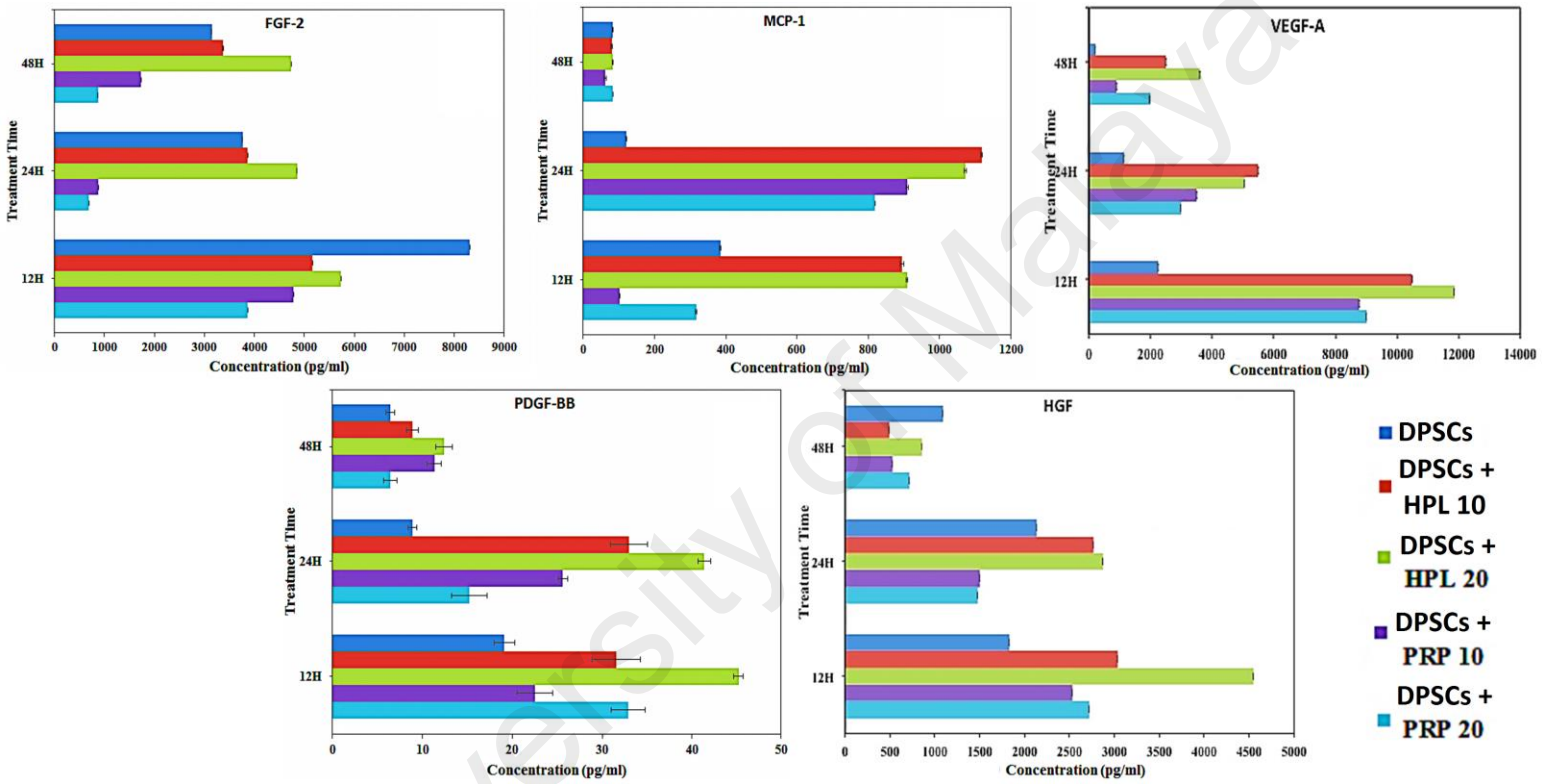

Figure 5.5: Concentration of growth factors released in DPSCs upon exposure to various concentrations of HPL and PRP. Exposure of DPSCs to various concentrations of HPL and PRP (10% and 20%) at 12h, 24h and 48h showed significantly ($p<0.05$) high release of growth factors at 12h and 24h in HPL 10% and 20% media groups when compared to control (DPSCs)

5.3.4 Effect of Tube formation capacity of DPSCs after treatment with various concentrations of HPL and PRP

DPSCs cultured with 20% HPL demonstrated the formation of tube-like structures at 12h, following a similar pattern as shown in control (ECM) group (Figure 5.6). DPSCs cultured in almost all concentrations of HPL and PRP showed evident network formation, but it was not as dense and interconnected, as seen in 20% HPL group. DPSCs cultured in FBS showed no evidence of tube-like network formation.

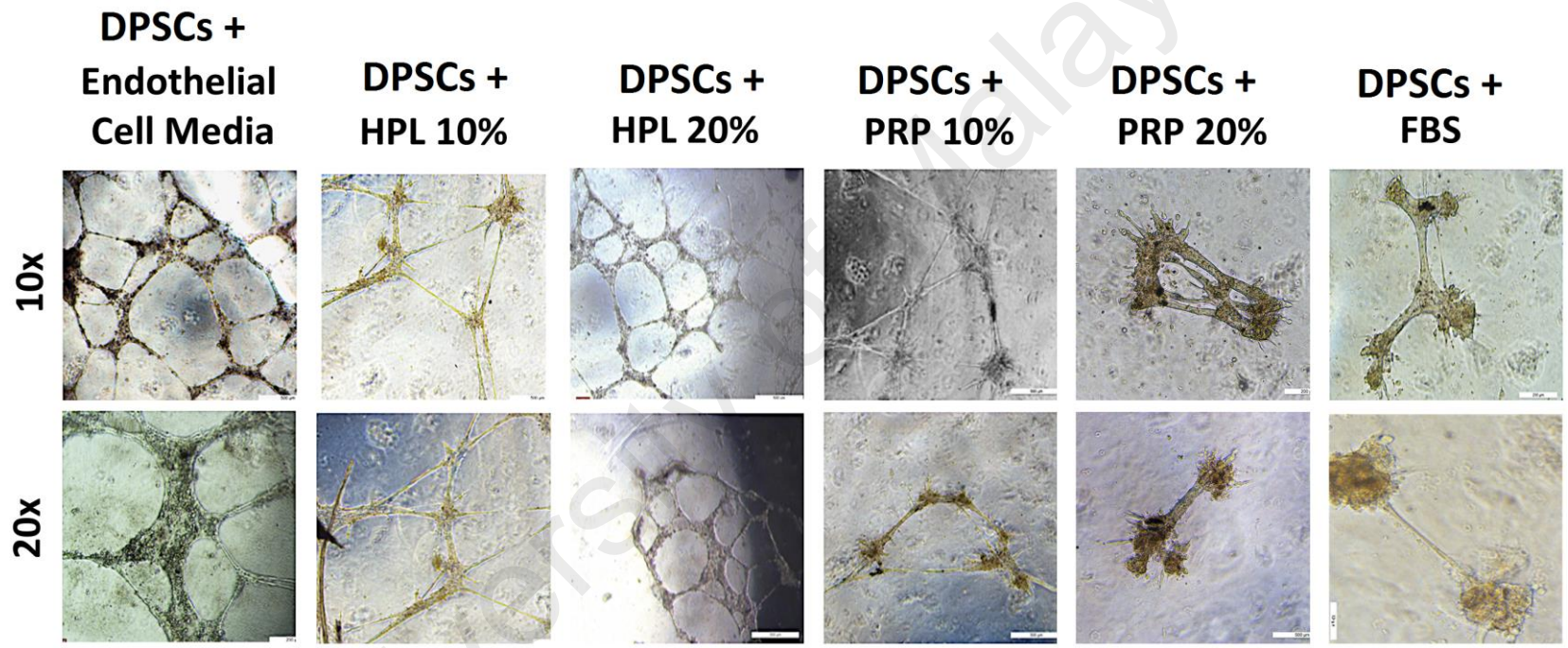

Figure 5.6: Tube formation assay of DPSCs on exposure to various concentrations of HPL and PRP. Scale bar = 100µm. Upon exposure of DPSCs to various concentrations of HPL and PRP showed evident tube like network formation in 20% HPL media group similar to that in control (ECM) media group.

5.4 Discussion

MSCs are currently in focus due to their potential in cell therapy and tissue regeneration. *In-vitro* stem cell culture and their transition to *in-vivo* application requires defined, safe, and animal component-free culture conditions (Atala, 2007) (Van der Valk et al., 2010) that can yield stem cell in higher numbers to the order of millions (Niu et al., 2008; Niu & Kolattukudy, 2009). However, most isolation and expansion protocols for clinical-scale production of MSCs utilize commercially available foetal bovine serum (FBS) (Pittenger et al., 1999), which has demonstrated to be a possible host of potential viral and prion transmission (Kocaoemer et al., 2007). As suggested by good cell culture practice (GCP), substitution of sera would lead to a better quality and reproducibility of experimental data (Mirjalili et al., 2005).

PRCs are autologous serums that contain abundant biologically active GFs (Balls et al., 2006; Gstraunthaler, 2006; Van der Valk et al., 2010). After appropriate pathogen inactivation PRCs demonstrated potential cell growth promoting and differentiation activity with reduced chances of infection transmission, when compared to animal-based serums (Qiao et al., 2017). They have also been demonstrated as an effective source of angiogenesis promoting GFs, which is a vital requisite for healing of inflamed tissue (Barsotti et al., 2013). Adequate vascularization of the tissue confers the supply of nutrition to the tissues (Nör, 2006). Hence, for successful regenerative therapy it is paramount to have sufficient blood supply to the target tissues. When the inflammatory phase has been initiated, the healing response requires angiogenesis that modulates the activation, proliferation, and migration of endothelial cells to establish new blood vessels from pre-existing vasculature (Oklu et al., 2010).

PRCs are known to contain a plethora of GFs and could have an augmented effect on angiogenic potential of DPSCs. However, effect of PRCs on DPSCs has not been explored widely. Hence, this study was designed to optimise the suitable concentration

of HPL and PRP to maintain viability of DPSCs, comparable to FBS, *in vitro* and to compare the angiogenic effect of HPL and PRP in DPSCs.

HPL used in this study was prepared by freezing and thawing of pooled PRP from eight healthy donors, since pooling of PRP from various donors reduces the individual variation (Irsch & Lin, 2011). Concentrations of HPL and PRP used in this study were selected based on references from the literature. A study done on wound healing model reported that both 10% and 20% of human platelet lysate (HPL) supported cell viability and migration in a co-culture of endothelial cells, monocytes, fibroblasts and keratinocytes. However, 20% HPL exhibited a significant angiogenic effect due to the presence of high concentration of angiogenesis promoting growth factors (Barsotti et al., 2013). Another study on periodontal (PDL) fibroblast demonstrated significantly high cell viability when PDL fibroblasts were treated with 5% PRP compared to 0.1% and 50% PRP. It was shown that remarkably reduced viability was evident at 50% PRP (Barsotti et al., 2013). A study on endothelial progenitor cells reported formation of microvascular network when treated with PL. However, effect of concentration was not reported in the study (Tavassoli-Hojjati et al., 2016).

Based on the findings from previous studies we selected a range of HPL and PRP concentrations to optimize the most suitable concentration to supports the DPSCs viability. The optimized concentrations were then investigated for their pro-angiogenic effect on DPSCs.

After culturing DPSCs in various concentrations of HPL and PRP, morphology of DPSCs was observed to be elongated and spindle shaped (Figure 5.4). There were no significant visible differences in morphology between the DPSCs cultured in media with HPL and PRP when compared to those cultured in media supplemented with FBS. These findings were partially in line with a study done on cryoprotective effect of PRP (Fortunato et al., 2016). It was also observed that at 20% HPL concentration DPSCs

started becoming expanded and flat, that closely resembles the morphology of endothelial cells. However, no studies in the literature have reported on the effect of higher concentrations of HPL on DPSCs morphology.

Cell viability results of the study showed that HPL at 10%, 15% and 20% supported high cell viability and was found to be comparable to FBS. This observation was in agreement with a study done on adipose derived stem cells (Goei et al., 2015). A significantly high viability was noticed in HPL treated DPSCs when compared to PRP; however, PRP at 5% concentration showed good percentage of viable cells Day 2 and Day 4. This viability promoting effect of lower concentrations of PRP and viability reducing effect at higher PRP concentrations may be explained by the fact that as cell surface receptors are completely invaded by a specific growth factor, the remaining unbounded growth mediators have no additional impact. On the contrary, in higher concentrations, an inhibitory effect on cell functions may be induced (Castegnaro et al., 2011). To understand the mechanism in detail an exploratory molecular pathway analysis is suggested, to mark which GF in specific triggers this feedback mechanism. These findings were in line with a similar study performed on BMSCs treated with various concentrations of platelet concentrates (Graziani et al., 2006).Viability results of our study were also supported by another study in the literature suggesting higher cell viability effect of 10% and 20% PL on a co culture of endothelial cells, monocytes, fibroblasts and keratinocytes (Fekete et al., 2012).Thus, the optimal concentration of the platelets should be selected according to the target cells as a determinant element. From the finding of this study it can be suggested that effect of HPL and PRP on cell viability was dose dependent and 10%, 15% and 20% HPL and 5% PRP hold the potential of replacing FBS as a media supplement in DPSCs culture.

On comparing the effect of treatment time of HPL and PRP on cell viability of DPSCs, a general decrease in viability of DPSCs was observed from Day 2 to Day 6 that

later exhibited an incremental pattern till Day 10. Initial decrease in viability might be due to the exhaustion of GFs in the media that in turn might have resulted in apoptosis leading to gradual fall in viability. However, cell viability was significantly lower in comparison to the viability observed at 12 and 24 hours..

Subsequent experiments in this study were undertaken to analyse growth factor release and tube formation capacity of DPSCs treated with two (10% and 20%) concentrations of HPL and PRP. Selection of concentrations for subsequent experiments was based on highest concentrations of PRCs exhibiting comparably high viability and reference from a previously performed similar study (Barsotti et al., 2013).

To evaluate the effect of two concentrations (10%and 20%) of HPL and PRP on tube formation capacity of DPSCs, a matrigel based *in vitro* assay, known as tube formation assay was performed. Results of tube formation assay revealed that HPL in 20% concentration showed a noteworthy tube formation capacity compared to all other treatment groups. This could be due to increased release of pro-angiogenic GFs in their active state in 20% HPL group compared to the PRP group. This can also be due to the reason that PRP requires prior activation to initiate the release of GFs that might have resulted in slow release of GFs in contrast to HPL, which is a ready pool of GFs in active state. Hence, the magnitude of GFs action could be insufficient to induce the angiogenic behaviour at lower concentrations. Finding in our study were in line with a similar study performed on endothelial cells cultured with platelet lysate gel (Barsotti et al., 2013). Results of tube formation assay of our study were also similar to another study done that investigated effect of human platelet lysate on wound healing (Fortunato et al., 2016). Hence, confirming 20% to be a potential HPL concentration to stimulate tube formation in DPSCs.

There has been extensive research done on the effect of commercially available individual GFs. However, there is less data accessible on the angiogenic effect of platelet

derivatives as HPL and PRP on DPSCs. Results of protein release in our study demonstrated that, amongst all media groups 20% HPL showed the highest release of all tested pro-angiogenic GFs. FGF-2 is known to have a key role in increasing cell migration and adhesion in ESCs (Barsotti et al., 2013), which are vital for cell growth and tissue remodelling. In our study, it was shown that along 10% and 20% HPL and control (FBS) group demonstrated significantly high release FGF-2. This might be attributed to the reason that commercially available FBS based culture media is selectively enhanced with cell growth inducing factors in high concentrations (Eiselleova et al., 2009; L Liu et al., 2016).

A study has shown that PDGF-BB plays an important role in microvascular formation in endothelial cells (Witzeneder et al., 2013) and promotes angiogenesis in MSCs (Battegay et al., 1994). In this study, it was observed that at higher (20%) HPL concentration PDGF-BB was released in significantly higher concentrations. These findings in our study are in line with another study done on wound healing model (Carmeliet, 2005).This finding also supports the high tube forming stimulatory effect of 20% HPL, since at this concentration HPL stimulates high release of PDGF-BB, that would have driven DPSCs to initiate tube like network formation. These findings in our study are supported by few studies in the literature that found similar results with individual effect of PDGF-BB on embryonic stem cells (Barsotti et al., 2013).

Another potential growth factor investigated in our study was VEGF. It is known to have high pro-angiogenic activity by recruiting ESCs in blood vessels during the process of angiogenesis (Fortunato et al., 2016; S Lange et al., 2009). It has also been reported that VEGF-A promotes stem cells proliferation and migration during tissue remodelling process (Beckermann et al., 2008). Results of our study have shown that VEGF-A release was significantly high in all the HPL and PRP media groups for initial 12 hours that subsequently dropped at 24 hours and beyond. This is suggestive of short

half-life of VEGF-A detected in cell culture media supernatant and cell lysate. These findings were partially in line with another study in the literature that had shown significant VEGF-A release by HPL at higher concentrations (Pons et al., 2008) and platelet rich plasma (Barsotti et al., 2013).

HGF is a pro-angiogenic growth factor (Bertrand‐Duchesne et al., 2010) that is also known to exert anti-inflammatory effect by regulating NFkB pathway (Xin et al., 2001). In this study HGF demonstrated a significantly high release at 24 hours by HPL and PRP groups at all concentrations, suggesting a cumulative effect. This finding in our study is in line with a similar study in literature involving cartilage cells (Bendinelli et al., 2010). However, highest release was demonstrated by 10% and 20% HPL. Our findings are in line with a similar study done with platelet lysate membrane (Anitua et al., 2005).

Lastly, we investigated the effect of MCP-1, which has been reported to stimulate the migration of macrophages (Babo et al., 2014) and stimulate VEGF mediated angiogenesis in MSCs (Niu & Kolattukudy, 2009). In this study it was observed that MCP-1 was secreted in significantly high concentration at 24 hours in both HPL and PRP group. However, highest release was exhibited at HPL 10% group, thus suggesting that cell – cell communication and migration could be achieved at lower 10% HPL, hence supporting 10% HPL to be a potential concentration that promotes cell growth and proliferation. No study in the literature could be found in support of this finding. However, enhanced MCP-1 release by PRP group is supported by a recent study on tendon cells (Hong et al., 2005).

Data in this study brings additional scientific support to probable clinical application of platelet derivatives, signifying the use of HPL and PRP for regenerative application. HPL would be able to substitute growth supplements such as FBS and GFs, with a dose-related effect, with peculiar characteristics at the higher HPL concentrations

evaluated. The use of a pool of healthy donors could minimize individual variability, encouraging application of PRC products. The development of controlled-release systems to protect exhaustion GFs and provide sustained delivery would enhance HPL efficiency for clinical application in healing and regeneration. This speculation needs to be investigated further. Nevertheless, the present study is valuable in determining the merits of using HPL as a culture supplement for DPSCs culture *in-vitro* to provide sufficient pre-differentiated cells, which can then be used for clinical trials. Analysis of the role of specific GFs present actively in PRCs can be a focus of future research. Another potential area for future exploration is to further understand the complex molecular mechanism underlying the action of PRCs in promoting desired differentiation of DPSCs.

5.5 Conclusion

Amongst all the tested concentrations of HPL and PRP, 5% PRP and 10%, 15% and 20% HPL were optimum to the maintain viability of DPSCs comparable to FBS. Hence, it is inferred that HPL and PRP in suggested concentrations can replace FBS as media supplement for DPSCs culture expansion *in-vitro*. 20% HPL is optimum to stimulate pro-angiogenesis in DPSCs. Hence, it is suggested to further elucidate the efficacy of 20% HPL for its pro-angiogenic effect on DPSCs in an animal model.

Further studies have been intended for the potential use of platelet rich concentrates in place of FBS in cell culture experiments requiring DPSCs expansion to the order of millions, especially prepared for clinical trials.

CHAPTER 6: ANGIOGENIC EFFECT OF PLATELET RICH CONCENTRATES ON INFLAMMATION INDUCED DENTAL PULP STEM CELLS (iDPSCs)

6.1 Introduction

Dental pulp is a unique tissue present within the core of the tooth and contains nerves, blood vessels, and connective tissue (Berkovitz et al., 2016). Dentine and enamel provides a strong mechanical support and protection to dental pulp from the microbial rich oral environment. If this rigid shell loses its structural integrity in events of microbiological, mechanical or chemical insult, the pulp becomes vulnerable to oral microenvironment and harsh external conditions leading to adverse biological consequences. This might provide pathways for microorganisms and their toxins to enter the pulp resulting in inflammation. If left untreated, this might progress to chronic inflammation and pulp necrosis (C Yu & Abbott, 2007). Most dental pulp infections are irreversible due to the lack of innate immunity of dental pulp. This is because dental pulp tissue has unique anatomical position and organization in the centre of the tooth enclosed in hard intact tissues called enamel and dentine (Andia & Rubio-Azpeitia, 2014). Dental pulp receives its only major blood supply from a small opening located at the root tip also known as apical foramen. Due to its anatomical location and minimum collateral blood supply, dental pulp has insufficient amount of blood derived tissue repair factors, crucial to suppress the inflammation and accelerate healing (Berkovitz et al., 2016). On the same note, due to minimal collateral blood supply there are insufficient numbers of immune cells that are required to clear necrotic tissues to further combat the infection. That in turn aids in reducing infection induced inflammation hence fostering tissue repair and healing (Huang et al., 2009b).

In conventional endodontic treatment, infected dental pulp is usually removed and replaced with inorganic materials (gutta percha) via root canal therapy. However, loss of tooth

vitality and filling of the root canal with an artificial material cause loss of a significant amount of dentin, hence increases fragility of the tooth rendering the tooth to be brittle and susceptible to postoperative fracture (Yang et al., 2016). Thus, an effective treatment strategy is required to regenerate vital and adequately vascularised tissue in order to treat dental pulp diseases. To achieve a successful and workable pulp regenerative therapy, adequate vascular supply to the tissue is a key requisite.

Dental pulp stem cell (DPSCs), by virtue of their high proliferative capacity and multipotent nature are considered the most suitable choice to initiate de novo pulp regeneration. Some *in vitro* and animal studies in the past have attempted regenerating dental pulp using stem cells from apical papilla, dental pulp and periodontal ligament stem cells (Huang et al., 2009b) in combination with scaffolds made of collagen (Chevallay et al., 2000), chitosan, PGA (Bäumchen et al., 2009),silk protein (Kim et al., 2008), and PLGA (Ohazama et al., 2004). Interestingly, DPSCs are capable of differentiating into endothelial cells and give rise to functional blood vessels (Sakai et al., 2012). Alongi et.al demonstrated that stem cells derived from inflamed dental pulp possess regenerative potential comparable to that of DPSCs derived from normal pulp (Alongi et al., 2010). This indicates that even in inflamed microenvironment DPSCs can initiate tissue healing when exposed to appropriate growth inducing microenvironment. In spite of achieving positive regenerative outcomes, one major challenge of all the attempted strategies was inability to attain functional angiogenesis that involves formation of small blood vessels de novo or from existing blood vessels. Development of improved angiogenic strategies is an important challenge in the field of dental pulp tissue engineering (Flávio Fernando Demarco et al., 2011).

Angiogenesis is a cellular process of forming new blood vessels and this complex molecular mechanism needs intricate interplay of diverse growth factors. These growth factors/morphogens induce cell signalling, influencing critical functions such as cell division, matrix synthesis, proliferation and angiogenesis (Jakse et al., 2003). A few pro-angiogenic

growth factors, i.e. Vascular Endothelial Growth Factor (VEGF), Fibroblastic Growth Factor (FGF), Platelet Derived Growth Factor (PDGF) and Hepatocyte Growth Factor (HGF) have been demonstrated to play a pivotal role in retinal angiogenesis (Praidou et al., 2010). It has been shown that VEGF and FGF are capable of enhancing neovascularization of inflamed human dental pulps (Mullane et al., 2008).

Majority of studies in the literature aiming to achieve angiogenesis using stem cells have employed single growth factor at a time that might limit the other aspects of angiogenesis process. The commercially available growth factors were mainly xenogeneic product and non-cost effective therapeutics to scale up for clinical uses. To address these challenges, there is a need for the development of strategies to harness autologous source of growth factors that is easily attainable on a cost effective mode. This in turn will provide a safer therapeutic regime without any xenogeneic or prion contamination.

Autologous bio products are one such alternative that can address the above issues. Past few studies, utilizing collagen and hydroxyapatite scaffolds could achieve positive results. However, yet again tissue revascularization was one such challenge they faced in their outcomes. Recently, platelet derived products have gained attention in the field of regenerative medicine and are currently paving their way in surgical and wound healing strategies. Autologous blood-derived products, such as platelet rich concentrates (PRCs), are key sources of growth factors involved in regeneration and tissue repair. PRCs contain concentrated cocktail of bioactive molecules that play essential role in fundamental processes, including angiogenesis, cell migration, metabolism and inflammation in pathological conditions (Andia & Maffulli, 2013). It can easily be prepared and form a three-dimensional fibrin matrix that acts as a scaffold (Anitua et al., 2006).

PRCs are well known to release myriad of growth factors. Currently, there are three major fractions of PRCs that have gained attention for their use in clinics and laboratories. Two of the fractions of PRC namely platelet rich plasma (PRP) and platelet rich fibrin (PRF)

are currently extensively used in clinics. However, efficacy of each fraction of PRC depends largely on the donor and preparation method. PRP requires prior activation to induce the release of growth factors. On the other hand, other two fractions of PRC, namely Platelet rich fibrin (PRF) and Human Platelet Lysate (HPL) are ready to use activated pool of growth factors and do not require prior activation before use. Hence, use of the latter therapeutics reduces the overall processing steps and time so to achieve minimal manipulation prior to their usage

It has been evident that PRCs have successfully been used in past for post-surgical tissue healing. PRCs are an efficient source of pool of growth factors. Their role in reducing inflammation, tissue-healing angiogenesis promoting effect has not been explored extensively. It has been reported in the past that a fraction of PRCs, known as platelet rich plasma (PRP) could successfully block the action of NFkB (Nuclear factor kappa-light-chain-enhancer of activated B cells) complex that is primarily responsible for inflammatory process. However, other fractions of PRCs are yet to be explored about their inflammation suppressing activity. To initiate tissue healing and regenerate a functional tissue complex like dental pulp, the construction of the biomimetic microenvironment is vital, where therapeutic agents can demonstrate inflammation suppressing and repair enhancing process *in vitro* and *in vivo*. Cells respond differently to physicochemical and mechanical properties of the microenvironment. The interactions between cells and the extra cellular matrix control differentiation, migration, and proliferation, as well as tissue remodelling (Kaushik et al., 2016). PRCs were shown to have antimicrobial properties, although the exact mechanism of action of interaction with microbial pathogens is not clearly known (Del Fabbro et al., 2011). Hence, it could be a useful natural substance for controlling infection and inflammation along with inducing tissue healing and regeneration, yielding a successful regenerative strategy.

Since last two decades, application of autologous platelet rich plasma (PRP) has been reported to be used as a therapeutic agent in many fields including; orthopaedics, sports

medicine, dentistry, neurosurgery, ophthalmology, urology, wound healing, cosmetic, cardiothoracic, and maxillofacial surgery (Heyeraas & Kvinnsland, 1991). Studies suggest that PRC treatment can modulate many of the cellular and physiological processes including inflammation, post-operative blood loss, infection, narcotic requirements, osteogenesis, wound, and soft tissue healing (Sampson et al., 2008).

In addition to direct effects on differentiation, angiogenesis and proliferation, PRP also has effect on wound healing via the chemotactic recruitment of cells and local control of the inflammatory environment. PRP have been reported to chemo tactically attract human MSCs (Teixeira et al., 2012). They have also showed promising chemotactic effect on circulation-derived cells to trigger the initial stages of tendon repair in a rat tendon injury model (Kajikawa et al., 2008). PRP has been shown to attract peripheral blood monocytes in a dose-dependent manner, directly altering pro-inflammatory cytokine release profile of monocytes (El-Sharkawy et al., 2007). Many studies in the past have reported on the capability PRP to mitigate inflammation. In an osteoarthritic study, it was shown that anti-inflammatory effect of PRP is mainly due to presence of growth factors HGF and PDGF-BB, that play an important role in modulating NFkB, a key cascade involved in inflammatory pathway (Van Buul et al., 2011).

In the past studies, researchers have investigated the effect of PRCs in different cell populations involved in wound healing. However, inflammation reducing and angiogenesis promoting potential of HPL and PRP on DPSCs have not been explored on a comparative mode. To gain insight into this field, our study aimed to compare the various concentrations of HPL and PRP for their proliferative and angiogenic potential on an inflamed dental pulp culture model.

6.2 Materials and Methods

Ethics approval for the sample collection and processing was obtained from Medical Ethics Committee, Faculty of Dentistry, University of Malaya {DFDP 1304/002 (P)}.

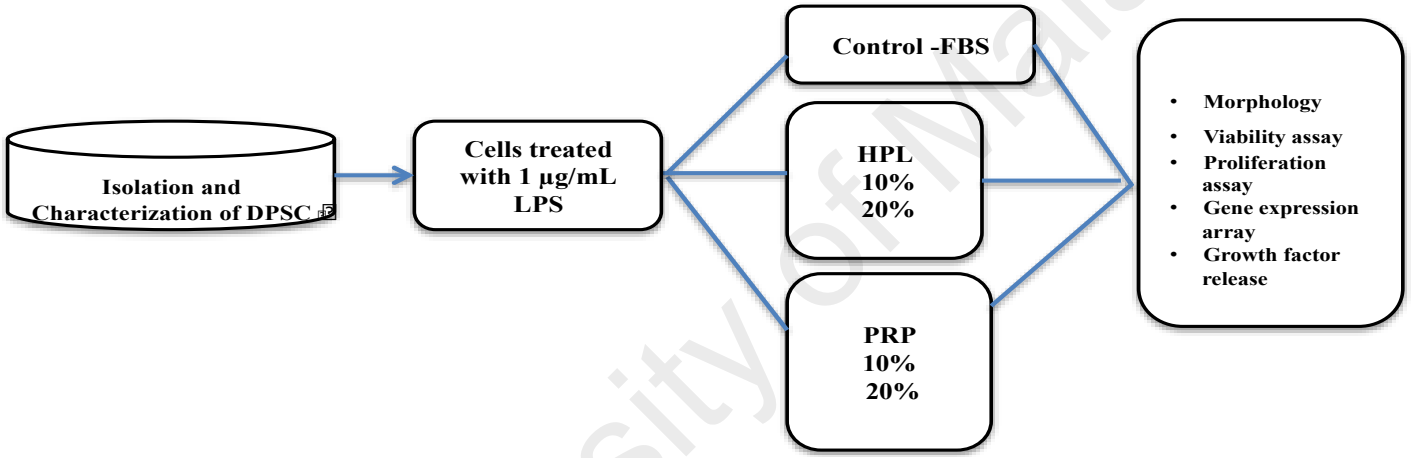

Figure 6.1: Flow of experiment. The study was commenced with isolation of DPSCs from dental pulp of healthy donors cultured up to sub-culture 3 and further treating them with 1 µg/mL of LPS from *E. coli* for 24 hours. The treated cells were then exposed to a series of concentrations of either HPL or PRP and were assessed for viability, proliferation, gene expression, and growth factors release. The experiments were compared with cells treated with FBS as control.

6.2.1 Treatment of DPSCs with LPS

DPSCs from adult dental pulp from five healthy donors (n=5) were isolated and expanded following the protocol as described in Chapter 3 Section 3.2.2 and 3.2.3. LPS media used in following experiments was prepared with 10mL of DMEM-KO and 10 µl of LPS from previously prepared LPS stock solution to achieve 1µg/mL LPS concentration (Chapter 4, Section 4.2.4.1). DPSCs yielded at passage three were seeded into 6-well plates at a seeding density of 2× 105 cell per well and incubated with complete culture media in a humidified incubator at 37°C and 5% CO2 for 24h. After 24 hours of incubation culture media from all the wells was discarded and DPSCs were washed twice with DPBS (Gibco) to remove any residual media. DPSCs in all wells were then incubated with 2mL of 1ug/mL of LPS media at 37°C and 5% CO2 in a humidified incubator for 24h.

6.2.2 Treatment of iDPSCs with HPL and PRP

10% and 20% of HPL and PRP supplemented culture medias used in the following experiments were prepared following the protocol as described Chapter 5, Section 5.2.3. Inflammation induced DPSCs (iDPSCs) from five donors (n=5) in Section 6.2.1 were washed twice with DPBS (Gibco, USA) to remove any residual culture media. Washed iDPSCs were then incubated with 10% and 20% of HPL and PRP supplemented media in a humidified incubator at 37°C and 5% CO2 for 24h. iDPSCs incubated with complete media was considered control. All culture media groups and control were tested in triplicates. Any morphological changes in iDPSCs treated with various concentrations of HPL and PRP were observed and recorded under an inverted microscope (Leica Microsystems, Germany) at 24h post-incubation.

6.2.3 Effect of various concentrations of HPL and PRP on viability of iDPSCs

DPSCs (n=5) at passage three were seeded into 96-well plates in triplicates at a density of 5 × 10^3 cell per well in 200 µl of complete culture media. After 24 hours of incubation at 37°C and 5% CO2 in a humidified incubator, complete culture media was discarded and DPSCs were washed thoroughly with DPBS (Gibco, USA) to remove any traces of culture media. DPSCs were then incubated with 200 µl of 1ug/mL of LPS media at 37°C and 5% CO2 in a humidified incubator for 24h. After 24h iDPSCs were then incubated with 200 µl of 10% and 20% of HPL and PRP culture media for 2,4,6,8 and 10 days. After each time point 100µl of DMEM-KO along with 10% Alamar Blue reagent (Thermo fisher scientific, USA) v/v was added to each well and plates were further incubated for 3 hours at 37°C and 5% CO2 in a humidified incubator. Absorbance was measured at 450nm with reference wavelength set at 590nm by Tecan (Infinite 200 PRO, Life sciences, Switzerland) microplate reader. Viability of iDPSCs was determined using Alamar Blue assay (Thermo fisher scientific, USA). Cell viability was calculated as percentage from the reduction in the absorbance recorded. Absorbance recorded for iDPSCs cultured in complete culture media was considered as a control and reference (normalized to 100%).

6.2.4 Effect of various concentrations of HPL and PRP on pro-angiogenic marker expression in iDPSCs

To investigate the expression of pro-angiogenic markers qPCR array was performed on the iDPSCs treated with 10% and 20% of HPL and PRP respectively for 24h. iDPSCs treated with FBS was considered as control. Total RNA was extracted from iDPSCs using the RNeasy plus Mini kit (Qiagen, Venlo, The Netherlands). Briefly, 1µg of RNA was reverse-transcribed into cDNA using All-in-One First Strand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA) and All-in-One qPCR Mix Kit (GeneCopoeia, Rockville, MD, USA) was added prior to real-time PCR using

ExProfile™ Human Angiogenesis Related Gene qPCR Array (GeneCopoeia, Rockville, MD, USA). The quality (A260/A280, A260/230) and concentration of the gDNA (ng/µl) was determined using the spectrophotometer ND-2000 (NanoDrop Technologies, Wilmington, DE, USA). The integrity of RNA was assessed using the Agilent Bioanalyzer-2100 (Agilent, Palo Alto, CA, USA) and only samples with RNA integrity number (RIN) more than 6 were used for cDNA synthesis and subsequently used for qRT-PCR analysis. The expression of the housekeeping gene GAPDH was used as reference for measurements. The ΔCt calculation method was used to evaluate the relative quantity of gene expression. Experiment was carried out on triplicate samples from three donors. The selection of pro-angiogenic markers tested in the study was done based on their participation in regulating the pro-angiogenesis and cell adhesion as reported in previous studies. Selection of cell surface markers used in this study to was done on the role in inducing angiogenesis during tissue healing as reported by previous studies (Table 6.1).

Table 6.1: Angiogenic surface markers with their functions

Surface marker	**Function**
ANGPT 1 (Angiopoetin-1)	Key role in vascular remodelling and angiogenesis (Hammond et al., 2009).
EREG (Epiregulin)	Encodes secreted epidermal growth factor receptor and involved in inflammation, wound healing, and cell proliferation (Witzenbichler et al., 1998)
FGF-2 (Fibroblast growth factor-2)	Potent angiogenesis inducers *in vivo* and *in vitro* by endothelial cell proliferation (Gao et al., 2015).
FIGF (C-Fos Induced Growth Factor) Vascular Endothelial Growth Factor D)	Key role in angiogenesis, lymph angiogenesis, and endothelial cell growth by activating VEGFR-2 and VEGFR-3 (Seghezzi et al., 1998)
VEGF-A (Vascular Endothelial Growth Factor –A)	Stimulates endothelial cells for invasion and migration (Orlandini et al., 1996).

PDGF-A (Platelet Derived Growth Factor Subunit A)	Regulates cell growth and division. Plays a significant role in stimulating growth of blood vessels from already existing blood vessel tissue (Sainson et al.).
TGFB1(Transforming growth factor-beta 1)	Prominent role in modulating angiogenesis (Sato et al., 1993).
JAG1(Jagged1)	Involved in an important pathway that initiates cell-cell signalling through notch receptor (Ferrari et al., 2009).
NRP2 (Neuropilin)	Potent mediators of neuronal and angiogenic guidance (Dufraine et al., 2008).
PLXDC1 (Plexin Domain Containing 1)	Overexpressed in human blood vessels (Geretti & Klagsbrun, 2007)
STAB (Stabilin-1)	Vital role in angiogenesis, lymphocyte homing and cell adhesion (Limb et al., 1996)
IL-8 (Interlukin-8)	Initiates vascularization of inflamed tissue during healing process.
CCL11(Eotaxin)	Induces chemotaxis of human microvascular endothelial cells (Adachi & Tsujimoto, 2002).
CCL2(C-CMotif Chemokine Ligand 2)	Induces angiogenesis brain endothelial cells (Salcedo et al., 2001)
CDH5 (Cadherin 5)	Key role in blood vessel morphogenesis (Stamatovic et al., 2006).
ITGB3 (Integrin Beta 3)	Regulates angiogenesis through Foxc2 transcription factor (Sauteur et al., 2014)
IFNA1 (Interferon alpha)1	Inhibits angiogenesis during blood vessel remodelling (Hayashi et al., 2008).
IL-Iβ (Interleukin 1 beta)	Mediates both acute and chronic inflammation and stimulates cell proliferation and tissue angiogenesis (Jablonska et al., 2010)
IL-6 (Interleukin-6)	crucial role in the biological mechanism of inflammatory-related diseases (Apte & Voronov, 2008).

TNF (Tumour Necrosis Factor)	Inhibits proliferation of endothelial cells and regulates the blood vessel growth (Fan et al., 2008).
CXCL1(Chemokine (C-X-C motif) ligand 1)	Induces microvascular endothelial cell migration and tube formation *in vitro* (Fajardo et al., 1992).
CXCL3 (Chemokine (C-X-C motif) ligand 3)	Recruitment and homing of specific hematopoietic cellular subsets during inflammatory conditions (Wang et al., 2006).
CXCL5 (Chemokine (C-X-C motif) ligand 5)	Modulates angiogenesis during intestinal inflammation (Billottet et al., 2013).

6.2.5 Effect of various concentrations of HPL and PRP on release of pro-angiogenic growth factors

Release of pro-angiogenic GFs, fibroblast growth factor (FGF), vascular endothelial growth factor-A (VEGF-A), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF-BB), and monocyte chemotactic protein-1 (MCP-1) using multiplex immunoassay (Procarta, Multiplex immunoassay, Affymetrix, ebioscience, USA). GFs used in the study were selected based on their involvement in cell proliferation, migration and angiogenic effect as reported by previous studies (Table 5.2). Experiment was conducted using commercially available multiplex immunoassays using reagents and detection antibodies from Affymetrix, ebioscience, USA.

6.2.5.1 Sample preparation for multiplex immunoassay

DPSCs at passage 3 (n=5) were seeded in T75 cell culture flasks at the seeding density of 2.1 x 106 and incubated with 10 mL complete culture media. After reaching 70% confluence, culture media from all the flasks was discarded and DPSCs were washed twice with DPBS (Gibco, USA) to remove any residual culture media. Washed DPSCs were then incubated with 10mL of 1ug/mL of LPS media at 37°C and 5% CO2 in a

humidified incubator for 24h. After 24h LPS media from all T75 flasks was aspirated and 2mL of aspirated media from one flask was stored at -80°C for multiplex immunoassay. iDPSCs were then washed twice with DPBS (Gibco, USA) to remove any traces of residual LPS media and incubated with 10mL of 10% and 20% of HPL and PRP culture media for 12h, 24h and 48h respectively in three replicates. iDPSCs incubated with complete media was considered as control.

For multiplex immunoassay, aspirated cell culture media supernatant and cell lysate from all the culture flasks were used. HPL and PRP supplemented media supernatant was aspirated from all T75 culture flasks and stored at -80ºC for multiplex immunoassay. iDPSCs lysate was prepared by scrapping the adherent cells from the flask bottom using a plastic cell scrapper. Cells were then transferred to 15mL centrifugation tube and centrifuged at 1500 rpm at 4ºC for 5 minutes. After discarding the supernatant, cells were re-suspended into 5mL of ice cold PBS and centrifuged again at 1500 rpm at 4ºC for 5 minutes. Supernatant was then removed without disturbing the cells and cell lysis buffer was added (Affymetrix, ebioscience, USA). Cell suspension is then pipetted up and down 8-10 times and incubated on ice for 5 minutes. Cell suspension was then transferred to 1.5mL micro centrifugation tube and centrifuged at 20000 rpm at bench top centrifuge for 10 min at four-degree C. Cell lysate obtained after centrifugation was used to run the ELISA assay. 650µl of each sample was used to carry out the assay that was performed using a standard antigen cocktail for standard curve preparation. GFs released in the supernatant and lysate were detected using detection antibodies (Affymetrix, ebioscience, USA). Quantification of GFs was done using Tecan microplate reader (Infinite 200 PRO, Life sciences, Switzerland) at absorbance 450nm, compared against the control. GFs were then detected in cell supernatants and cell lysates in triplicates of three donors.

6.2.6 Statistical Analysis

All experiments were conducted on samples from three separate donors. Data was analysed using SPSS 22.0 software. Results were expressed as mean and standard deviation of three replicates wells for all samples and controls. Statistical analysis was done using independent student's t-test. Values with $p<0.05$ were considered significant.

6.3 Results

6.3.1 Effect of HPL and PRP treatment on morphology of iDPSCs

Morphology of iDPSCs was observed to be elongated spindle shaped, resembling typical MSC morphology, that remained largely unchanged after treatment with all concentrations of HPL and PRP and no apparent morphological signs of increased apoptosis could be detected at 24 hours on iDPSCs using light microscopy.

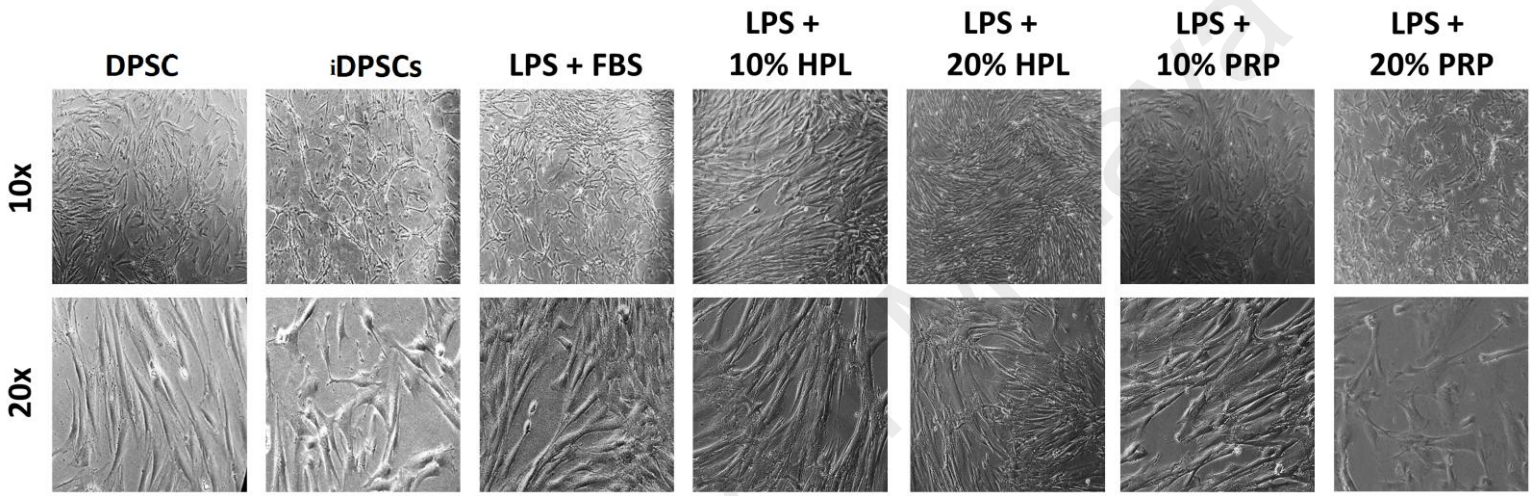

Figure 6.2: Morphology of iDPSCs upon exposure to various concentrations of HPL and PRP at 48h Scale bar = 100µm. Upon exposure of iDPSCs to various HPL and PRP concentrations (10% and 20%) at 48h showed that iDPSCs morphology remained elongated and spindle shaped at all concentration and time points.

6.3.2 Viability of iDPSCs after treatment with various concentrations of HPL and PRP

Experiment for cell viability was carried for 96h and observations were recorded at 24h, 48h and 96h (Figure 6.3). It was observed that no significant difference in viability was observed at 24h for all tested concentrations of HPL and PRP. At 48h only 20% HPL showed significantly high viability when compared to other media groups. At 96h a remarkable ($p<0.05$) increase in viability was evident in all media groups. However, 20% HPL demonstrated significantly ($p<0.05$) high viability compared to all other media groups. No significant difference in viability was observed at 48h and 96h for 20% HPL group. No significant change in viability was observed in iDPSCs treated with complete media (FBS) at all time points.

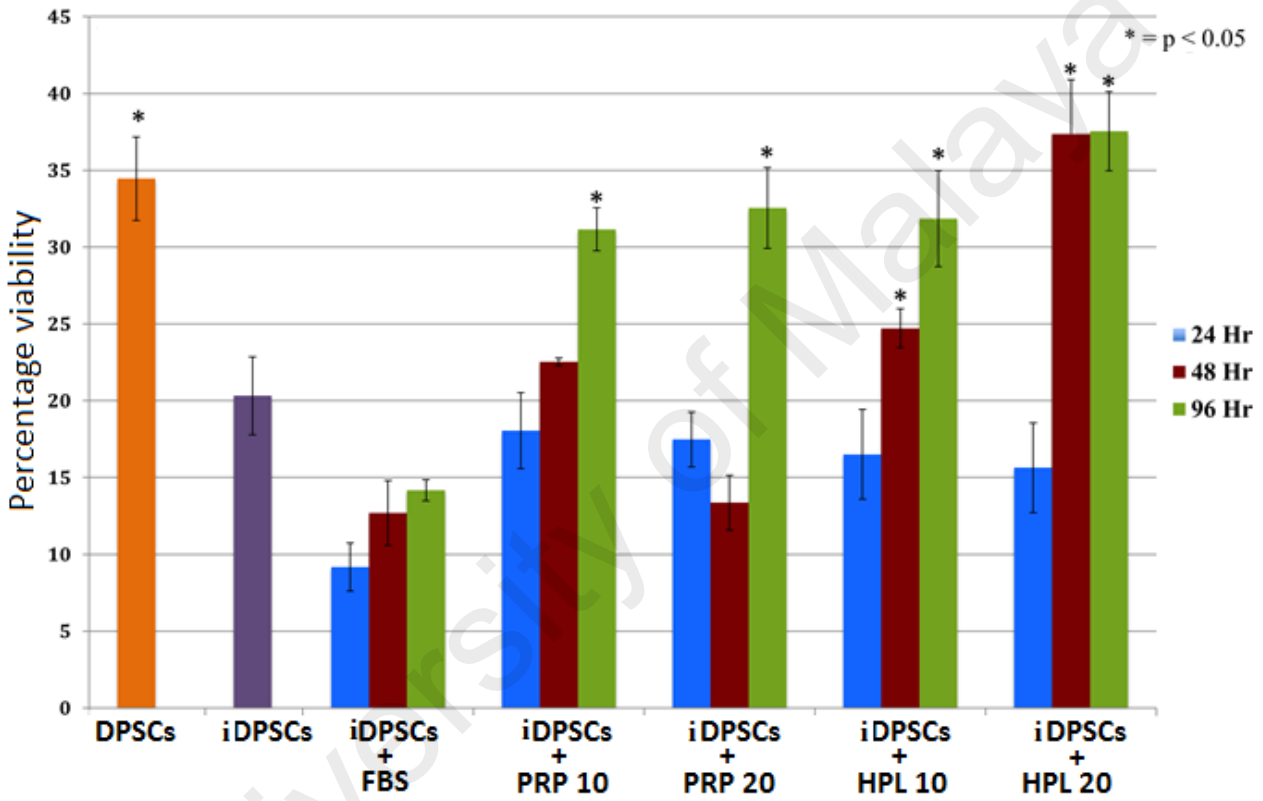

Figure 6.3: Percentage cell viability of iDPSCs upon exposure to various concentrations of HPL and PRP at five different time points. Exposure of iDPSCs to various concentrations of HPL and PRP (10% and 20%) at 24h, 48h and 96h showed significantly ($p<0.05$) high viability at Day 2 and Day 4 for 10%, 15% and 20% HPL media groups.

6.3.3 Pro-angiogenic marker expression after treatment with various concentrations of HPL and PRP

To investigate the potential of HPL and PRP in inducing angiogenesis process in iDPSCs model, a panel of genes were used to assess the expression of angiogenic, adhesion molecules and cytokine markers using qPCR profiler assay platform. It was noted that in the presence of 20% of HPL, the gene expression was augmented at least by three-fold in 10 out of 12 angiogenic markers such as ANGPT1, EREG, FGF2, FIGF, VEGF-A, and IGF-1 (Fig. 6.4, $p<0.05$). Though treatment with 20% of PRP showed increment of the aforementioned genes, their expression was significantly lower as compared to those treated with 20% of HPL. Interestingly, the expression of PDGF-A, TGFβ2, and STAB1 showed unique profile in which their expression upon exposure with 20% PRP was distinctly lower as compared to those exposed with FBS. The expression profile of genes related to adhesion molecules showed mixed pattern in which four out of seven genes namely NRP2, CCL2, CDH5, and ITGB3 presented significant augmentation (at least two-fold; Fig. 6.5 $p<0.05$) with the treatment of 20% HPL. Whereas, two genes (BAI1 and IL8) showed similar expression pattern, while the remaining gene, CCL11 showed significant augmentation in cells treated with 20% PRP (Fig. 6.6; $p<0.05$). Similarly, the expression profile of genes related to cytokines also presented mixed patterns.

In general, expression of the selected genes showed significant increase in the treatment group of either HPL or PRP except in the expression of Interferon A (IFNA1). To be precise, seven out of nine cytokine molecules that play a key role cell to cell signalling and migration namely IFNB1, IL1B, IL-6, TNF, CXCL10, CXCL3, and CXCL5 showed significant elevation by at least two-fold as compared to FBS treated cells ($p<0.05$). Expression of CXCL1 on the other hand was shown to have distinctly

increased in cells treated with 20% PRP. Expression of IFNA1 revealed remarkably lower expression pattern as compared to cells treated with FBS. This might be due to the reason that expression of IFNA1 required a different growth factors that were either not present in the treatment media, and if present might be in in insufficient amount, indicating some degree of inhibitory mechanism. This part has not been investigated in this study.

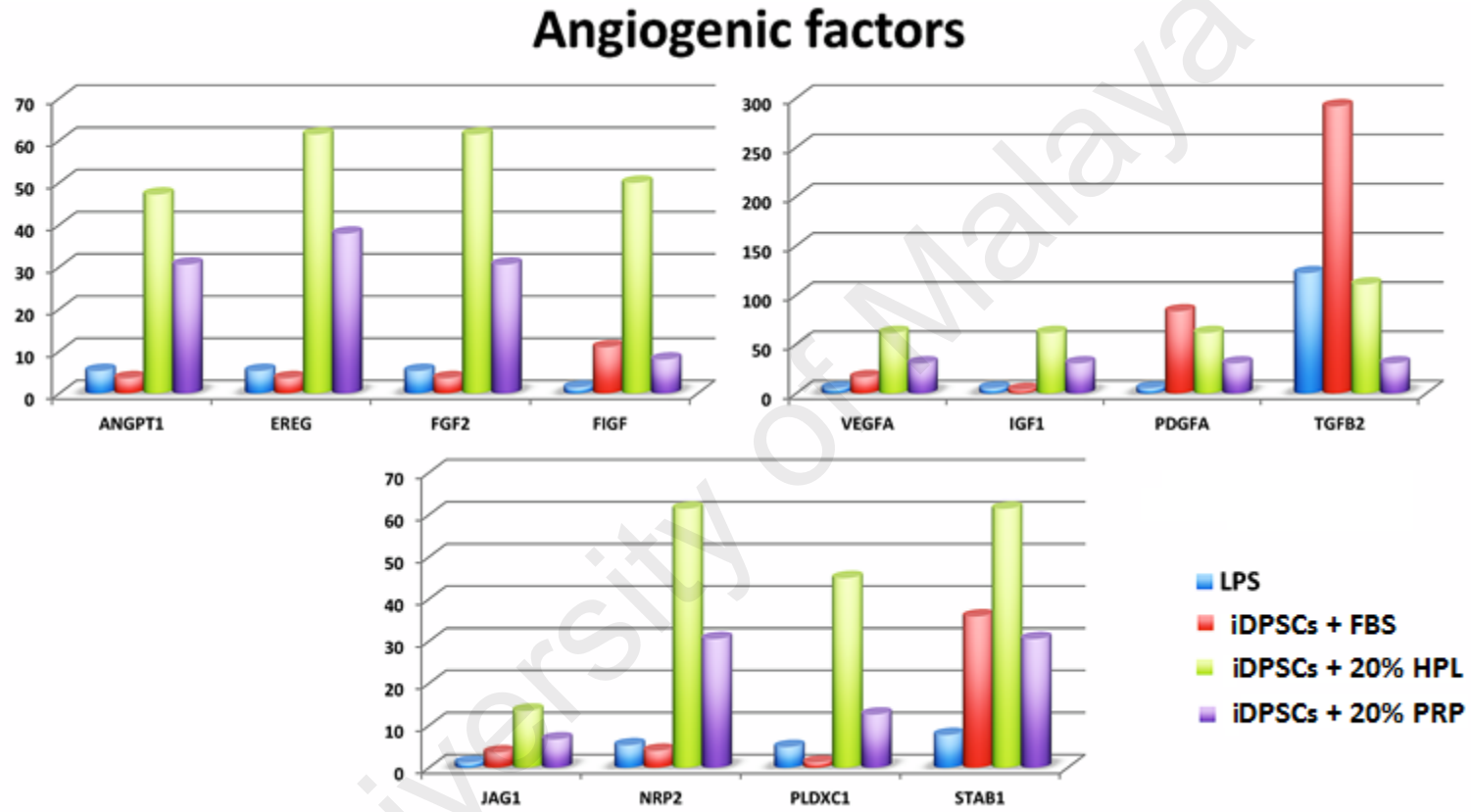

Figure 6.4 : Relative fold change in expression of angiogenic markers in iDPSCs upon exposure to (20%) HPL and PRP 24h. Exposure of iDPSCs to (20%) HPL and PRP 24h showed significantly high fold change in gene expression in iDPSCs treated with 20% HPL media group compared to other media groups.

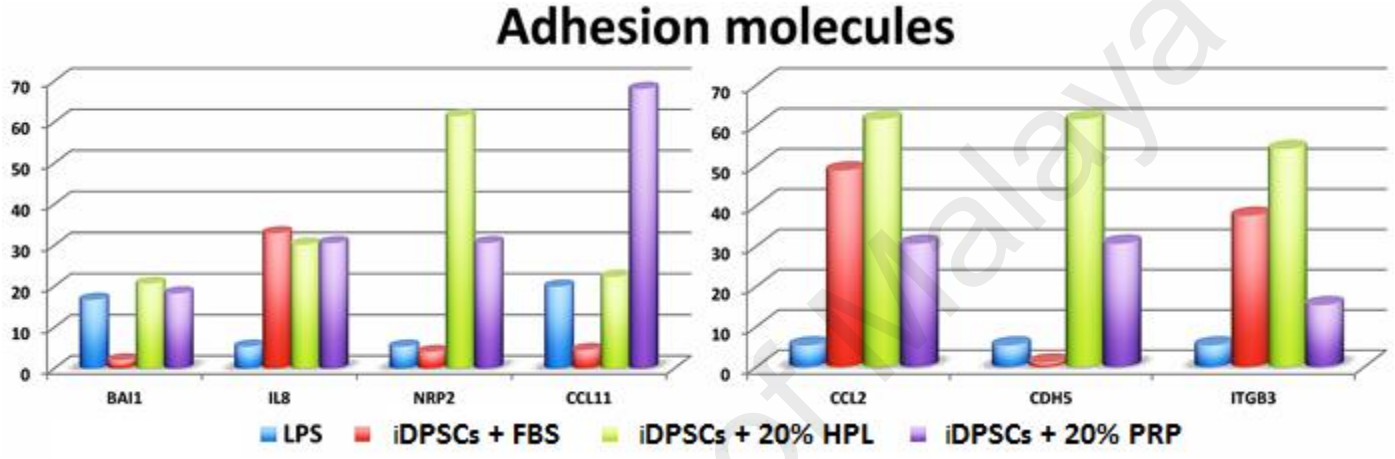

Figure 6.5: Relative fold change in expression of cell adhesion markers in iDPSCs upon exposure to (20%) HPL and PRP 24h. Exposure of iDPSCs to (20%) HPL and PRP 24h showed significantly high fold change in gene expression in iDPSCs treated with 20% HPL media group compared to other media groups.

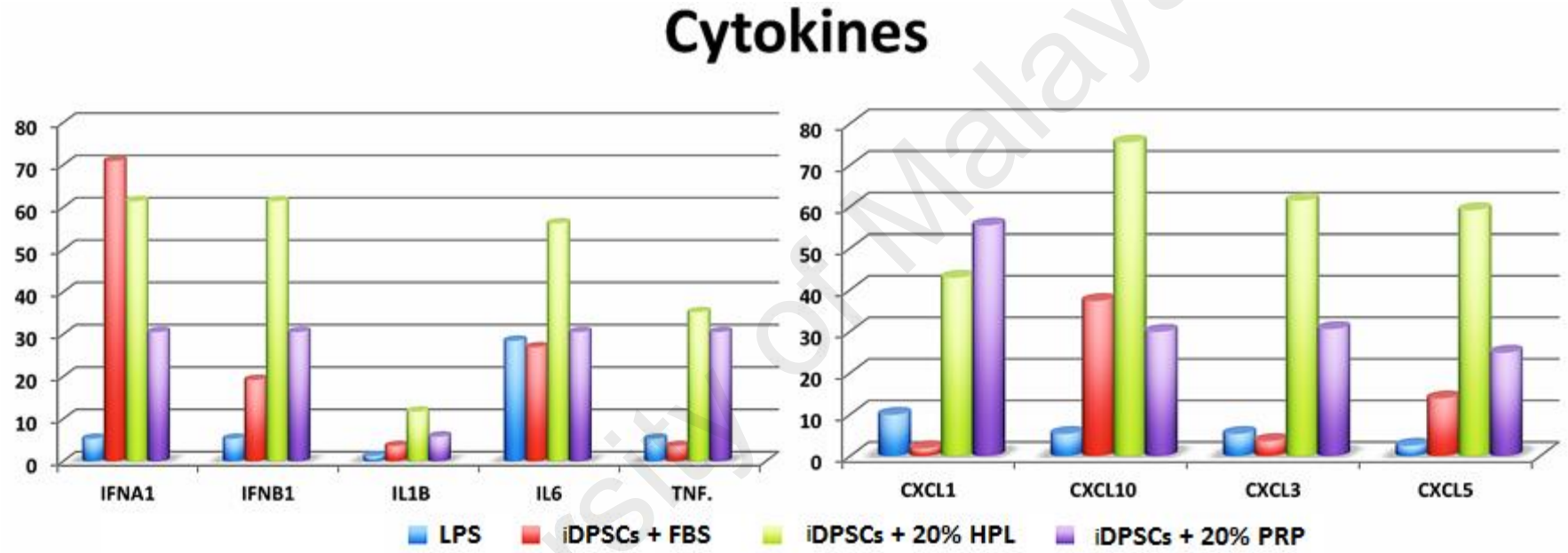

Figure 6.6: Relative fold change in expression of cell adhesion markers in iDPSCs upon exposure to (20%) HPL and PRP 24h. Exposure of iDPSCs to (20%) HPL and PRP 24h showed significantly high fold change in gene expression in iDPSCs treated with 20% HPL media group compared to other media groups.

6.3.4 Growth factors release after treatment with various concentrations of HPL and PRP

Additionally, the release levels of angiogenic and healing promoting growth factors such as FGF-2, MCP-1, PDGF-BB, HGF and VEGF-A were assessed using ELISA based procarta multiplex immunoassay. It was noted that the presence of HPL augmented the release of these proteins as compared to PRP group at all-time points. Another noteworthy finding was the release of these factors were shown to decrease over time regardless of the concentration the cells were exposed, possibly indicating reduced actively available amount of growth factors in supernatant or lysate as time increased.

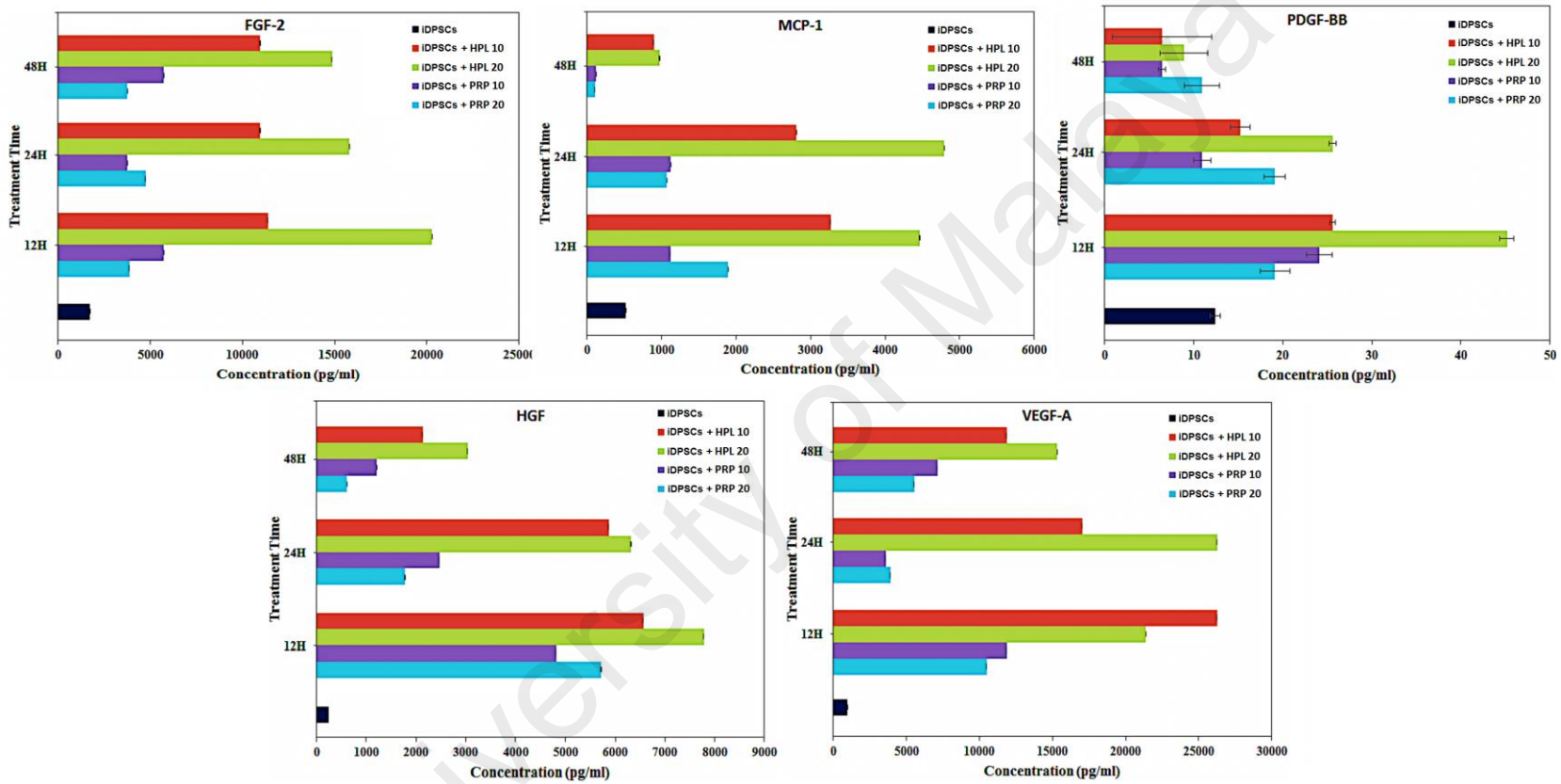

Figure 6.7: **Concentration of growth factors released in iDPSCs upon exposure to various concentrations of HPL and PRP**. Exposure of iDPSCs to (20%) HPL and PRP at 24h showed significantly ($p<0.05$) high release of growth factors in iDPSCs treated with 20%HPL media group compared to other media groups.

The FGF-2 expression was the highest (at least by 75-fold as compared to iDPSCs) with the presence of 20% of HPL at 24th hour, which then displayed decrement pattern over time. Similar expression pattern was also observed with HGF and PDGF-BB whereby their expression was the highest at 24th hour before declining over time. Although, 10% HPL had demonstrated significantly high release of all the growth factors at all times, however the release was significantly less than 20% HPL. Though the expression pattern in HPL was clear and predictable, it was rather irregular in those exposed with PRP. For instance, the expression of FGF2 at 10% PRP treatment revealed decrement from 6200 pg/mL at 12 hours to 4000 pg/mL at 24th hour followed by increment to 5800 pg/mL at 48th hour. Similarly, expression of FGF2 at 24th hour under exposure of 20% PRP was increased from 3500 pg/mL at 12 hours to 5200 pg/mL, before declining back to 3200 pg/mL at 48th hour. The same pattern was also observed in the expression of VEGF-A. It was observed that VEGF-A expression at 24th hour on exposure with 10% PRP displayed decrement from 12500 pg/mL to 3500 pg/mL which then rose to 6500 pg/mL Under the exposure of 20% PRP, expression of VEGF-A at 24th hour was decreased from 11000 pg/mL to 4000 pg/mL before rising back to 5100 pg/mL. As a whole, the protein expression revealed the efficacy of HPL as compared to PRP at all-time points, suggesting the usage of HPL for future works.

6.4 Discussion

Tissue healing and regeneration process involves a series of complex biological events, manifested through the complex cellular processes including cell growth, proliferation and paracrine actions. Growth factors play a vital role as controller of basic cell functions by their paracrine action. They initiate cell-to-cell communication in which cell produces a signal to trigger nearby cells leading to alteration or differentiation of

those cells. Majority of angiogenic growth factors regulate cellular processes through paracrine actions.

Many studies have highlighted role of PRCs in growth factor release and its significance in regulating inflammation and chemotaxis related biological events (Rowland et al., 2014). PRCs had been considered for the management of inflammation and pain in conditions like osteoarthritis, sports injuries and procedures like sinus lift surgery (El-Sharkawy et al., 2007; Y Zhu et al., 2013). In this study, effect of two types of PRCs namely HPL and PRP was investigated for their pro-angiogenic effect on iDPSCs. The assessment of the morphological changes of iDPSCs treated with HPL and PRP groups did not show any significant morphological changes when compared to control (FBS) group. Findings of the present study were in line with a similar study performed on bone marrow and adipose tissue derived stem cells (C-y Li et al., 2015). Results of cell viability in this study showed that amongst all tested media groups, 20% HPL significantly supported the viability of iDPSCs. This indicates that 20% HPL is the optimum concentration to maintain the cell viability of DPSCs exposed to inflamed microenvironment. The findings in our study were in line with similar studies done on adipose derived stem cells (ADSCs) (D Forte et al., 2015)and BMMSCs (Crespo-Diaz et al., 2011).

To determine the pro-angiogenic effects of HPL and PRP on iDPSCs we investigated the expression of pro-angiogenic and cell adhesion marker expression using qPCR array. The rationale behind the selection of specific markers was based on their angiogenesis promoting role, as reported by studies mentioned in Table 6.1. Cell adhesion markers like NRP2, CCL11, CCL2 and CD5 directly affect cell adhesion and migration during angiogenesis process. NRP2 is a potent mediator of angiogenesis (Geretti & Klagsbrun, 2007). CCL2 and CCL11 reported to have a chemotactic effect on human micro vascular endothelial cells to induce angiogenesis during tissue healing

(Stamatovic et al., 2006). CDH5 a potent marker of angiogenesis has shown to have a key role in vascular remodeling (Sauteur et al., 2014). Another set of markers investigated in this study was pro-angiogenic surface markers namely ANGPT1, EREG, FGF2, FIGF, VEGF-A, and IGF-1. In the literature, it is reported that ANGPT family has key roles in vascular development and angiogenesis in endothelial cells (Witzenbichler et al., 1998). EREG has been reported to have a vital role in inflammation, wound healing, and cell proliferation in DPSCs (Gao et al., 2015). FGF2 and VEGF-A are potent pro-angiogenic markers that have established role in regulating angiogenesis during healing process involving endothelial cells (Seghezzi et al., 1998).

In our study it was observed that 10% and 20% HPL demonstrated high expression of pro-angiogenic surface markers. However, 20% HPL had shown a significantly higher expression compared to all other media groups. Findings in our study were in agreement with the literature on endothelial cells and human umbilical vein endothelial cells (Fortunato et al., 2016; Oliveira et al., 2015). This proposition was also supported by a study, where it was reported that CCL5, a mediator of inflammatory regulation process, helps to modify the inflammatory microenvironment towards repair and healing by slowing down the inflammatory process progression (El-Sharkawy et al., 2007).

To further elucidate our findings, release of angiogenic GFs were investigated at protein level. In this study we investigated five key angiogenesis promoting GFs namely, FGF-2, MCP-1, PDGF-BB, HGF and VEGF-A. In this study, it was observed that release of PDGF-BB was remarkably increased in iDPSCs treated with 20% HPL, suggesting the onset of tissue remodeling by stimulating extracellular matrix formation, in line with a similar study done on effect of PDGF-BB on MSCs (Middleton et al., 2012). In order to yield a successful healing and regenerative repair of an inflamed site, the healing agent or media intended to be applied at inflamed site should be able to attain both anti-inflammatory and the angiogenic activities. Studies in the past have reflected upon the

anti-inflammatory action of HGF by blocking the NFkB cascade that is mainly responsible for inflammation progression (Galliera et al., 2011). In the present study, 20% HPL demonstrated significantly high release of HGF compared to all other tested media groups. The findings of our study were in line with the results obtained by study performed in wound healing model treated with human platelet lysate in two different concentrations. It was demonstrated that HPL at concentration of 20% induced NFkB pathway and showed the highest release of trophic growth factors in the population of monocyte and fibroblasts (Barsotti et al., 2013). In another similar study done on inflamed articular cartilage it was observed that release of HGF in chondrocytes, directly impairs the NFkB activity, which is a critical regulator of the inflammatory process (Bendinelli et al., 2010). Another study by El. Sharkawy et al. reported that PDGF-BB significantly stimulated adherence of periodontal ligament cells to root surfaces and decreased lipopolysaccharide inhibition of gingival fibroblast proliferation (El-Sharkawy et al., 2007).

In this study it was shown that 10% and 20% HPL and 5% PRP significantly increased cell viability. However, 20% PRP did not show a higher viability compared to other concentrations of HPL and PRP tested in the study. This might be due to the reason that, PRP requires biological activation to initiate efficient release of growth factors from the platelets. There was an initial release of growth factors due to the degranulation of platelets. That might have occurred due to the interaction with culture media components and repeated physical manipulation during the experiment process. Hence, in case of PRP the pro-angiogenic growth factors might not be in active state in media, compared to HPL, where growth factors are readily present in active state in media. However, we speculate it could not reach its full potential in terms of releasing active growth factors.

The non-significant effect of 10% HPL could be explained by a lower amount of pro-angiogenic growth factors. In order to induce angiogenesis, high concentrations of

angiogenesis promoting growth factors are required, that might not have reached with 10% HPL media group.

Expression of variety of pro-angiogenic and cell adhesion markers along with differential release of trophic factors directly correlates with the ability of HPL to stimulate healing and angiogenesis in iDPSCs. Hence, 20% HPL could be considered suitable in promoting angiogenesis process. As HPL efficacy is dependent on its availability to the inflamed or injured tissue, a direct delivery at the effected site through controlled-release systems would enhance HPL biological effects, protecting growth factors from enzymatic degradation and providing sustained delivery without loss of bioactivity (Barsotti et al., 2013). Reportedly, GFs released from PRP showed antimicrobial activity against *E. coli* and *S. aureus*, evidently beneficial in the clinical environment (Losi et al., 2013).

These two actions, namely inflammation reducing and angiogenesis promoting effect in combination is anticipated to efficiently promote healing and angiogenesis in inflamed dental pulp. Furthermore, these data indicate that these effects are dose-dependent. In this study, 20% HPL have shown to provide the most optimal environment to induce angiogenesis in iDPSCs through a balance between viability maintenance and pro-angiogenesis, that is eventually imperative for healing and regeneration of pulp. This also suggests that HPL, containing pool of growth factors, at higher concentration is likely to exhibit anti-inflammation in the cellular system in an inflamed microenvironment that was not investigated in this study. These findings therefore provide convincing evidence and useful data for the application of autologous and allogeneic HPL as a potent tool to initiate angiogenesis in inflamed DPSCs.

These findings reflect that there is a great hope that angiogenesis, which is a critical component of pulp regeneration, could be achieved by employing regulated release of growth factors from HPL. Hence, it would be appropriate to state that

therapeutic intervention with growth factors from HPL may provide possibilities for control of cellular processes for healing and angiogenesis during repair of inflamed tissue.

6.5 Conclusion

Within the limitation of the current study, it was found that 20% HPL is optimum to stimulate angiogenesis in iDPSCs while maintaining the cell viability as evidenced by increased expression of pro-angiogenic factors at gene and protein level. This data brings further scientific support to possible application of 20% HPL in regenerative applications involving DPSCs in inflamed state. Understanding the molecular mechanism responsible for triggering specific healing cascade, behind the action of HPL in inflamed microenvironment could be a potential focus for future research.

CHAPTER 7: SUMMARY OF FINDINGS AND CONCLUSION

Overall aim of this study was to investigate the angiogenesis promoting potential of two PRC preparations, namely, HPL and PRP. Specifically, to evaluate their angiogenic and tissue repairing potential on DPSCs cultured in LPS induced microenvironment.. Finally, it was observed that LPS induced DPSCs showed higher viability and angiogenic activity when treated with higher concentrations of HPL.

7.1 Behaviour of DPSCs in LPS Microenvironment

The first part of this study was commenced to determine the MSC like characteristics of isolated adult DPSCs. It was found that DPSCs population was mesenchymal in nature, evidenced by plastic adherence, MSC surface markers expression and trilineage differentiation potential. In the second part of the study, optimization of suitable concentration and treatment time of LPS was done to induce inflamed microenvironment for DPSCs. Bacterial lipopolysaccharide (LPS) at different concentrations and treatment time was used to induce inflammation in DPSCs. LPS at 1µg/mL concentration for 24h was chosen to be the optimum concentration to induce inflammation in DPSCs, evidenced by pro-inflammatory cytokines expression at gene and protein level, while maintaining DPSCs viability and mesenchymal characteristics.

7.2 The Quest for Dose Dependent Effect of PRCs on Normal DPSCs

In this study, optimum concentration of HPL and PPR, that could maintain viability and have pro-angiogenic effect on DPSCs were established. It was found that 5% PRP, 10%, 15% and 20% HPL were optimum to maintain the DPSCs viability

evidenced by significantly high DPSCs viability in the 5% PRP, 10%, 15% and 20% HPL group compared to the of control (complete media) group. It was also observed that 20% HPL had significantly high pro-angiogenic effect on DPSCs compared to all tested media groups, evidenced by high tube formation capacity in functional angiogenesis and increased expression of pro-angiogenic markers at gene and protein level.

7.3 20% HPL is proposed to be the optimum concentration of PRC for inducing angiogenesis in iDPSCs

Amongst all tested groups, HPL treatment showed sufficient cell viability at the highest concentrations tested (10% and 20%). While 10% and 20% HPL increased cell viability, only 20% platelet lysate was able to significantly promote angiogenic activity ($p<0.05$ vs. all other concentrations tested). 20% HPL induced a significant increase of high cell viability and gene expression profile of pro-angiogenic and cell adhesion markers was highest in HPL group at 12th and 24th hour thus indicating their progressive angiogenesis. However, this function gradually reduced at 48th hour of treatment.

7.4 Clinical Significance

Our study showed that 10% HPL is suitable to be used as a FBS replacement for large-scale expansion of DPSCs in- vitro that can be further applied for clinical trials. In addition, 20% HPL is proposed to be suitable to exhibit pro-angiogenic effect on iDPSCs. Hence, suggesting its use for filling partially pulpectomized root canals where it can stimulate remnant pulp tissue in the root canal to induce angiogenesis and healing by virtue of pool of bioactive growth factors. In a completely pulpectomized root canal it may stimulate the stem cells present at the apical area to stimulate tissue regeneration inside the canal. HPL, being a derivative of fibrin, has a tendency to gel in absence of anticoagulant. Hence, it can act as an extracellular matrix for stem cells to grow and proliferate inside the root canal.

7.5 Limitations of Study

a. LPS induced inflammation in DPSCs could be validated by wider array of proteins and by using confirmatory functional assays.
b. In our study we used only one cell population i.e. DPSCs, however, in dental pulp, there is a diverse cell population, that might had reacted differently to various treatment used in the study. This aspect was not investigated in the study.
c. Immunological response of DPSCs to various treatments like LPS, HPL and PRP were not investigated.
d. Cell apoptosis and cytotoxicity assay of DPSCs was not performed for all the treatments used in the study.

7.6 Future Recommendations

Much anticipated extended work on the study might include:

a. Validating the LPS induced inflammation by other available advanced techniques.
b. Investigating the molecular mechanism underlying the LPS induced inflammation in DPSCs.
c. Validating the HPL induced angiogenesis by further extending the work using more advanced functional assays in three-dimensional model.
d. Analysis of specific growth factor(s) responsible for HPL induced angiogenesis.
e. An exploration into the anti-inflammatory effect of specific growth factors released from PRCs and molecular mechanism behind that.
f. To duplicate this study in an animal model to investigate if any immunogenic reaction gets triggered in response to allogenic HPL and PRP.
g. A study to support the sustained release of growth factors from PRCs should be designed to enhance the therapeutic potential of PRCs.

7.7 Conclusion

Isolated DPSCs population in our study were confirmed to be mesenchymal by MSC marker expression and trilineage differentiation potential. From the present study it can be interpreted that 1µg/mL of LPS treatment for 24 hours is most optimum to induce inflammation in DPSCs *in-vitro*. Amongst, the two types of PRCs tested we could comprehend that 5% PRP and 10%, 15% and 20% HPL demonstrated significantly higher viability of DPSCs than FBS *in-vitro*. For potential pro-angiogenic effect on DPSCs, 20% HPL was found to be optimum amongst all tested PRCs. 20% HPL demonstrated significantly high cell viability and pro-angiogenic activity on DPSCs in inflamed state (iDPSCs).

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications from the current project

1. Dental Pulp Tissue Engineering and Regenerative Endodontic Therapy. *Biomaterials for Oral and Dental Tissue Engineering, 2017, Elsevier publishers*. Bindal, P., Abu Kasim, N. H , Ramasamy TS, Dabbagh. A, K. Moharamzadeh, Lin, C.

2. Neuro-Fuzzy method for predicting the viability of stem cells treated at different time-concentration conditions. *Technology and Health 2017*
   Bindal,P., Bindal,U., Lin,C.W, Kasim,N.H.,RamasamyTS, Dabbagh A., Salwana E., Shamshirband S.

3. Bindal, P., Gnanasegaran. N., Abu Kasim, N. H, Ramasamy T S, Lin, C. Immunological properties of DPSCs in an inflamed microenvironment. *Saudi Journal of Biological Sciences (Under review)*

Other Publications

1. **Bindal, P.**, Lin, C. W., Bindal, U., Safi, S. Z., Zainuddin, Z. & Lionel, A. (2015) 'Dental Treatment and Special Needs Patients (SNPs): Dentist's Point of View in Selected Cities of Malaysia.', *Biomed Res-India Biomedical Research*, 26(261), pp. 152–156. Available at: www.biomedres.info (Accessed: 20 March 2017).

2. **Bindal, P.**, Bindal, U., Dabbagh, A., Ramanathan, A. & Ginjupalli, K. (2015) 'Comparative Effects of Turmeric, Coffee, and Chewable Tobacco on the Colour Stability of Tooth-coloured Restorative Materials', *Open Journal of Dentistry and Oral Medicine*. Horizon Research Publishing, 3(3), pp. 59–67. doi: 10.13189/OJDOM.2015.030301.

3. Abu Kasim, N. H., Abu Kassim, N. L., Razak, A. A. A., Abdullah, H., **Bindal, P.**, Che' Abdul Aziz, Z. A., Sulaiman, E., Farook, M. S., Gonzalez, M. A. G., Thong, Y. L., Ahmad, N. A., Naimie, Z., Abdullah, M., Lui, J. L. & Abdul Aziz, A. (2014) 'Pairing as an instructional strategy to promote soft skills amongst clinical dental students', *European Journal of Dental Education*, 18(1), pp. 51–57. doi: 10.1111/eje.12058.

4. **Bindal, P.** (2014) 'Labial Impressions: A Tool for Identification', *Journal of Forensic Research*. OMICS International, 5(3). doi: 10.4172/2157-7145.1000226.

5. **Bindal, P.**, Bindal, U., Safi, S. & Hussain, K. (2014) 'Dental students' perceptions on patients with HIV or hepatitis B infection', *Annual Research &*. Available at: http://search.proquest.com/openview/66703af211f15e54c7e1be9c975aef77/1?pq-origsite=gscholar&cbl=626448 (Accessed: 20 March 2017).

6. Ahmad, N. A., Naimie, Z., Lui, J. L., Aziz, A. A., Abdullah, M., Abu Kasim, N. H., Abu Kassim, N. L., Toh, C. G., Thong, Y. L., Abdul Razak, A. A., Abdullah, H., Che' Ab Aziz, Z. A., Sulaiman, E., Gonzalez, M. A. G. & **Bindal, P.** (2012) 'Clinical pairing revisited: a study at the University of Malaya, Malaysia.', *Journal of dental education*, 76(10), pp. 1377–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23066138 (Accessed: 20 March 2017).

7. **Bindal, P.**, Lin, C., Bindal, U., Safi, S., Zainuddin, Z., & Lionel, A. (1980) 'Dental Treatment and Special Needs Patients (SNPs): Dentist's Point of View in Selected Cities of Malaysia.', *Biomedical Research*. Medknow Publications. Available at: http://www.biomedres.info/abstract/dental-treatment-and-special-needs-patients-snps-dentists-point-ofrnview-in-selected-cities-of-malaysia-830.htmL (Accessed: 20 March 2017

Awards

1. Best Presentation award at Federation Dental International conference 2016, Poland.
2. Scholarship awarded under High Impact Research Grant Scheme, Ministry of Higher Education, Malaysia
3. Oral presentation – The 2015 Tissue Engineering Congress: London, United Kingdom
4. Poster presentation – Effect of Bleaching on Colour of Esthetic Restorative Materials Stained with Food Simulating Solutions, 35th Asia Pacific Dental Congress, 2013, Kuala Lumpur
5. Poster presentation – Effect of food simulating solutions on colour of aesthetic restorative biomaterials, International Association of Dental Research conference, 2012, Bangi.