A NOVEL PROCESS USING HUMAN SERA FOR THE PRODUCTION OF SECRETOME AND POTENTIALLY CLINICAL GRADE MESENCHYMAL STEM CELL FOR REGENERATIVE THERAPY

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FACULTY OF DENTISTRY
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ABSTRACT

Introduction: Cell-based regenerative therapies offer tremendous hope to many individuals suffering from degenerative diseases. Mesenchymal stem cells (MSCs) are considered as an attractive source of stem cells for regenerative therapies. Like MSCs, cell culture supernatants and secretomes from peripheral blood mononuclear cells (PBMCs) have also shown regenerative potential. *In vitro* expansion of MSCs and culture of PBMCs are critical to obtain cells and secretomes with more regenerative potential. Usually, xenogeneic sera and purified recombinant proteins supplemented media are used for *in vitro* culture that may cause xeno-contamination and priming of cells eventually affect the regenerative outcomes. *Objectives:* Firstly, to compare the ability of pooled human serum (pHS) and foetal bovine serum (FBS), as supplement for the production of MSCs with more regenerative potential. Secondly, to assess the effect of autologous human serum (AuHS) and FBS in producing secretome from PBMCs. *Methods:* Stem cells from human extracted deciduous teeth (SHED) was used as a source of MSCs. SHED (n=3) was cultured with either pHS or FBS supplement to compare their suitability in maintaining the regenerative potential and immunomodulatory properties during *in vitro* expansion. The PBMCs (n=7) were cultured with either AuHS, FBS or without any serum supplement to measure viability and differentiation. Cytokines present in the secretome (n=6) were analysed. Ingenuity Pathway Analysis (IPA) were performed to predict the up/down-regulation of biological functions related to regeneration process. *Results:* SHED showed the characteristics of MSCs such as plastic adherence, expression of specific cell surface markers, and tri-lineage differentiation. Expanded SHED (n=3) showed significantly ($p<0.05$) higher proliferation in pHS medium compared to FBS medium. Significantly lower proportion of flattened cells was observed in pHS medium compared to FBS medium (FBS: 7%, pHS: 3%). Furthermore, migration of SHED in pHS medium was found more

directional. Presence of selected 10 paracrine factors known for their proliferation and migration potential was detected in the human sera (n=6) that were used to produce pHS, none of which were detected in FBS. SHED expanded in pHS or FBS media were able to survive in the presence of complement and immune cells. IPA predicted results showed the suitability of pHS over FBS for the expansion of SHED to maintain their regenerative potential and immunomodulatory properties. Culture of PBMCs showed that AuHS supported viability of PBMCs until 96 hours of incubation. While with the FBS supplement, the viability of PBMCs was significantly reduced at 96 hours compared to those at 0 and 24 hours of incubation ($p<0.05$). A significantly higher content of EGF was detected in FBS secretome collected after 24 hours ($p<0.05$) compared to AuHS or basal medium secretome. While, AuHS secretome contained significantly higher amount of HGF after 24 ($p<0.05$) and 96 hours ($p<0.01$), and VEGF-A at 24 hours ($p<0.05$) compared to those in FBS secretome. SDF-1A was not detected in the FBS secretomes collected after either 24 or 96 hours. *Conclusions:* pHS has been shown to be better at supporting SHED to maintain its self-renewal capability, homogeneity and immunomodulatory properties. Besides, AuHS seems to favour cytokine composition of the secretomes with better regenerative potential.

ABSTRAK

Pengenalan: Terapi penjanaan semula berasaskan sel menawarkan harapan yang tinggi kepada ramai individu yang mengalami penyakit-penyakit degeneratif. Sel induk *mesenchymal* (MSCs) dianggap sebagai sumber yang menarik bagi terapi penjanaan semula. Seperti MSCs, *supernatants* dan *secretome* yang terhasil dari proses pengkluturan sel-sel mononuklear darah periferi (PBMCs) juga telah menunjukkan potensi untuk penjanaan semula. Adalah kritikal untuk mendapatkan sel-sel dan *secretome* yang mempunyai potensi penjanaan semula yang lebih tinggi semasa proses pengembangan MSCs secara in vitro dan mengkluturan PBMCs. Biasanya, serum *xenogeneic* dan protein rekombinan tulen yang digunakan sebagai bahan tambahan dalam media untuk pengembangan sel secara in vitro boleh menyebabkan pencemaran *xeno* dan penyebuan sel, akhirnya ia menjejaskan hasil penjanaan semula. *Objektif:* Pertama, untuk membandingkan keupayaan serum manusia yang terkumpul (pHS) dan serum janin lembu (FBS), sebagai bahan tambahan untuk meghasilkan MSC yang mempunyai potensi penjanaan semula yang lebih tinggi. Kedua, untuk menilai kesan serum manusia autologus (AuHS) dan FBS dalam penghasilkan *secretome* dari pengkulturan PBMCs. *Kaedah:* Sel induk dari gigi susu manusia yang telah dicabut (SHED) digunakan sebagai sumber MSC. SHED (n=3) dikultur sama ada dalam pHS atau media yang ditambah FBS untuk membandingkan kesesuaian media bagi mengekalkan potensi penjanaan semula dan sifat immunomodulatori semasa pengembangan sel secara in vitro. PBMCs (n=7) dikultur sama ada dalam AuHS, FBS atau media tanpa serum tambahan bagi menentukan daya kebolehhidupan dan pembezaan sel. Sitokin yang terhasil dalam *secretome* (n=6) telah dianalisa. Ramalan *up/down-regulation* fungsi biologi yang berkaitan dengan proses penjanaan semula dibuat menggunakan Ingenuity Analisis Pathway (IPA). *Keputusan:* SHED menunjukkan ciri-ciri MSC seperti lekapan plastik, ekspresi specific penanda

permukaan sel, dan pembezaan *tri-lineage*. SHED yang dikembangkan dalam pHS (n=3) menunjukkan sifat proliferasi yang signifikan ($p<0.5$) berbanding media FBS. Sel-sel leper yang diperhatikan dalam pHS adalah jauh lebih rendah dan signifikan berbanding dengan sel dalam media yang ditambah dengan FBS (FBS: 7%, PHS: 3%). Tambahan pula, penghijrahan SHED dalam pHS didapati lebih terarah. Kehadiran 10 *paracrine factors ter*pilih yang berkait rapat dengan potensi proliferasi dan penghijrahan dikesan dalam serum manusia (n=6) yang digunakan untuk penghasilan pHS, tetapi tidak dikesan dalam FBS. SHED yang dikembangkan dalam pHS mampu bertahan dengan kehadiran komplemen dan sel-sel imun berbanding FBS. IPA meramalkan kesesuaian pHS berbanding FBS untuk pengembangan SHED dan mampu mengekalkan potensi penjanaan semula dan sifat immunomodulatorinya. Pengkulturan PBMCs menunjukkan bahawa AuHS menyokong kebolehhidupan sel apabila diinkubasikan sehingga 96 jam. Walaubagaimanapun kebolehhidupan PBMCs yang diinkubasikan selama 96 jam menurun secara signifikan ($p<0.05$) apabila dikultur dalam media yang ditambah dengan FBS berbanding dengan data inkubasi selama 0 dan 24 jam. Kandungan EGF adalah jauh lebih tinggi dikesan dalam *secretome* FBS yang dikumpulkan selepas 24 jam ($p<0.05$) berbanding AuHS atau *secretome* media asas. Manakala, *secretome* AuHS yang dikumpulkan selepas 24 dan 96 jam masing-masing mengandungi HGF dengan jumlah jauh lebih tinggi, $p<0.05$ dan $p<0.01$, dan kandungan VEGF-A juga lebih tinggi selepas 24 jam ($p<0.05$) berbanding dengan *secretome* FBS. SDF-1A juga tidak dikesan dalam *secretome* FBS yang dikumpul sama ada selepas 24 atau 96 jam. *Kesimpulan:* pHS telah menunjukkan kebolehan yang lebih baik dalam membantu SHED untuk mengekalkan keupayaan pembaharuan diri (*self-renewal*), kehomogenan dan sifat immunomodulatorinya. Selain itu, AuHS jelas menunjukkan penghasilan *secretome* yang berkomposisikan sitokin yang menpunyai potensi penjanaan semula yang lebih baik.

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LIST OF SYMBOLS AND ABBREVIATIONS

AD	:	Adipose derived
ADCC	:	Antibody dependent cell-mediated cytotoxicity
Ag	:	Antigen
Ang-1	:	Angiopoietin-1
AuHS	:	Autologous human serum
BDNF	:	Brain-derived neurotrophic factor
BM	:	Bone marrow
CCR	:	Chemokine receptor
CDC	:	Complement-dependent cytotoxicity
CEJ	:	Cement-enamel junction
CPD	:	Cumulative population doubling
Creb	:	cAMP response element-binding protein
CSCs	:	Cardiac stem cells
DCs	:	Dendritic cell
Dlx3	:	Distal-less homeobox 3
DPSC	:	Dental pulp stem cells
E3UL	:	E3 ubiquitin ligase
EGF	:	Epidermal growth factor
ERK	:	Extracellular-signal-regulated kinases
ESCs	:	Embryonic stem cell
FAK	:	Focal adhesion kinase
FBS	:	Foetal bovine serum
FC	:	Flattened
Fcer1	:	High-affinity IgE receptor

FGF-2	:	Basic fibroblast growth factor
FSS	:	Flat spindle-shaped
G-CSF	:	Granulocyte colony stimulating factor
GLUT	:	Glucose transporter
GM-CSF	:	Granulocyte macrophage colony stimulating factor
HGF	:	Hepatocyte growth factor
HIF	:	Hypoxia inducible factor
HLA-G	:	Human leukocyte antigen G
HMGB1	:	High-mobility group box 1 protein
HoS	:	Horse serum
HPH	:	HIF-1 prolyl-hydroxylases
HRE	:	Hypoxia-response element
HS	:	Human serum
HSCs	:	Hematopoietic stem cell
HSP	:	Heat shock protein
ICAM1	:	Intracellular adhesion molecule 1
IDO	:	Indolamin-2,3-dioxygenase
IGF-1	:	Insulin-like growth factor-1
IL	:	Interleukin
INF-γ	:	Interferon gamma
IPA	:	Ingenuity Pathway Analysis
iPSCs	:	Induced pluripotent stem cell
ISCT	:	International Society for Cellular Therapy
Jnk	:	c-Jun N-terminal kinase
LDH	:	Lactate dehydrogenase
LIF	:	Leukaemia inhibitory factor

M-CSF	:	Macrophage colony stimulating factor
MHC	:	Major histocompatibility complex
MSCs	:	Mesenchymal stem cell
MSX1	:	Msh homeobox 1
MSX2	:	Msh homeobox 2
NADH	:	Nicotinamide adenine dinucleotide
Neu5GC	:	N-glycolylneuraminic acid
NFκB	:	Nuclear factor kappa B
NK	:	Natural killer
NO	:	Nitric oxide
NSCs	:	Neural stem cells
ODD	:	Oxygen-dependent degradation domain
PBMCs	:	Peripheral blood mononuclear cells
PD	:	Population doubling
PDGF	:	Platelet derived growth factor
PDK	:	Pyruvate dehydrogenase kinase
PDT	:	Population doubling time
PGE2	:	Prostaglandin E2
pHS	:	Pooled human serum
PMD	:	Placental matrix-derived
ROS	:	Reactive oxygen species
RS	:	Rapidly self-renewing
SCF	:	Stem cell factor
SDF	:	Stromal cell-derived factor
SHED	:	Stem cells from the pulp of human extracted deciduous teeth
SM	:	Supplemented medium

SMMs	:	Skeletal muscle myoblasts
SS	:	Spindle-shaped
T_C	:	Cytotoxic T cells
TGF	:	Transforming growth factor
T_H	:	Helper T cells
TNF-α	:	Tumour necrosis factor alpha
T_{regs}	:	Regulatory T cells
TREM1	:	Triggering receptor expressed on myeloid cells 1
UCB	:	Umbilical cord blood
VCAM1	:	Vascular cell adhesion molecule 1
VEGF	:	Vascular endothelial growth factor
VHL	:	von Hippel Lindau protein
WJ	:	Wharton's jelly

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CHAPTER 1: INTRODUCTION

1.1 Introduction

The promising role of regenerative therapy is becoming more conceivable in addressing the unmet needs of treating degenerative diseases through conventional medicine. Diseases such as diabetes, myocardial infarction, spinal cord injury, stroke, Parkinson's and Alzheimer's diseases have become more prevalent with increasing life expectancy. It has been estimated that in the United States alone, approximately 1 in 3 individuals would benefit from regenerative therapy during their lifetime (Harris, 2009).

Self-renewal and multi-potency are the key hallmarks of stem cells, permitting them to act as the fundamental units maintaining growth, homeostasis and repair of many tissues. These two key features establish stem cells as the most promising tool for regenerative medicine (Rehman, 2010; Wagers & Weissman, 2004). Among the different types of stem cells, mesenchymal stem cells (MSCs) or multipotent mesenchymal stromal cells (Dominici et al., 2006) are considered as a potential tool to treat degenerative diseases. This is due to their multipotent differentiative capacity (Govindasamy et al., 2011a; Sasaki et al., 2008; Toma et al., 2002) with trophic activity (Caplan & Dennis, 2006; Zhang et al., 2007), potent immunosuppressive effects (Aggarwal & Pittenger, 2005; Chen et al., 2006; Nauta & Fibbe, 2007), and ability to induce vascularisation (Martens et al., 2006). Moreover, controversies surround the use of embryonic stem cells (ESCs) are also not applicable for MSCs (Robertson, 2010). These properties have fascinated and encouraged researchers to push the frontiers of regenerative medicine, utilizing MSCs to treat a large variety of pathologies; including traumatic lesions (Richardson et al., 2010), stroke (Doeppner & Hermann, 2010), autoimmune diseases (Siegel et al., 2009), tumour (Kosztowski et al., 2009), musculoskeletal and cardiac disorders (Miyahara et al., 2006).

MSCs can be efficiently isolated from tissues such as bone marrow (Shi & Gronthos, 2003), adipose tissue (Lund et al., 2009), umbilical cord blood (Erices et al., 2000), and dental pulp (Govindasamy et al., 2010; Shi & Gronthos, 2003). Recently, derivation of MSCs from induced pluripotent stem cells (iPSCs) has been reported (Lian et al., 2010; Liu et al., 2012b; Villa-Diaz et al., 2012; Zou et al., 2013). Hence, iPSCs may resolve patient-specific MSCs scarcity (Lian et al., 2010; Villa-Diaz et al., 2012; Zou et al., 2013). MSCs from these sources can be used for cell-based therapy and tissue engineering. As a source of MSCs, in this study stem cells from the pulp of human extracted deciduous teeth (SHED) is being used, since it is readily accessible, have a large donor pool, and pose no risk of discomfort for the donor (Govindasamy et al., 2011a; Wang et al., 2012a). Moreover, SHED share similar characteristics with bone marrow (BM)-MSCs (Govindasamy et al., 2010).

For each regenerative therapy, 50-400 million MSCs are required (Carlsson et al., 2015; Estrada et al., 2013; Levy et al., 2015; Mathiasen et al., 2013; Tan et al., 2012; Weiss et al., 2013). Despite the various sources, presence of very low number of MSCs within the harvested tissues (Aust et al., 2004; Pittenger et al., 1999) makes it impractical to isolate such a large number of MSCs from a single donor (Haque et al., 2015). Thus, regardless of the sources, *ex vivo* expansion of MSCs prior to transplantation is required to yield enough MSCs for cell-based therapy (Haque et al., 2013; Pittenger et al., 1999).

In clinical trials, usually *ex vivo* expanded MSCs are being transplanted to assess their efficacy in treating degenerative diseases (Connick et al., 2011; Tewarie et al., 2009), reducing acute rejection of transplanted organs (Tan et al., 2012), and in preventing and treating graft-versus-host disease (Le Blanc et al., 2008; Ringden et al., 2006). Sometimes the expanded cells are induced to differentiate into a particular cell

type and then the pre-differentiated cells are transplanted for the regeneration of particular tissues or organs (Mohamadnejad et al., 2010). After transplantation, tissue-specific migration and engraftment determine the success of cell-based regenerative therapy. Several *in vitro*, *in vivo* and clinical studies reported encouraging regenerative potential of MSCs (Azizi et al., 1998; Jin et al., 2002; Kim & Cho, 2013; Shake et al., 2002). Although MSCs based clinical trials reported significant short term regenerative benefits, the reported number of engrafted cells in the defected tissues is very low (Copland, 2011; Volarevic et al., 2011). The low number of engrafted MSCs is considered to be a major drawback for long term functional benefits from transplanted MSCs (Malliaras & Marban, 2011; Volarevic et al., 2011). In order to enhance engraftment efficiency several techniques such as intra-arterial delivery instead of intravenous delivery to avoid accumulation of MSCs in the lung, and modification of cell surface molecules through chemical, genetic and coating techniques to promote selective particular organs or tissues have been developed (Kean et al., 2013). However, these strategies overshadowed the importance of the culture environment and media composition that could possibly resolve the issue of low MSCs engraftment.

From isolation to engraftment, the MSCs pass through two different environmental conditions (Figure 1.1); *in vitro* culture condition (from isolation to transplantation) and *in vivo* or physiological condition (prior to isolation and post transplantation). Studies reported that culture environment such as hypoxia or normoxia have an influential effect on cellular aging and chemokine marker expression during *in vitro* expansion that may affect homing and engraftment of MSCs following transplantation (Estrada et al., 2012; Estrada et al., 2013; Haque et al., 2015).

Culture media also play a vital role in maintaining cellular proliferation, aging and migration of MSCs. For decades xenogeneic sera are being used to supplement *in vitro*

cell culture media (Gstraunthaler, 2003). These xenogeneic sera contain xenoantigen that may trigger hyper-immunogenicity to the expanded MSCs (Haque et al., 2015; Sakamoto et al., 2007). This hyper immunogenicity may lead to acute rejection of transplanted xeno-contaminated MSCs (Haque et al., 2015; Komoda et al., 2010; Li & Lin, 2012). To overcome the issue of xeno-contamination the use of serum free media was proposed; requiring purified or recombinant protein supplement to maintain safety, reproducibility and consistency of cells in culture (Corotchi et al., 2013; Mujaj et al., 2010; Patrikoski et al., 2013; Simoes et al., 2013). However, most of the serum free media supplemented with recombinant or purified proteins do not support isolation of MSCs (Crapnell et al., 2013; Rashi, 2012).

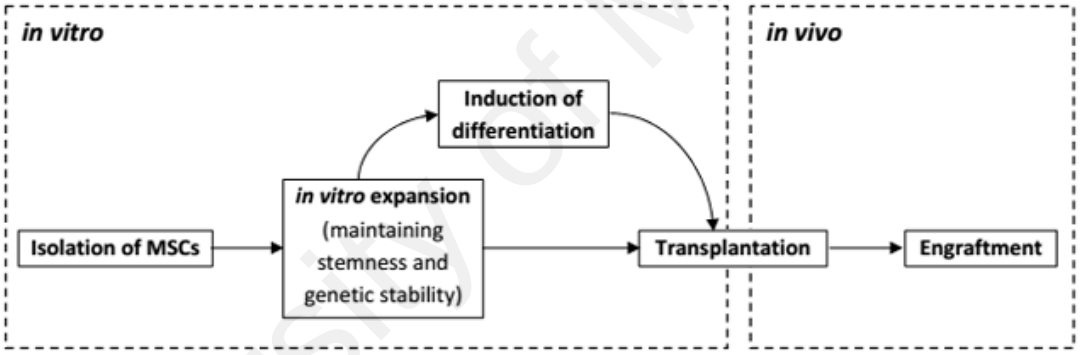

Figure 1.1: Steps involved in MSCs based therapy (Haque et al., 2013)

Like MSCs, secretomes or cell culture supernatants are also considered as potential tool for regenerative therapy. The growing evidence on the role of paracrine factors (cytokines, chemokines and growth factors) in regeneration of affected organs has led to the introduction of cell culture supernatants or secretomes as a new therapeutic tool of regenerative medicine. Regenerative potential of secretomes from stem and progenitor cells has been reported in the treatment of neuronal disorders (Pires et al., 2014), vascular diseases (Dao et al., 2013), and cutaneous wounds (Yew et al., 2011). Like other adult stem cells secretomes (Madrigal et al., 2014), regenerative potential of the

secretome of peripheral blood mononuclear cells (PBMCs) have also been reported recently (Mildner et al., 2013).

In vitro PBMCs culture media need to be supplemented with foetal bovine serum (FBS) or purified proteins, as they are prone to die without any supplementation (Zhang et al., 2004). For decades FBS is being used as *in vitro* cell culture media supplement, providing proteins, growth factors, hormones, lipids, vitamins, attachment factors and other important trace elements those are needed for the survival and proliferation of cells in culture (Gstraunthaler, 2003). Composition of FBS varies from lot-to-lot, and xenoantigen such as N-glycolylneuraminic acid (Neu5GC) present in the FBS has the potential to trigger immune response (Lindroos et al., 2009). Hence, the use of serum free media was proposed; requiring purified or recombinant protein supplement to maintain safety, reproducibility and consistency of cells in culture (Haque et al., 2015; Mujaj et al., 2010). However, the serum free media supplemented with recombinant or purified proteins potentially regulate the secretomes composition by modulating autocrine and paracrine signalling pathways (Mirshahi et al., 2000).

In the last decade, several studies reported the potential of human serum, plasma and/or platelet lysate as replacement for FBS (Aldahmash et al., 2011; Jonsdottir-Buch et al., 2013; Lin et al., 2005; Shahdadfar et al., 2005). Autologous human serum (AuHS) has been reported to have positive effect on the proliferation (Kobayashi et al., 2005; Mizuno et al., 2006), differentiation potential (Kobayashi et al., 2005; Shahdadfar et al., 2005; Stute et al., 2004), genetic stability (Dahl et al., 2008; Shahdadfar et al., 2005), immunomodulation (Perez-Ilzarbe et al., 2009), and motility (Kobayashi et al., 2005) of MSCs compared to FBS. (Perez-Ilzarbe et al., 2009). Allogeneic human serum and human cord blood serum are other suitable alternatives to FBS (Aldahmash et al., 2011; Bieback et al., 2012; Jung et al., 2009). Pooled allogeneic human serum (pHS) from

adult AB-blood donors and pooled cord blood serum reported to maintain differentiation potential, motility and immunosuppressive properties of MSCs (Cooper et al., 2010; Kobayashi et al., 2005; Le Blanc et al., 2007; Phadnis et al., 2006; Poloni et al., 2009; Tateishi et al., 2008; Turnovcova et al., 2009). Superiority of platelet lysate over FBS in maintaining growth potential, genetic stability, immunomodulatory properties, and differentiation potential of MSCs have also been reported (Capelli et al., 2007; Crespo-Diaz et al., 2011; Govindasamy et al., 2011b; Griffiths et al., 2013; Jonsdottir-Buch et al., 2013; Perez-Ilzarbe et al., 2009; Trojahn Kolle et al., 2013; Vasanthan et al., 2014). Media supplements from human source might prove useful for the production of secretome from PBMCs as well.

1.2 Research questions

The overarching goal of our research is to obtain MSCs and secretomes with high regenerative potential. The literature revealed that poor engraftment possibly leads to undesirable outcomes of cell-based regenerative therapies. This study focuses on the following research questions;

i. What are the solutions to address the issues of poor MSCs engraftment?
ii. Could media supplement from human source such as pooled human serum, platelet lysate be used to address the issues related to engraftment?
iii. Could media supplement from human source maintain immunomodulatory property of MSCs?
iv. What are the limitation of using xenogeneic serum and purified proteins for the production of secretome from PBMCs?
v. Is AuHS a good microenvironment for cells to produce secretomes with superior regenerative paracrine factors profile?

1.3 Aim and objectives

In alignment with the overarching goal, this study is divided into four interrelated aims;

Aim I: Isolation and characterization of SHED.

Objectives:

a) To isolate SHED from human extracted deciduous dental pulp tissue.
b) To characterise and confirm the MSCs like properties of SHED.
c) To determine the growth kinetics of SHED.

Aim II: Use of pHS as an alternative to FBS to produce SHED with more regenerative potential.

Objectives:

a) To determine the growth kinetics and metabolic activity of SHED when expanded in either FBS or pHS supplemented medium *in vitro*.
b) To determine the morphology of SHED when expanded in either FBS or pHS supplemented medium *in vitro*.
c) To determine the migration pattern of SHED when expanded in either FBS or pHS supplemented medium *in vitro*.

Aim III: Effect of FBS and pHS on the immunomodulatory properties of SHED.

Objectives:

a) To determine the effect of complement on SHED expanded either in FBS or pHS supplemented medium.
b) To determine the effect of monocytes on SHED expanded either in FBS or pHS supplemented medium.
c) To determine the effect of lymphocyte on SHED expanded either in FBS or pHS supplemented medium.

Aim IV: Effect of FBS and Autologous human serum (AuHS) on the production of secretome from PBMCs.

Objectives:

a) To determine the viability of PBMCs cultured either in FBS or AuHS supplemented medium.

b) To determine the differentiation of PBMCs cultured either in FBS or AuHS supplemented medium.

c) To determine the cytokine composition of the supernatant from PBMCs cultured either in FBS or AuHS supplemented medium.

1.4 Research framework

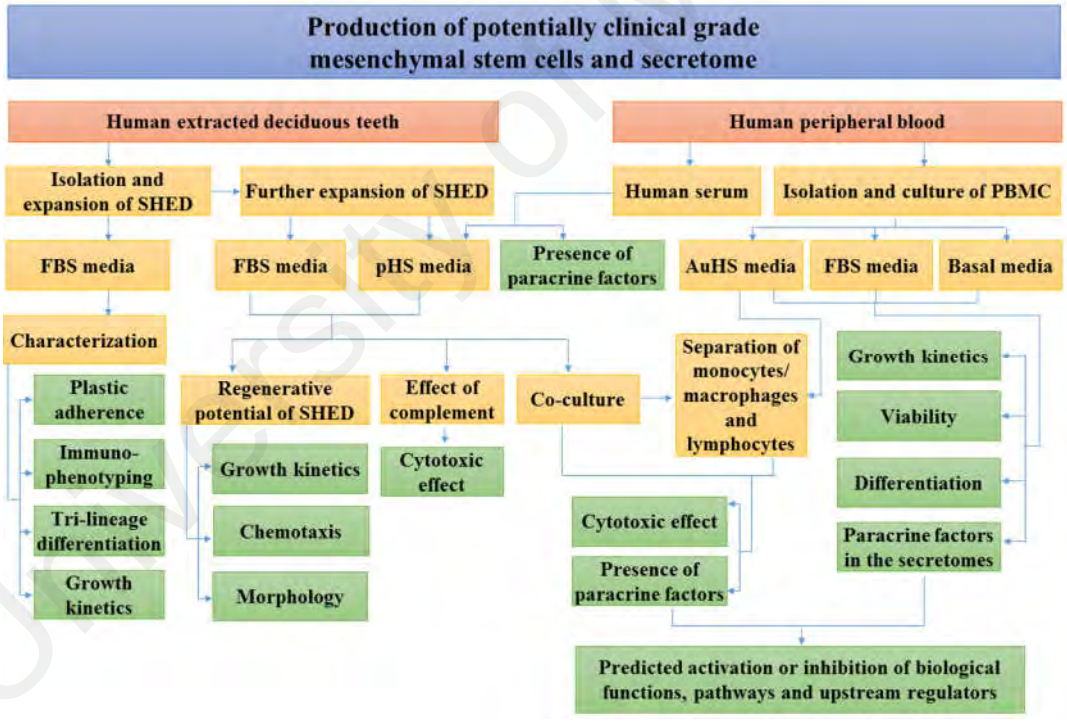

Figure 1.2: Work flowchart of this study to produce clinical grade SHED and secretome from PBMCs (SHED, stem cells from human extracted deciduous teeth; PBMC, peripheral blood mononuclear cells; FBS, foetal bovine serum; pHS, pooled human serum; AuHS, autologous human serum)

CHAPTER 2: REVIEW OF LITERATURE

2.1 Stem Cells

2.1.1 Types of stem cells

Self-renewal and plasticity to differentiate into different cell types are the key features of stem cells (Rehman, 2010; Wagers, 2012). On the basis of the plasticity or lineage potential stem cells can be divided into four broad subgroups: totipotent, pluripotent, multipotent and unipotent (Callihan et al., 2011). A cell having the potentiality to produce an entire new individual is known as totipotent cell. The zygote and blastomeres from the early cleavage of zygote fall into this category (Triller Vrtovec & Vrtovec, 2007; Tsonis, 2007). ESCs are marked as the source of pluripotent stem cells. These cells exhibit the ability to differentiate into any cell type of the adult organism (Johnson et al., 2008). In recent years, a new type of pluripotent stem cells has been generated by chemically reprogramming the adult somatic cells, which is known as iPSCs (Chen et al., 2011; Takahashi et al., 2007). Multipotent stem cells are those having the capability to differentiate into more than one cell types. Hematopoietic stem cells (HSCs) and MSCs are the two most common sources of multipotent stem cells (Phinney & Prockop, 2007; Zou et al., 2012). The final types of stem cells; unipotent stem cells or adult lineage-committed progenitor cells exhibit the potential to differentiate into a particular cell population (Can, 2008; Holterman & Rudnicki, 2005; Ousset et al., 2012). Almost all the organs or tissues in the human body such as myosatellite cells of muscles, endothelial progenitors and luminal stem cells have their lineage committed progenitor cells (da Silva Meirelles et al., 2006; Pleniceanu et al., 2010).

2.1.2 Adult stem cell function within the body

Lineage committed progenitor cells or unipotent stem cells are involved in maintaining homeostasis by replenishing the lost cells with new cells during the process of turnover (da Silva Meirelles et al., 2006; Fuchs & Chen, 2013). In addition to these tissue specific progenitor cells, different parts of the human body such as bone-marrow, adipose tissue, and dental pulp contain MSCs which are also involved in maintaining homeostasis (Erices et al., 2000; Govindasamy et al., 2010; Korbling & Estrov, 2003; Lund et al., 2009; Shi & Gronthos, 2003). Several researchers reported that there is an increase in the number of MSCs in peripheral blood of the patients suffering from skeletal muscle injury (Ramírez et al., 2006) and osteoporosis (Carbonare et al., 2009). Higher numbers of circulatory stem cells and progenitor cells have also been observed in patients immediately following ischemic stroke and myocardial infarction (Jung et al., 2008; Kucia et al., 2006; Paczkowska et al., 2009; Ripa et al., 2007; Wang et al., 2006; Yip et al., 2008). These events denote the importance of adult stem cells in repairing the diseased or injured organs. In many conditions such as myocardial infarction, stroke, and spinal cord injuries, natural regenerative process alone is not sufficient to repair the diseased or injured organ (Hatzistergos et al., 2010) and this could be due to the inability of body to supply higher number of cells. Thus supplementing or substituting current treatment modalities with stem cells therapy could be considered.

2.1.3 Stem cells sources for regenerative therapy

Due to its pluripotency, ESCs are considered as the best tool for tissue regeneration (Harris, 2009; Levi et al., 2012). However, ethical issues over the use of ESCs (Brock, 2010; Robertson, 2010) lead researchers to search for suitable replacements. In the recent years, the potential of iPSCs has been explored as they share similar

characteristics of ESCs (Okita et al., 2013; Takahashi et al., 2007; Wang et al., 2012b). Nevertheless, there are some issues (e.g. epigenetic memory, teratoma formation, immunogenicity) related to the therapeutic potentials of iPSCs has yet to be resolved (Hayden, 2011; Kim et al., 2010; Polo et al., 2010). Meanwhile, multipotency, immunomodulatory effects, trophic functions, vasculogenesis potential as well as large donor pool make MSCs an attractive source of stem cells for regenerative therapy (Caplan, 2013; Caplan & Correa, 2011; Govindasamy et al., 2011a).

MSCs are generally obtained from placenta, bone marrow, human muscle, adipocytes and other tissues (Mohyeldin et al., 2010; Prockop et al., 2003). However, scarcity of the source and the invasive procedures required to isolate and culture are among the limiting factors for their use. As an alternative to minimize those limitations SHED has been considered to be an appealing source for MSCs. SHED, are readily accessible, have a large donor pool, and pose no risk of discomfort for the donor (Govindasamy et al., 2011a; Wang et al., 2012a). Controversies surround the ESCs are also not applicable when harvesting SHED. Moreover, these cells share similar functions with BM-MSCs and has been shown to be able to differentiate into osteoblasts, adipocytes, and neurogenic cell types *in vitro* (Govindasamy et al., 2010).

2.1.4 Mesenchymal stem cells based clinical trials

In the last two decades, several *in vitro* and animal studies have elucidated the tremendous therapeutic potential of MSCs (Azizi et al., 1998; Jin et al., 2002; Komatsu et al., 2010; Sato et al., 2012; Shake et al., 2002; Xu et al., 2012). This has led researchers to conduct clinical trials in an attempt to bring MSCs from bench to bedside. On 04/05/2016, the public clinical trial database http://clinicaltrials.gov reported that 456 clinical trials are evaluating the therapeutic safety and efficacy of MSCs. Of the 456 clinical trials, 126 have been completed and the majority of these trials were in phase I,

phase II, or a combination of phases I and II (Figure 2.1). Among the 126 clinical trials that has been documented as completed, only 37 trials have published data (Table 2.1).

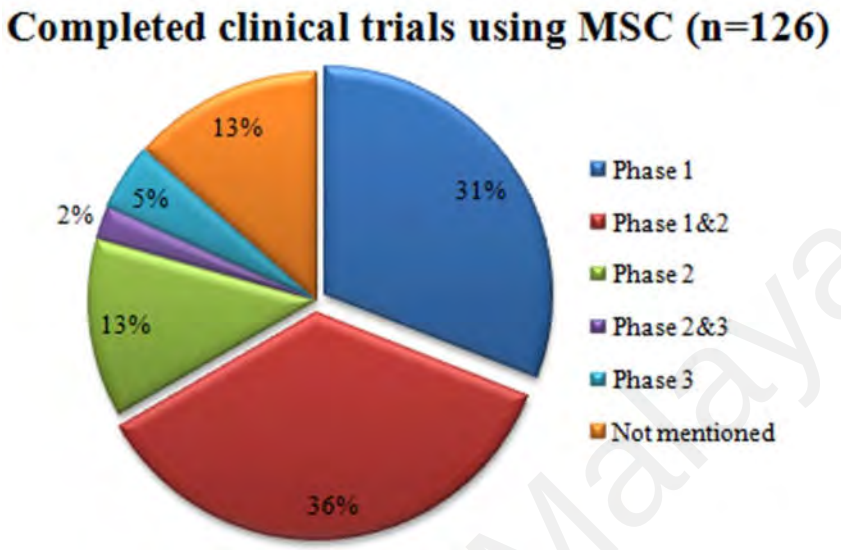

Figure 2.1: Completed MSC based clinical trial by clinical phase

Table 2.1: List of completed clinical trials using *ex vivo* expanded MSCs

Clinical trial No.	Source of MSC	Serum Supplement	Disease Treated	Dose No. of treatment	Route of Administration	Phase	Design	References
NCT01343511	Allo WJ	-	Autism	1×10^6 cells/ kg BW Multiple	Intravenous (2 times) Intrathecal (2 times)	I & II	Non-randomized, Safety/efficacy study, Parallel assignment, Open Label	(Lv et al., 2013)
NCT01068951	Au BM	HPL	Type 1 diabetes	$2.1–3.6\times10^6$ cells/ kg BW Single	Intravenous	-	Randomized, Efficacy study, Parallel assignment, Open label	(Carlsson et al., 2015)
NCT00587990	Au BM	-	Myocardial infarction	2×10^7 cells Multiple	Transepicardial	I & II	Randomized, Safety/efficacy study, Parallel assignment, Double blind (Subject, investigator)	(Karantalis et al., 2014)
NCT01392105	Au BM	FBS	Myocardial infarction	1×10^6 cells/ kg BW Single	Intracoronary	II & III	Randomized, Safety/efficacy study, Parallel assignment, Open label	(Lee et al., 2014)
NCT00883727	Allo BM	-	Myocardial infarction	-	Intravenous	I & II	Randomized, Safety/efficacy study, Parallel assignment, Double blind (Subject, caregiver, investigator)	(Chullikana et al., 2015)
NCT00114452	Allo BM	-	Myocardial infarction	$0.5/1.6/5\times10^6$ cells/kg BW Single	Intravenous	I	Randomized, Safety study, Parallel assignment, Double blind (Subject, Caregiver, Investigator, Outcomes assessor)	(Hare et al., 2009)
NCT01291329	Allo WJ	FBS	Myocardial infarction	6×10^6 cells Single	Intracoronary	II	Randomized, Safety/efficacy study, Parallel assignment, Double blind (Subject, caregiver, investigator, outcomes assessor)	(Gao et al., 2015)
NCT00260338	Au BM	FBS	Coronary artery disease	- -	Intra-myocardial	I & II	Non-randomized, Safety/efficacy study, Single group assignment, Open Label	(Mathiasen et al., 2013)
NCT01087996	Au BM Allo BM	-	Ischemic cardiomyopathy	$20/100/200\times10^6$ cells Single	Transendocardial	I & II	Randomized, Safety/efficacy study, Parallel assignment, Open label	(Hare et al., 2012)
NCT00768066	Au BM	-	Ischemic cardiomyopathy	$1/2\times10^8$ cells Single	Transendocardial	I & II	Randomized, Safety/efficacy study, Parallel assignment, Double blind (Subject, investigator)	(Heldman et al., 2014)
NCT00644410	Au BM	FBS	Ischaemic heart failure	$2\text{-}4\times10^7$ cells Multiple	Intra-myocardial	I & II	Randomized, Safety/efficacy study, Parallel assignment, Double blind (Subject, caregiver, investigator, outcomes assessor)	(Mathiasen et al., 2015)
NCT01274975	Au AD	FBS	Spinal cord injury	400×10^6 cells Single	Intravenous	I	Randomized, Safety study, Single group assignment, Open label	(Ra et al., 2011)
NCT01325103	Au BM	FBS	Spinal cord injury	5×10^6 cells/cm^3 lesion Single	Intralesional	I	Non-randomized, Safety/efficacy study, Single group assignment, Open label	(Mendonca et al., 2014)
NCT00816803	Au BM	Serum free	Spinal cord injury	2×10^6 cells/ kg BW Multiple	Lumbar puncture	I & II	Safety/Efficacy Study, Parallel Assignment, Single Blind (Outcomes Assessor)	(El-Kheir et al., 2013)
NCT01183728	Au BM	-	Osteoarthritis	4×10^7 cells Single	Intra-articular	I & II	Safety/efficacy study, Single group assignment, Open label	(Orozco et al., 2013, 2014)
NCT01586312	Allo BM	FBS	Osteoarthritis	4×10^7 cells Single	Intra-articular	I & II	Randomized, Safety/efficacy study, Parallel assignment, Double blind (Subject, Outcomes assessor)	(Vega et al., 2015)
NCT01436058	Au BM	FBS	Osteoarthritis	5×10^5 cells/kg BW Single	Intra-articular	I	Safety/efficacy study, Single group assignment, Open label	(Emadedin et al., 2015)
NCT01207661	Au BM	FBS	Osteoarthritis	5×10^5 cells/kg BW Single	Intra-articular	I	Safety/efficacy study, Single group assignment, Open label	(Emadedin et al., 2015)
NCT01499056	Au BM	FBS	Osteoarthritis	5×10^5 cells/kg BW Single	Intra-articular	I	Safety/efficacy study, Single group assignment, Open label	(Emadedin et al., 2015)

Table 2.1 continued

Clinical trial No.	Source of MSC	Serum Supplement	Disease Treated	Dose No. of treatment	Route of Administration	Phase	Design	References
NCT00225095	Allo BM	-	Osteoarthritis	5×10^7 cells Single	Intra-articular	I & II	Randomized, Safety/efficacy study, Parallel assignment, Double blind (Subject, investigator, outcomes assessor)	(Vangsness et al., 2014)
NCT01300598	Au AD	FBS	Degenerative arthritis.	$1/5/10 \times 10^7$ cells Single	Intra-articular	I & II	Non-randomized, Safety/efficacy study, Single group assignment, Open label	(Jo et al., 2014)
NCT00187018	Allo BM	FBS	Osteogenesis imperfect	$0.68\text{-}2.75\times10^3$ cells/kg BW Single	Intravenous	-	Non-Randomized, Safety/Efficacy Study, Single Group Assignment, Open Label	(Otsuru et al., 2012)
NCT00504803	Allo BM	Irradiated FBS	Graft-versus-host-disease	- Single	Intravenous	II	Non-randomized, Safety/efficacy study, Single group assignment, Open label	(Baron et al., 2010a)
NCT00823316	Allo UCB	FBS	Graft rejection and graft-versus-host-disease	$1 \& 5 \times10^6$ cells/ kg BW Single	Intravenous	I & II	Randomized, Safety/efficacy study, Parallel assignment, Open label	(Lee et al., 2013)
NCT00658073	Au BM	-	Renal transplant rejection	$1\text{-}2\times10^6$ cells/ kg BW Twice	Intravenous	-	Randomized, Efficacy study, Parallel assignment, Open label	(Tan et al., 2012)
NCT00734396	Au BM	FBS	Renal transplant rejection	1×10^6 cells/ kg BW Twice	Intravenous	I & II	Non-randomized, Safety/efficacy study, Single group assignment, Open label	(Reinders et al., 2013)
NCT00883870	Allo BM	-	Critical limb ischemia	2×10^6 cells/kg BW Single	Intramuscular	I & II	Randomized, Safety/efficacy study, Parallel assignment, Double blind (Subject, caregiver, investigator)	(Gupta et al., 2013)
NCT01065337	Au BM	FBS, HoS	Critical limb ischaemia	-	Intramuscular or Intraarterial	II	Randomized, Safety/efficacy study, Parallel assignment, Open label	(Kirana et al., 2012)
NCT00911365	Au BM	FBS	Multiple system atrophy	40×10^6 cells Multiple	Intraarterial (1 time) Intravenous (3 times)	II	Randomized, Parallel assignment, Single blind (subject)	(Lee et al., 2012b)
NCT00683722	Allo BM	-	Coronary obstructive pulmonary disorder.	100×10^6 cells Multiple	Intravenous	II	Randomized, Safety/efficacy study, Parallel assignment, Double blind (subject, caregiver, investigator, outcomes assessor)	(Weiss et al., 2013)
NCT00956891	Au BM	FBS	Liver failure	$\approx100\times10^6$ cells Single	Hepatic artery	-	Case Control, retrospective	(Peng et al., 2011)
NCT01144962	Allo BM	FBS	Crohn's Disease	$1/3/9 \times10^7$ cells Single	Local injection	I & II	Randomized, Safety/efficacy study, Parallel assignment, Double blind (Investigator, Outcomes assessor)	(Molendijk et al., 2015)
NCT01309061	Au AD	-	Romberg's Disease	1 x 107 cells Single	Intramuscular	II	Randomized, Safety/efficacy study, Single group assignment, Open label	(Koh et al., 2012)
NCT01297205	Allo UCB	FBS	Bronchopulmonary dysplasia	$1/2 \times 10^7$ cells/kg BW Single	Intratracheal	I	Safety/efficacy study, Single group assignment, Open label	(Chang et al., 2014)
NCT02395029	Allo PMD	-	Peyronie's Disease	-	Intracavernosal or intralesional	I	Safety/Efficacy study, Single group assignment, Open label	(Levy et al., 2015)
NCT00395200	Au BM	FBS	Multiple Sclerosis	$1\text{-}2\times10^6$ cells/ kg BW Single	Intravenous	I & II	Non-randomized, Safety/efficacy study, Single group assignment, Open label	(Connick et al., 2012; Connick et al., 2011)
NCT01297972	Allo BM	FBS	Aplastic anemia	1×10^6 cells/kg BW Multiple	Intravenous	I & II	Safety/Efficacy study, Single group assignment, Open label	(Cle et al., 2015)

Au- Autologous; Allo- Allogeneic; BM- Bone marrow; UCB- Umbilical cord blood; AD- Adipose derived; WJ- Wharton's jelly; PMD- Placental matrix-derived; FBS- Foetal bovine serum; HoS- Horse serum.

2.1.5 Drawbacks of mesenchymal stem cells when used as a therapeutic agent in clinical trials

For each regenerative therapy 50-400 million MSCs are required (Carlsson et al., 2015; Estrada et al., 2013; Levy et al., 2015; Mathiasen et al., 2013; Tan et al., 2012; Weiss et al., 2013). The presence of MSCs within the tissues is very low that makes it impractical to isolate such a large number of MSCs from a single donor. Thus, *ex vivo* expansion of MSCs prior transplantation is an inevitable option (Pittenger et al., 1999; Sun et al., 2008).

For clinical trials, MSCs are mostly expanded in xenogeneic serum supplemented media and the use of these MSCs (both autologous and allogeneic) for therapeutic purposes has been proven safe (Baron et al., 2010a; Connick et al., 2012; Connick et al., 2011; El-Kheir et al., 2013; Gupta et al., 2013; Hare et al., 2012; Hare et al., 2009; Lee et al., 2012b; Lee et al., 2013; Otsuru et al., 2012; Peng et al., 2011; Reinders et al., 2013; Tan et al., 2012). Clinical trials that utilises autologous MSCs for the treatment of multiple system atrophy, renal transplant rejection, multiple sclerosis, ischemic cardiomyopathy, spinal cord injury and liver failure have shown short term regenerative benefits or partial improvement of the patients' condition (Connick et al., 2012; Connick et al., 2011; El-Kheir et al., 2013; Hare et al., 2012; Lee et al., 2012b; Peng et al., 2011; Reinders et al., 2013; Tan et al., 2012). In addition, clinical trials based on allogeneic MSCs have also been shown significant increase in the overall survival of graft-versus-host disease patients; improved forced expiration volume and global symptom score, and reduced infarct size in cardiovascular disease patients; improved Ankle Brachial Pressure Index in critical limb ischemia patients; and increased osteopoetic cell engraftment in patient with osteogenesis imperfecta (Baron et al., 2010a; Gupta et al., 2013; Hare et al., 2012; Hare et al., 2009; Lee et al., 2013; Otsuru et al., 2012). However, none of these trials have been reported the long term benefits of

MSCs therapy. Several researchers have reported that low number of engrafted MSCs as a major drawback for long term functional benefits (Malliaras & Marban, 2011; Volarevic et al., 2011). Different strategies has been used in an attempt to minimize this drawback; such as intra-arterial delivery instead of intravenous delivery to avoid accumulation of MSCs in the lung (Guo et al., 2013; Lu et al., 2013b); and modification of cell surface molecules through chemical, genetic and coating techniques to promote selective adherence to particular organs or tissues (Kean et al., 2013). Several modifications in *ex vivo* or *in vitro* culture environment have also been given due attention to overcome the problem of inadequate engraftment of MSCs such as culturing MSCs in hypoxic environment for partial (Liu et al., 2012a) or entire (Saller et al., 2012) period of time; and culturing MSCs in medium that mimics the hypoxic condition (Hung et al., 2012). However, all these attempts overshadowed the role of media supplements on the engraftment of *ex vivo* expanded MSCs.

2.1.5.1 Aging of mesenchymal stem cells during *in vitro* or *ex vivo* expansion

In standard culture conditions, MSCs reach senescence after a limited number of cell division (Estrada et al., 2013). Cellular aging or replicative senescence affects proliferation and differentiation potentials of stem cells (Bonab et al., 2006; Nauta & Fibbe, 2007; Schellenberg et al., 2011; Wagner et al., 2008). Senescence can be triggered by gradual loss of telomere repeat sequences, DNA damage and de-repression of the *INK4/ARF* locus (Collado et al., 2007). Without any detectable telomere loss, oxidative stress-induced premature senescence may also take place in cultured cells (Ho et al., 2011; Kuilman et al., 2010).

Previous study has also shown that highly confluent MSCs (100%) aged faster than the cells passaged at lower confluency (60-70%) (Ho et al., 2011). During *in vitro* culture, MSCs obtained from highly dense population exhibited prolonged population

doubling time, higher expression of senescence associated β-galactosidase, and increased cell cycle arrest along with increased intracellular reactive oxygen species (ROS) (Ho et al., 2011). Studies have also reported that ambient culture environment causes higher ROS generation within cultured cells including MSCs compared to hypoxic culture environment (2-5%), and ROS is also responsible for faster telomere shortening and cellular senescence (Estrada et al., 2012; Estrada et al., 2013).

Cell morphology and proliferation are also considered as important indicators of cellular aging (Sethe et al., 2006). Proliferation and morphology of MSCs play vital role in the success of regenerative therapy. Better engraftment of rapidly proliferating MSCs (Lee et al., 2006) or MSCs from early passage (passage 1) (Jin et al., 2011) has been reported. Thus, during MSCs' *ex vivo* expansion, its high proliferation rate should be maintained.

Researchers also reported the correlation between the stemness and morphological differences of MSCs. On the basis of morphology and proliferation potential researchers divided MSCs into the four groups namely rapidly self-renewing (RS), flat spindle-shaped (FSS), spindle-shaped (SS), and flattened (FC) (Colter et al., 2001; Haasters et al., 2009; Prockop et al., 2001; Saller et al., 2012). RS cells are small, triangular or star shaped; FSS are triangular and elongated; SS cells are fibroblast like elongated and spindle shaped; FC cells are large, cuboidal and flattened with prominent nucleus (Haasters et al., 2009; Saller et al., 2012). Among these morphologically defined cell types, it has been reported that FC have the lowest self-renewable capacity (Colter et al., 2001), whereas the highest stemness was shown in RS cells (Haasters et al., 2009; Prockop et al., 2001).

Growth factors namely vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF)-BB, basic fibroblast growth factor (FGF-2), hepatocyte growth

factor (HGF) and epidermal growth factor (EGF) have been reported to increase proliferation of MSCs (Rodrigues et al., 2010). FGF-2 was found to maintain long telomeres without up-regulation of telomerase expression (Bianchi et al., 2003; Yanada et al., 2006). However, the possible effect of these growth factors in reducing differentiation potential and priming of MSCs should be taken into consideration when used in regenerative therapy (Handorf & Li, 2011; Mastri et al., 2014; Prasanna et al., 2010).

Evidences suggest that aging of MSCs in culture is inevitable. It might not be possible to stop the aging process completely, yet it can be delayed and reduced by using appropriate growth factors and manipulating the culture practice and environment. As the success of stem cell-based therapy depends on both the self-renewal and differentiation of the transplanted cells following engraftment (Limb & Daniels, 2008), it is essential to produce MSCs with higher proportion of RS cell population.

2.1.5.2 Effect of xenogeneic serum on engraftment of transplanted mesenchymal stem cells

Following transplantation, homing of cells to the targeted organ or site of inflammation and starting reproduction of cells is known as 'engraftment' (Chavakis et al., 2008). Engraftment of MSCs are commonly identified by using bioluminescence, GFP and chimerism assays (Baron et al., 2010b; Cle et al., 2015; Enoki et al., 2010). Site-specific trafficking and engraftment of transplanted MSCs are important in cell-based regenerative therapy. These events are assisted by the affinity of chemokine receptors on MSCs (CXCR4, CXCR7, CX3CR1) to the chemokines (stromal cell-derived factor 1, SDF-1; fractalkine) (Baek et al., 2011; Liu et al., 2012a; Ponte et al., 2007; Smith et al., 2012; Song et al., 2011; Wu & Zhao, 2012). Loss of these

chemokine receptors during their *in vitro* or *ex vivo* expansion (Honczarenko et al., 2006) is believed to affect the regenerative outcomes. Growth factors like PDGF-AB, PDGF-BB, insulin-like growth factor-1 (IGF-1), HGF, EGF and angiopoietin-1 (Ang-1) work as chemoattractants for MSCs (Fiedler et al., 2006; Forte et al., 2006; Phipps et al., 2012; Ponte et al., 2007; Tamama et al., 2006). Inflammatory cytokine such as tumour necrosis factor alpha (TNF-α) also helps migration of MSCs towards the site of chemokines (Baek et al., 2011; Ponte et al., 2007). All these paracrine signalling molecules are of primary importance for tissue specific migration and engraftment of MSCs. *In vivo* composition of these paracrine factors may vary depending on the type and stage of pathological conditions. Once isolated and expanded in *ex vivo* culture media, MSCs needed to embrace different cytokine composition, depending on the type of media supplements. In other words, media supplementation with xenogeneic serum and purified proteins or growth factors do not match the *in situ* cytokine composition in the serum of patients undergoing stem cell-based regenerative therapy. Therefore, paracrine signals to *ex vivo* expanded MSCs in those media supplement might cause improper trafficking consequently poor homing and engraftment.

2.1.5.3 Post-transplantation hyper-immunogenicity to mesenchymal stem cells cultured in xenogeneic serum

MSCs are able to prevent expression of co-stimulatory molecules such as CD40, CD80, CD83 and CD86 and induce expression of inhibitory molecules such as B7-H1, B7-H4 and human leukocyte antigen G (HLA-G). At the same time, MSCs were reported to secrete soluble factors such as prostaglandin E2 (PGE2), transforming growth factor (TGF)-β, interleukin 10 (IL-10), nitric oxide (NO), HGF and indolamine-2,3-dioxygenase (IDO). These properties render MSCs to inhibit proliferation and function of cytotoxic T cells (T_C), natural killer (NK) cells and B cells, as well as

prevent differentiation of monocytes into antigen-presenting dendritic cells (DCs). Notably, IDO plays an important role in activating immunosuppressive regulatory T cells (T_{regs}), facilitating differentiation of monocytes into M2 macrophages, and inhibit helper T cells (T_H) and T_C cells (Francois et al., 2012; Gebler et al., 2012; Plock et al., 2013). These immunomodulatory properties, makes MSCs a "universal donor" for stem cell-based regenerative therapy (Klyushnenkova et al., 2005).

In contrast, MSCs are described as immune evasive rather than immune privileged since differentiated MSCs or MSCs treated with interferon gamma (INF-γ) exhibit significantly higher expression of MHC class I and MHC class II. If mismatched, these MHC class I and MHC class II act as a source of hyper-immunogenicity thus the "universal donor" role of MSCs becomes questionable (Ankrum et al., 2014; Le Blanc et al., 2003). Besides, MSCs expanded in FBS supplemented media can be contaminated with bovine proteins that remains after multiple washings (Spees et al., 2004). MSCs contaminated with Neu5Gc xenoantigen (Heiskanen et al., 2006; Komoda et al., 2010) originating from FBS potentially cause immunological reaction after transplantation with anti-Neu5Gc antibodies present in human serum (Ghaderi et al., 2010; Zhu & Hurst, 2002). Binding of anti-Neu5Gc antibody present in the human serum to xenoantigen Neu5Gc may cause post-transplantation lyses of the MSCs (Figure 2.2). Antibody dependent lysis of MSCs may take place in two ways: (i) complement-dependent cytotoxicity (CDC) and (ii) NK cell-based antibody dependent cell-mediated cytotoxicity (ADCC).

CDC to MSCs regardless of their source (autologous or allogeneic) has been reported in both *in vivo* and *in vitro* studies (Komoda et al., 2010; Li & Lin, 2012). However, CDC was less in autologous MSCs and this effect was greatly reduced when CD55 was highly expressed by MSCs (Li & Lin, 2012). In contrast, MSCs that showed

expression of complement regulatory proteins such as CD46, CD55, and CD59 have been reported to be resistant to CDC (Komoda et al., 2010). The role of MSCs secreted factor H on inhibiting complement activation has also been reported (Tu et al., 2010). For cell-mediated cytotoxicity, higher phagocytic activity and ADCC was reported for the Neu5Gc-contaminated MSCs. In addition, reduced Neu5Gc contamination was reported to reduce cell-mediated phagocytosis and lysis of the MSCs expanded in human serum supplemented medium (Komoda et al., 2010). Thus, CDC and ADCC to xeno-contaminated MSCs may lead to acute rejection of transplanted cells (Heiskanen et al., 2006; Komoda et al., 2010; Padler-Karavani & Varki, 2011). Therefore, the effect of xenogeneic serum on poor engraftment of transplanted MSCs regardless of autologous or allogeneic source should not be disregarded. Moreover, FBS supplemented media are potential source of viral or bacterial infections (Dedrick, 1997) and prions transmission (Cobo et al., 2006).

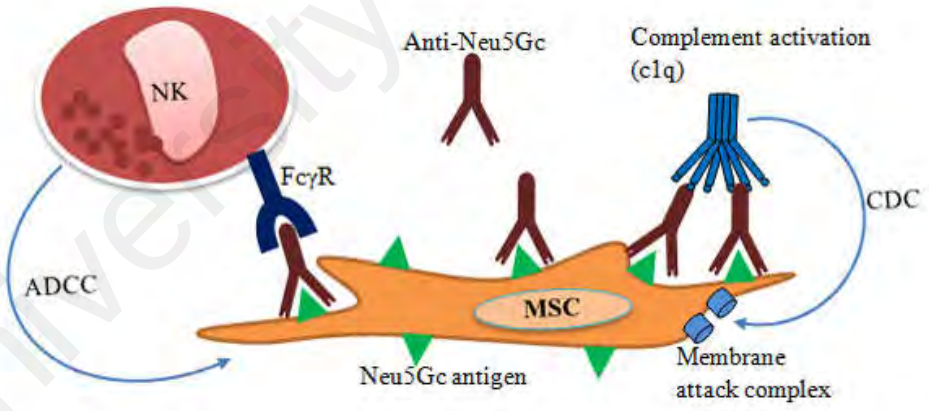

Figure 2.2: Immune response to transplanted xeno-contaminated MSCs. N-glycolylneuraminic acid (Neu5Gc) in FBS contaminates MSCs during *ex vivo* expansion. Anti-Neu5Gc antibody present in human serum may bind to the xeno-contaminated MSCs following transplantation. As a result, natural killer (NK) cells may bind to the antibody coated cells through Fc-gamma receptors (FcγR) and cause lysis by antibody dependent cell-mediated cytotoxicity (ADCC). Anti-Neu5Gc antibody may also activate complement-dependent cytotoxicity (CDC) and cause lysis through membrane attack complex (Haque et al., 2015)

2.1.6 Approaches to enhance engraftment and regenerative benefits of mesenchymal stem cells

In recent years, researchers have modified the culture media and environment (Figure 2.3) to improve engraftment efficiency of transplanted MSCs. Such modifications have shown partial improvement in the characteristics of MSCs. These modified culture techniques have both advantages and limitations in producing clinical grade MSCs with high engraftment potential.

Serum Supplement	FBS	FBS	Xeno-Free	Allogeneic	Platelet Lysate	Autologous
Oxygen Tension	19-21%	2-5%	19-21%	19-21%	19-21%	19-21%
Xeno-Contamination	√	√	×	×	×	×
Prion Contamination	√	√	×	×	×	×
Supports 1° MSC Expansion	√	√	?	√	√	√
Proliferation	↓↑	↑	↓↑	↑	↑	↑
Senescence	↑	↓	NA	?	↓	NA
Genetic Stability	↓	↑	NA	NA	↑	↑
Motility	↓	NA	NA	↑	NA	↑
Suitability for Transplantation	?	?	√	√	√	√

Figure 2.3: Effect of culture media supplement on *in vitro* or *ex vivo* expansion of MSCs, and their suitability for clinical applications. FBS, allogeneic serum (pooled AB-serum), platelet lysate and autologous serum supplemented media support isolation and expansion of MSCs. Presence of xenoantigen in FBS make its use controversial. Although xeno-free media do not support isolation, they support further expansion of MSCs isolated in any serum supplemented media. MSCs expanded in xeno-free media and media supplemented with platelet lysate, pooled allogeneic AB-serum or autologous serum are considered appropriate for regenerative therapy as they are free from any xeno-contamination. Abbreviations are: MSC, Mesenchymal stem cells; FBS, foetal bovine serum. [↓= decrease; ↑= increase; ↓↑= regular/unchanged; ×=absent; √ = present; ?= controversial; NA= data not available; (Haque et al., 2015)]

2.1.6.1 Expansion of mesenchymal stem cells in xeno-free media

Since the very beginning of the development of synthetic cell culture medium by Harry Eagle in 1955, researchers have been eyeing for suitable supplement to support cell viability and expansion. Animal serum especially FBS have been widely using to

supplement media, as it provides almost all the necessary nutrients needed for the survival and proliferation of cells in culture condition (Gstraunthaler, 2003; Whitford, 2005). However, the uncertainty over the composition and concentration of paracrine factors in FBS, possibility of disease transmission, and Neu5GC mediated hyper-immunogenicity (Gstraunthaler, 2003; Lindroos et al., 2009) are considered as drawbacks of FBS when used for isolation and expansion of stem cells for therapeutic purposes (Komoda et al., 2010; Schallmoser et al., 2007; Spees et al., 2004). Hence, xeno-free media or well-defined serum free media are being used as an alternative (Corotchi et al., 2013; Patrikoski et al., 2013; Simoes et al., 2013). Usually xeno-free media require different types of growth factors as supplement: recombinant human PDGF-BB, FGF-2 and transforming growth factor (TGF) -β1 (Chase et al., 2010). MSCs in both growth factors supplemented serum free media and FBS supplemented media showed similar growth kinetics and differentiation potential during *in vitro* expansion (Chase et al., 2010; Crapnell et al., 2013; Rashi, 2012). However, limitation of xeno-free medium in supporting primary culture or isolating MSCs have also been reported (Crapnell et al., 2013; Rashi, 2012). Indeed, after isolation of MSCs in any serum supplemented medium, they can be further expanded and differentiated in xeno-free media (Crapnell et al., 2013; Rashi, 2012). Moreover, xeno-free media does not offer solutions for early senescence, telomere shortening, and loss of chemokine receptors that are needed for site-specific migration, engraftment and long term regeneration benefits.

2.1.6.2 Human serum and platelet lysate for expansion of mesenchymal stem cells

In the search for a solution to the problems related to severe immunogenicity to xeno-contamination caused by FBS, and limited isolation and expansion of MSCs in serum free media, researchers have proposed human serum, plasma and/or platelet

lysate as possible alternatives (Aldahmash et al., 2011; Jonsdottir-Buch et al., 2013; Lin et al., 2005; Shahdadfar et al., 2005). The potential of autologous human serum in supporting *in vitro* isolation and expansion of MSCs has gained considerable attention in the last decade (Mizuno et al., 2006; Perez-Ilzarbe et al., 2009; Shahdadfar et al., 2005; Stute et al., 2004). Autologous human serum has been reported to have positive effect on the proliferation (Kobayashi et al., 2005; Mizuno et al., 2006) and differentiation potential of MSCs (Kobayashi et al., 2005; Shahdadfar et al., 2005; Stute et al., 2004). MSCs cultured in autologous human serum have shown more stable gene expressions (Dahl et al., 2008; Shahdadfar et al., 2005) and higher motility (Kobayashi et al., 2005) compared to MSCs cultured in FBS. Moreover, MSCs cultured in autologous serum has been reported to be more effective in immunomodulation as it significantly decreased the percentage of INF-γ producing activated T cells compared to MSC cultured in FBS (Perez-Ilzarbe et al., 2009). Nonetheless, collection of blood from the elderly, diseased and inflamed patients could be a limiting factor for serum preparation for the *ex vivo* expansion of MSCs prior to transplantation (Cooper et al., 2010; Jung et al., 2009; Stute et al., 2004).

In addition to the autologous serum, allogeneic human serum and human cord blood serum has also been considered as suitable alternative to FBS (Aldahmash et al., 2011; Bieback et al., 2012; Jung et al., 2009). However, it has been reported that allogeneic serum supplement used during *in vitro* expansion of MSCs could cause over expression of genes that are responsible for growth arrest and cell death (Shahdadfar et al., 2005). As opposed to that pooled allogeneic serum from adult AB-blood donors and pooled cord blood serum support isolation and expansion of MSCs while maintaining its differentiation potentials, motility and immunosuppressive property (Cooper et al., 2010; Kobayashi et al., 2005; Le Blanc et al., 2007; Phadnis et al., 2006; Poloni et al., 2009; Tateishi et al., 2008; Turnovcova et al., 2009). Lower level of hemagglutinin in

pooled cord blood serum compared to adult serum, and lack of A and B hemagglutinin in pooled allogeneic AB-serum was attributed to be behind this observation (Turnovcova et al., 2009). Platelet lysate was considered as an alternative to FBS because of its superiority in maintaining growth potential, genetic stability, immunomodulatory properties, and differentiation potential (Capelli et al., 2007; Crespo-Diaz et al., 2011; Govindasamy et al., 2011b; Griffiths et al., 2013; Jonsdottir-Buch et al., 2013; Perez-Ilzarbe et al., 2009; Trojahn Kolle et al., 2013; Vasanthan et al., 2014). However, to produce clinical grade MSCs, the utilization of human serum and platelets free of infectious agents are of vital importance to prevent any possibility of disease transmission.

2.1.6.3 Transient adaptation of expanded mesenchymal stem cells in autologous serum for transplantation

Despite the advantages of using platelet lysate or allogeneic serum for *ex vivo* expansion, the microenvironment of the culture media varies significantly compared to that of the patients' diseased organ. Hence, to render the *ex vivo* expanded MSCs accustomed with new microenvironment upon transplantation, incubation of the MSCs in well-defined or xeno-free media supplemented with freshly prepared autologous serum might be a worthwhile effort (Figure 2.4). Regeneration is a complex process and a large number of autocrine and paracrine signalling factors play a vital role in promoting this (Vunjak-Novakovic & Scadden, 2011; Wagers, 2012). Effect of paracrine factors on enhancing the chemotaxis and site-specific migration of MSCs have been reported (Fiedler et al., 2002; Forte et al., 2006; Ji et al., 2004; Ponte et al., 2007). Furthermore, enhanced site-specific migration potential has been shown in MSCs pre-incubated with inflammatory cytokine TNF-α (Baek et al., 2011; Ponte et al., 2007). In recent years, researchers have acknowledged that the regenerative properties

of microvesicles (also known as exosomes) have been overlooked for years (Aliotta et al., 2010; Ratajczak et al., 2012). Microvesicles are small (30-1000 nm) membranous vesicles released from the activated healthy cells or damaged cells during membrane blebbing (Aliotta et al., 2010; Ratajczak et al., 2006; Sabin & Kikyo, 2014; Yuan et al., 2009). Rozmyslowicz et al. reported the transfer of CXCR4 receptor from the surface of platelets or megakaryocytes to the surface of CD4+/CXCR4-null cells through microvesicles (Rozmyslowicz et al., 2003). Microvesicles are also able to transfer mRNA and miRNA from the cell of origin to the receiver cells (Aliotta et al., 2010; Biancone et al., 2012; Camussi et al., 2010; Mrvar-Brecko et al., 2010). Induced epigenetic changes following internalization of microvesicles by receiver cells have been recognized as a universal phenomenon (Aliotta et al., 2010; Deregibus et al., 2007; Lee et al., 2012c; Quesenberry et al., 2010; Ratajczak et al., 2006).

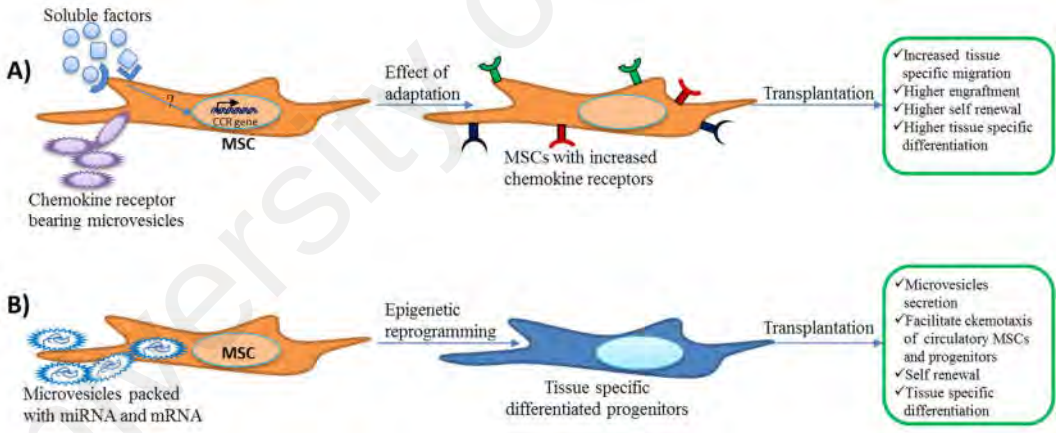

Figure 2.4: Possible effects of adaptation of expanded MSCs in autologous serum supplemented media on engraftment and regenerative efficiency. A) Cytokines and other soluble factors present in the freshly prepared autologous serum may increase chemokine receptor (CCR) expression on MSCs. Microvesicles present in the serum may deliver chemokine receptors that might enhance chemotactic properties of incubated MSCs. Expression of chemokine receptors may facilitate tissue specific migration and further regenerative benefits. B) In addition, mRNA or miRNA packed in microvesicles may be delivered to MSCs during incubation that could aid in tissue specific differentiation. Upon transplantation, these tissue specific differentiated cells may produce microvesicles similar to the cells within the injured tissues. This may help tissue specific migration of circulatory progenitors or MSCs and enhance regenerative outcomes (Haque et al., 2015)

Several human and animal studies reported the increase of inflammatory paracrine factors and microvesicles in blood circulation following stroke and ischemic heart disease (Drimal et al., 2008; Ferrarese et al., 1999; Intiso et al., 2004; Lionetti et al., 2010; Ratajczak et al., 2012; Ripa et al., 2007; Wang et al., 2006). If the expanded MSCs are meant for transplantation in such pathological conditions where inflammatory paracrine factors and circulatory microvesicles are increased, positive response of the transplanted cells to the host microenvironment is highly important for successful regenerative therapy.

Notably, chemokines and inflammatory cytokines in the patients' freshly prepared autologous serum have the potential to enhance migratory potential of MSCs by inducing the expression of chemokine markers during incubation (Baek et al., 2011; Ferrarese et al., 1999; Ponte et al., 2007; Wang et al., 2006). Meanwhile, microvesicles present in the patients' autologous serum could enhance MSCs' migratory properties by delivering chemokine markers and as well as potentially cause epigenetic changes of MSCs by transferring host mRNA or miRNA (Aliotta et al., 2010; Camussi et al., 2010; Lee et al., 2012c; Mrvar-Brecko et al., 2010; Quesenberry et al., 2010; Ratajczak et al., 2006; Sabin & Kikyo, 2014; Yuan et al., 2009). Expression of chemokine markers on MSCs, transiently incubated in autologous serum, may facilitate tissue specific migration and engraftment. At the same time, the tissue specific modified cell population may produce microvesicles similar to that of injured tissues and organs (Quesenberry et al., 2010) following engraftment. In turn, it might facilitate the migration and homing of circulatory MSCs and prevent apoptosis of cells in injured tissues or organs (Ratajczak et al., 2012). Since the number of circulatory MSCs and progenitor cells in circulation was found to be increased within 24 hours following stroke and myocardial infarction (Kucia et al., 2006; Paczkowska et al., 2009; Wang et

al., 2006), incubation of MSCs for similar time period, i.e., 24 hours, would be considered sufficient for the transient *ex vivo* adaptation of the expanded MSCs.

2.1.6.4 Hypoxic condition for genetic stability and stemness of mesenchymal stem cells

Tissues where MSCs reside in are hypoxic in nature (Eliasson & Jönsson, 2010; Harrison et al., 2002; Mohyeldin et al., 2010; Panchision, 2009). *In vitro* hypoxic culture conditions (2-5% oxygen) help MSCs to grow faster while maintaining homogeneity, differentiation potential, increased chemokine receptors expression and retard the cellular aging process as well (Basciano et al., 2011; Estrada et al., 2012; Estrada et al., 2013; Haque et al., 2013; Saller et al., 2012). Biosafety issue related to aneuploidy in expanded MSCs caused by oxidative stress (Estrada et al., 2013) can also be resolved by using hypoxic culture conditions (Haque et al., 2013). Hypoxia inducible factor (HIF) especially HIF-1 plays an important role in maintaining the regenerative potential at hypoxic environment.

The HIF-1β subunit of a heterodimeric transcription factor HIF-1 (HIF-1α and HIF-1β) (Fong, 2008; Wang et al., 1995) is non-responsive to oxygen, whereas HIF-1α is an oxygen labile protein. Therefore, under ambient condition the HIF-1α subunit is usually synthesized and degraded rapidly, whereas under hypoxic conditions their break down is delayed (Fong, 2008; Weidemann & Johnson, 2008). Degradation of HIF-1α under ambient culture condition (Figure 2.5) is regulated by HIF-1 prolyl-hydroxylases (HPH) (Jaakkola et al., 2001). HIF-1 prolyl-hydroxylases (HPH) in presence of oxygen, iron and α-ketoglutarate; hydroxylate the proline residues 402 and 564 of the oxygen-dependent degradation domain (ODD) of HIF1α (Hon et al., 2002; Metzen et al., 2003), which in turn induce a conformational change of HIFα, thus allowing von Hippel Lindau protein (VHL) to bind with it (Pasarica et al., 2009). Consequently, VHL binds

to a complex that serves as E3 ubiquitin ligase (E3UL), and ubiquitinylate HIF-1α for degradation in proteasome (Ivan et al., 2001; Maxwell et al., 1999; Sharp & Bernaudin, 2004).

In contrast, under hypoxic conditions the prolyl-hydroxylation process is suppressed due to lack of oxygen that allows HIF-1α accumulation and nuclear translocation to occur (Weidemann & Johnson, 2008). After nuclear translocation it binds with HIF-1β to form the heterodimer. Then the HIF-1 heterodimer binds to a hypoxia-response element (HRE) in the target genes, associated with co-activators such as CBP/p300 and regulates the transcription (Figure 2.5) of as many as 70 genes involved in metabolism, angiogenesis, invasion/metastasis and cell fate (Brahimi-Horn & Pouyssegur, 2007).

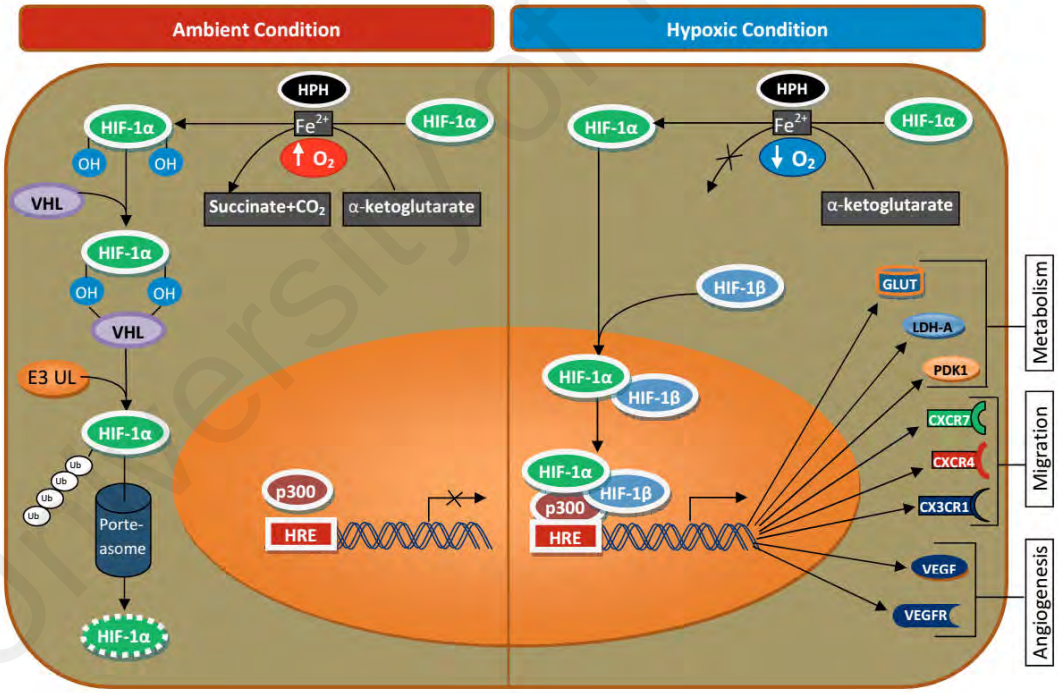

Figure 2.5: Regulation of transcription by HIF-1 during ambient and hypoxic condition. HIF, hypoxia-inducible factor; HPH, HIF-1 prolyl-hydroxylases; VHL, von Hippel Lindau; E3UL, E3 ubiquitin ligase; HRE, hypoxia-response element; GLUT, Glucose transporter, LDH, Lactate dehydrogenase; PDK, pyruvate dehydrogenase kinase (Haque et al. 2013)

Besides, through Notch signalling, HIF-1α regulate the expression of genes (e.g. HES and HEY) that maintain proliferation of cells (Haque et al., 2013). To provide MSCs natural niche like oxygen concentration isolation, expansion and adaptation of MSCs should be done in hypoxic (2-5% oxygen) conditions. This culture environment will facilitate proliferation, site-specific migration, and prevent early aging of MSCs. Moreover, hypoxic culture environment may increase bio-safety by reducing aneuploidy (Estrada et al., 2013).

2.2 Regenerative potential of supernatants or secretomes

Proteins secreted by cell, tissue or organism under certain condition or at a particular time is expressed as 'secretome' (Hathout, 2007). Usually the secretomes help to maintain cell growth, cell differentiation, apoptosis, immune defence and tumorigenesis (Hathout, 2007; Zwickl et al., 2005). Paracrine factors present in the secretomes may also help to inhibit apoptosis of cells in the damaged organs, induce proliferation of progenitor or stem cells, and induce neovascularization to supply nutrient to the affected tissue (Ratajczak et al., 2012). The properties of paracrine factors in the secretomes has generated interest amongst researchers to study its regenerative potential and introduced cell culture supernatants or secretomes as new therapeutic tool of regenerative medicine (Newman et al., 2013; Ratajczak et al., 2012; Stoddart et al., 2015).

2.2.1 Supernatant or secretome from adult stem cells

Stem cells mobilized into the circulation from their physiological niche following activation and secrete several paracrine factors needed to maintain homeostasis within the body (Ratajczak et al., 2012). Hence, just after removal or isolation of adult stem cells from their natural niche for *in vitro* or *ex vivo* expansion they became activated, causing the secretion of different types of paracrine factors in the culture media (Ratajczak et al., 2012). To date, secretomes from several types of adult stem cells

namely skeletal muscle myoblasts (SMMs), neural stem cells (NSCs), cardiac stem cells (CSCs), and MSCs from different sources have been studied and their regenerative potential reported (An et al., 2015; Newman et al., 2013; Park et al., 2010; Perez-Ilzarbe et al., 2008; Pires et al., 2014; Salgado et al., 2015; Stastna et al., 2009; Stastna et al., 2010).

2.2.2 Cell culture supernatant or secretome from peripheral blood mononuclear cells

Like other adult stem cells secretomes, regenerative potential of the secretome of PBMCs have also been reported recently (Altmann et al., 2014; Hoetzenecker et al., 2012; Hoetzenecker et al., 2013; Lichtenauer et al., 2011; Mildner et al., 2013). In these studies, researchers analysed the regenerative potential of secretomes from healthy and apoptotic PBMCs. Hoetzenecker et al., (2013) and Mildner et al., (2013) reported the potential of secretomes from healthy PBMCs to treat autoimmune myocarditis and skin ulcer respectively (Hoetzenecker et al., 2013; Mildner et al., 2013). The potential of secretomes from apoptotic PBMCs to treat myocardial infarction and stroke have also been reported (Altmann et al., 2014; Hoetzenecker et al., 2012; Lichtenauer et al., 2011).

2.2.3 Limitations of secretome production from peripheral blood mononuclear cells

Till now PBMCs are cultured in FBS supplemented medium or serum free medium supplemented with purified proteins or growth factors for the production of secretomes (Altmann et al., 2014; Hoetzenecker et al., 2012; Hoetzenecker et al., 2013; Lichtenauer et al., 2011; Mildner et al., 2013). As FBS is contaminated with xenoantigen (Heiskanen et al., 2007; Komoda et al., 2010) its suitability for the production of clinical grade

secretomes is controversial. Serum free medium supplemented with growth factors are not suitable for the production of secretome from PBMCs, as the growth factors regulate the production of cytokines by modulating the autocrine and paracrine pathway (Weijers et al., 2011).

On the basis of the above literature we proposed an ideal approach to produce potentially clinical grade MCSs that could be divided into two steps. Firstly, isolation of MSCs in FBS supplemented media followed by expansion in pHS supplemented media in hypoxic conditions. Secondly, transient adaptation of them in the recipients' own sera (AuHS) prior to transplantation might provide additional regenerative benefits. To address the issue of suitability of media supplementation, we also propose the use of AuHS for the production of secretome from PBMCs. As cytokines gradient play an important role in the regeneration process, combine use of MSCs and secretome therapy might prove more effective in the process of regeneration (Figure 2.6).

Figure 2.6: Proposed therapeutic strategy to improve the regeneration process using MSCs and secretome. (MSCs, mesenchymal stem cells; FBS, foetal bovine serum; PBMCs, peripheral blood mononuclear cells; AuHS, autologous human serum)

CHAPTER 3: ISOLATION AND CHARACTERIZATION OF STEM CELLS FROM HUMAN EXTRACTED DECIDUOUS TEETH (SHED)

3.1 Introduction

Mesenchymal stem cells (MSC) are considered to be a potentially good tool for regenerative medicine. MSCs can be isolated from several tissues within the body such as bone marrow, adipose tissue and umbilical cord (Erices et al., 2000; Lund et al., 2009; Shi & Gronthos, 2003). Presence of stem cells in the dental pulp was firstly reported by Gronthos et al. (Gronthos et al., 2000). In 2003, Shi and Gronthos reported stem cells from the perivascular region of the dental pulp as MSCs (Shi & Gronthos, 2003). Afterwards, several *in vitro* studies have shown the self-renewal and multipotent differentiative capability of dental pulp derived stem cells (Arthur et al., 2008; d'Aquino et al., 2007; Govindasamy et al., 2011a; Kadar et al., 2009; Vasanthan et al., 2014; Zhang et al., 2006).

Dental pulp stem cells from the permanent teeth has been referred to as DPSC, and stem cells from the human exfoliated deciduous teeth as SHED (Miura et al., 2003; Suchanek et al., 2010; Wang et al., 2012a). In this study, SHED is used as a source of MSCs as it has large donor pool and poses no risk to the donors. However, the deciduous teeth used in this study were not exfoliated but those that has been indicated for extraction. Higher proliferation and differentiation potential of SHED compared to DPSC has been reported (Govindasamy et al., 2010; Wang et al., 2012a). Studies also reported that age has an effect on the stemness of MSCs (Alt et al., 2012; Asumda, 2013; Chen et al., 2016). As the SHEDs were collected from very young donors it may provide cells with better regenerative properties.

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 of MSCs (Dominici et al., 2006). Thus, the main aim of this study is to isolate and characterise SHED from primary source. To achieve the aim, the specific objectives of this study are as follows:

a) To isolate SHED from human extracted deciduous dental pulp tissue.
b) To characterise and confirm the MSCs like properties of SHED.
c) To determine the growth kinetics of SHED.

3.2 Materials and Methods

3.2.1 Ethics approval for the collection of human extracted deciduous teeth

Sample collection procedure for the current research was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Reference #DF CO1107/0066[L]). All the samples were obtained following informed written consent from the guardian of the young donors.

3.2.2 Media preparation

Different types of media and buffers are required for stem cell isolation, culture and cryopreservation. Composition and procedure to prepare those media are described further.

3.2.2.1 Preparation of transportation medium

Transportation medium is required to transport the extirpated dental pulp. Transportation medium was prepared by using the KnockOut^{TM} DMEM medium (Gibco, Grand Island, NY, USA) supplemented with 20% (v/v) FBS (Gibco), 10 mM Glutamax (Gibco), and 200 units/ml of Penicilline-Streptomycin (Gibco). The complete transportation medium was sterilized by filtration through a 0.2 μm membrane filter (Thermo Scientific, Mexico) and stored at 4°C for further use.

3.2.2.2 Preparation of washing buffer

Washing buffer was prepared by mixing equal volume of DPBS (Gibco) and Penicillin-Streptomycin (Gibco). Prepared buffer was sterilized by filtration through a 0.2 μm membrane filter (Thermo Scientific). Always the freshly prepared washing buffer was used.

3.2.2.3 Preparation of cell culture medium

SHED culture medium was prepared by using KnockOut^{TM} DMEM (Gibco) supplemented with 10% (v/v) FBS (Gibco), 2 mM Glutamax (Gibco), and 50 units/ml Penicillin-Streptomycin (Gibco). The complete medium was sterilized by filtration through a 0.2 μm membrane filter (Thermo Scientific) and stored at 4°C for further use.

3.2.2.4 Preparation of solution for tissue digestion

Tissue digestion solution is needed to digest the tissue to isolate stem cells. In this study 0.1% collagenase type I (Gibco) solution was used. This solution was prepared by dissolving 100 mg collagenase type I (Gibco) in 99 ml DPBS without Ca^{2+} and Mg^{2+} (Gibco) and 100 units/ml Penicillin-Streptomycin (Gibco). Prepared solution was sterilized by filtration through a 0.2 μm membrane filter (Thermo Scientific) and stored at 4^0C for further use.

3.2.2.5 Preparation of freezing medium

Freezing medium is needed to cryopreserve the stem cells. Freezing medium was prepared by mixing 90% FBS (Gibco) and 10% DMSO (Sigma-Aldrich, Steinheim, Germany). Freezing medium was sterilized by filtration through a 0.2 μm membrane filter (Thermo Scientific). Always the freshly prepared freezing medium was used to cryopreserve the samples for further use.

3.2.3 Isolation and expansion of stem cells from human extracted deciduous teeth

Extracted clinically sound intact deciduous molars were collected from healthy children (n=3; ages 5-9 years) who were undergoing a planned serial extraction for management of occlusion at the Department of Paediatric Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya. SHED were isolated according to the established procedure described before (Aziz et al., 2015; Govindasamy et al., 2010).

In brief, following extraction, the root surfaces of teeth were cleaned with povidone-iodine (Sigma Aldrich, St Louis, MO, USA) and the teeth were then placed into sterile solution prior to sectioning. Teeth were sectioned at the cement-enamel junction (CEJ) using a diamond rotary disc and the dental pulp was extirpated with an endodontic broach and transferred into the transportation medium (Figure 3.1). Following three times washing with washing buffer, the dental pulp was minced by using sterile scalpel followed by enzymatic digestion in tissue digestion solution for 40 minutes at 37°C. After that Collagenase was neutralized using complete culture medium and was centrifuged at 1250 rpm for 6 minutes. Following centrifugation, the pellet was resuspended in complete culture medium and seeded in T25 flasks (BD Bioscience, Franklin Lakes, NJ, USA). Flasks were left at 37°C and 5% CO_2 in humidified chambers.

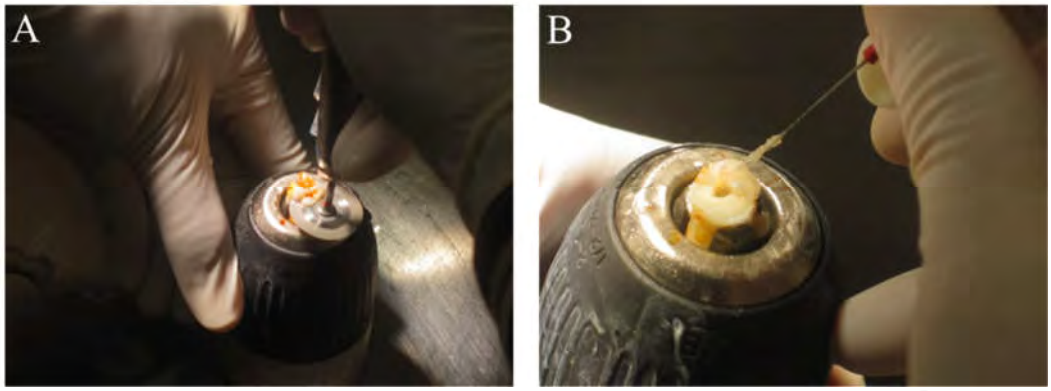

Figure 3.1: Isolation of dental pulp. A) sectioning of tooth by using diamond rotary disc, B) extirpation of dental pulp with an endodontic broach

3.2.4 Identification of MSC like properties of stem cells from human extracted deciduous teeth

According to the International Society for Cellular Therapy (ISCT) MSC should have adherence to plastic, specific surface antigen (Ag) expression, and multipotent differentiation potential (Dominici et al., 2006).

3.2.4.1 Plastic adherence of stem cells from human extracted deciduous teeth

Plastic adherence of SHED was confirmed by viewing the culture flask under inverted microscope (Primo Vert, Carl Zeiss, Jena, Germany).

3.2.4.2 Expression of specific surface antigen on stem cells from human extracted deciduous teeth

Expression of specific surface antigen on SHED (at passage 3) was ascertained by staining cells with human MSC phenotyping kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and using MACSQuant® Analyser 10 flow cytometer (Miltenyi Biotec).

In brief, after reaching 70% confluency SHED were dissociated using TrypLE^{TM} express (Gibco). Number of nucleated cells were counted using trypan blue (Gibco) and aliquoted (1×10^6 cells each) into seven pre-labelled (i.e. 1, 2, PerCP, PE, APC, FITC

and blank) in microcentrifuge tubes. Cells were centrifuged at 300×g for 10 minutes and supernatants were aspirated completely. Cells in the tube labelled 'blank' were resuspended into 500 µl of buffer (2% FBS containing DPBS) and kept in a container containing ice. Cells in the other six tubes were resuspended into 100 µl buffer. After that, 10 µl of the MSC phenotyping cocktail (anti-human: CD14-PerCP, CD20-PerCP, CD34-PerCP, CD45-PerCP, CD73-APC, CD90-FITC, CD105-PE), 10 µl of the isotype control cocktail (anti-human: mouse IgG1-FITC, mouse IgG1-PE, mouse IgG1-APC, mouse IgG1-PerCP, mouse IgG2a-PerCP), 10 µl of anti-human CD73-Biotin, 10 µl of anti-human CD105-PE, 10 µl of anti-human CD73-APC, and 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 4 °C. Cells were washed by adding 1 ml of buffer and centrifuging at 300×g for 10 minutes. Supernatants were aspirated completely. Except for the tube labelled PerCP, cell pellet in all other tubes were resuspended into 500 µl of buffer and kept in a container filled with ice. 10 µl of anti-Biotin-PerCP was added to the tube labelled PerCP and cells were mixed gently. Following incubation for 10 minutes in the dark at 4 °C, cells were washed by adding 1 ml of buffer and centrifuging at 300×g for 10 minutes. Supernatant was aspirated completely and cells were resuspended in 500 µl buffer. Tubes labelled 1 and 2 were used to analyse the expression of specific antigen on SHED and other tubes were used to compensate instrument.

3.2.4.3 Multipotent differentiation capacity of stem cells from human extracted deciduous teeth

Multipotent (adipogenic, chondrogenic and osteogenic) differentiation capacity was confirmed by inducing the differentiation of SHED in StemPro® Adipogenesis Differentiation Kit (Gibco), StemPro® Osteogenesis Differentiation Kit (Gibco), and

StemPro® Chondrogenesis Differentiation Kit (Gibco). At 70-80% confluency, cells were stimulated with complete adiopogenesis/osteogenesis/chondrogenesis differentiation medium (StemPro® Adipocyte/Osteocyte/Chondrocyte Differentiation Basal Medium 90 ml, StemPro® Adipocyte/Osteocyte/Chondrocyte Supplement 10 ml, Penicillin-Streptomycin 50 units/ml). For adipogenic and chondrogenic differentiation, cells were maintained in the differentiation medium until day 14, and for osteogenic differentiation cells were maintained until day 21. At every three days interval, cells were fed with fresh differentiation medium.

For adipogenic differentiation analysis, on day 14 cells were fixed with 10% formaldehyde (Sigma) for 30-40 minutes. Then formaldehyde was aspirated completely, and cells were washed with DPBS (Gibco) and ddH_2O twice each. Then the cells were stained with 0.18% (w/v) Oil Red O (Sigma) solution for 50 minutes.

Chondrogenic differentiation was analysed on day 14. First, cells were fixed with 10% formaldehyde (Sigma) for 10 minutes. Then formaldehyde was aspirated completely, and cells were washed twice with DPBS (Gibco). Finally, the cells were stained with 0.1% (w/v) Safranin O (Sigma) solution.

In order to show osteogenic differentiation, on day 21 cells were fixed with 10% formaldehyde (Sigma) for 30-40 minutes. Then formaldehyde was aspirated completely, and cells were washed twice with DPBS (Gibco). Then the cells were stained with 2% (w/v) Alizarin Red S (Sigma) solution. For all differentiation assays, SHED cultured in complete proliferation medium were used as the negative control.

3.2.5 Cell proliferation and doubling time

Cell proliferation assays of freshly isolated and cryopreserved (1.5-2 years) SHED were performed in the appropriate complete media as described above. Cells (n=3) at

70-80% confluence were dissociated using TrypLE™ express (Gibco) and live cells were counted by using trypan blue (Gibco) dye exclusion method. Then, SHED were plated at a density of 1000 cells/cm^2 into new 25 cm^2 tissue culture flasks and incubated at 37°C and 5% CO_2 in humidified chambers. Their confluence was determined under an inverted microscope (Primo Vert, Carl Zeiss). Three separate, independent replications of each experiment were performed to determine the population doubling time (PDT) for each cell culture until passage 5. Data were analysed and plotted using Microsoft Excel. PDT in hours was measured using the following formula:

$$T2 = \frac{t}{\log_2 (\frac{N}{N_0} + 1)}$$

where N_0 is the number of cells at the beginning of observation, N is the increase in the number of cells during the period of time of the length t, and T2 is the doubling time (Korzyńska & Zychowicz, 2008).

Population doubling (PD) at each passage was calculated from the cell count by using the following equation:

$$X = \frac{\log_{10} (N_H) - \log_{10} (N_I)}{\log_{10} (2)}$$

where X = population doublings, N_I = inoculum number, and N_H = cell harvest number. To obtain the cumulative population doubling (CPD), the PD increase at current passage was added to the PD of previous passages (Cristofalo et al., 1998; Li et al., 2015).

Cumulative cell number was calculated by multiplying the initial seeding cell number of 100,000 cells at passage 1 by the fold expansion at each passage till passage 5 (Dolley-Sonneville et al., 2013).

3.3 Results

3.3.1 Isolation of stem cells from human extracted deciduous teeth

Between day 3 to day 5 of incubation, growth of cells (n=3) was observed under an inverted microscope (Primo Vert, Carl Zeiss). Typically, on day 14 the first subculture could be established.

3.3.2 Mesenchymal stem cells like characteristics of stem cells from human extracted deciduous teeth

Homogenous monolayer of adherent and spindle-shaped SHED of different passages (Figure 3.2 A-C) were observed under an inverted microscope (Primo Vert, Carl Zeiss) thus confirming its plastic adhering capability. Flow cytometry data showed that 95% of the cultured SHED expressed MSC positive markers (CD73, CD90, CD105) and 3% of the cultured SHED expressed MSC negative markers (CD14, CD20, CD34, CD45) (Figure 3.2 D). In the presence of induced differentiation kit (Gibco) SHED were able to be differentiated into adipocytes, chondrocytes and osteocytes indicating their multipotent differentiation capacity (Figure 3.2 E-G).

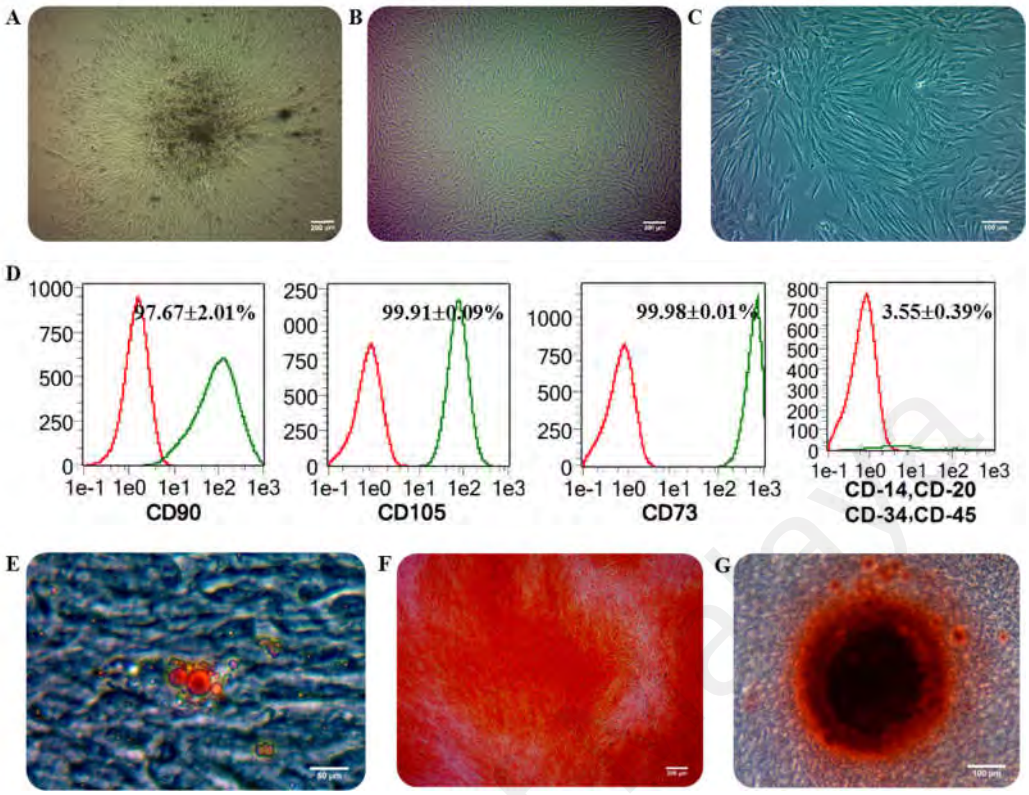

Figure 3.2: Identification of SHED. (A-C) Plastic adherence of SHED. (A) SHEDs growing from primary dental pulp tissue on day 7. (B) Confluent homogenous monolayer of spindle shaped SHED, passage 3. (C) Spindle shaped SHED after 72 hours of incubation, passage 5. (D) Expression of specific surface antigen on SHED. (E-G) Tri-lineage differentiation of SHED. SHEDs were cultured in chondrogenic and adipogenic differentiation medium for 14 days, and in osteogenic differentiation medium for 21 days. (E) On day 14 adipogenic differentiation was checked by staining with 'Oil red O'. (F) On day 14 chondrogenic differentiation was checked by staining the cells with 'Safranin-O'. (G) On day 21 osteogenic differentiation was checked by staining with 'Alizarin Red'. Photomicrographs (A-C, E-G) were taken using inverted microscope (Primo Vert, Carl Zeiss). Scale bars: A,B,F- 200 µm; C,G- 100 µm; E-50 µm

3.3.3 Growth kinetics of stem cells from human extracted deciduous teeth

Population doubling time of freshly isolated samples (n=3, P1-P5) and cryopreserved samples (n=3, P2-P5) were calculated. No significant difference between the population doubling time of fresh samples and cryopreserved samples was detected (Figure 3.3 A). Cumulative population doubling (Figure 3.3 B) and cumulative cell number (Figure 3.3 C) of freshly isolated samples until passage 5 (day 58) were computed as well. This

result indicates that by passage 2 (day 34) it is possible for SHED to yield adequate number (about 1.5×10^{10}) of cells for transplantation.

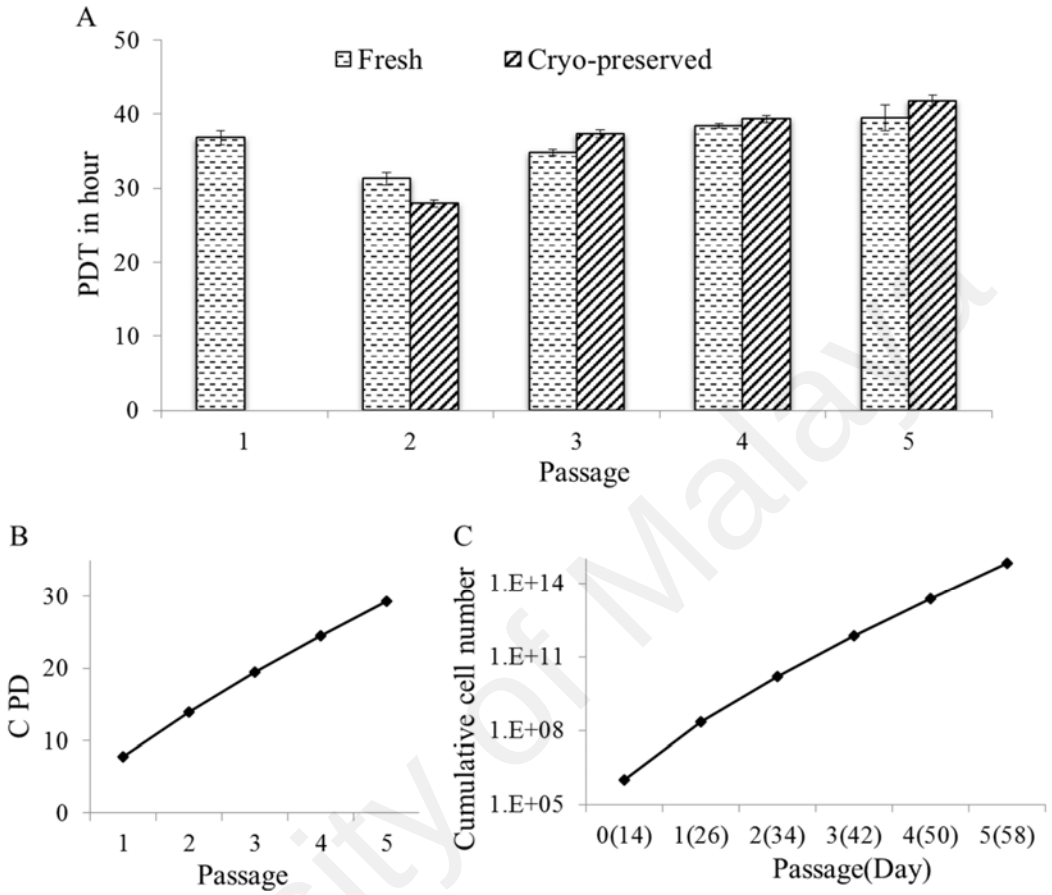

Figure 3.3: Growth kinetics of SHED. (A) Population doubling time (PDT) of freshly isolated sample (n=3, passage 1 to passage 5) and cryopreserved samples (n=3, passage2 to passage5). (B) Cumulative population doubling (CPD) and (C) Cumulative cell number of SHED untill passage 5

3.4 Discussion

Basic characterization confirmed MSCs like properties of SHED that has been isolated from primary dental pulp tissues (Figure 3.2). Although SHED showed tri-lineage (osteogenic, chondrogenic and adipogenic) differentiation potential, their differentiation towards adipocyte was poor. This result concurred with previous studies (Eslaminejad et al., 2015; Gronthos et al., 2000; Koyama et al., 2009). Reciprocal relationship between adipogenic and osteogenic differentiation was found in MSCs

including SHED (Cheng et al., 2003; Ichida et al., 2004; Koyama et al., 2009). In MSCs, higher expression of osteogenic differentiation inducing genes, namely, Msh homeobox 1 (MSX1), Msh homeobox 2 (MSX2), distal-less homeobox 3 (Dlx3) and distal-less homeobox 5 (Dlx5) reported to have adipogenic differentiation suppression capability (Cheng et al., 2003; Fujii et al., 2015; Goto et al., 2016; Ichida et al., 2004; Lee et al., 2012a). Hence, comparatively higher osteogenic differentiation potential might be the cause behind weak adipogenic differentiation of SHED. However, further studies are needed for confirmation.

The population doubling time (31-41 hours) of freshly isolated SHED (P1-P5) were found to be competitive (Figure 3.3 A) compared to MSCs isolated for from other sources namely, bone marrow (40-70 hours), adipose tissue (≈45 hours), umbilical cord (≈24 hours), Wharton's jelly (≈40 hours), and placenta (≈55 hours) (Li et al., 2014; Lu et al., 2006). The short (1.5-2 years) cryopreservation time of SHED showed no significant difference in population doubling time (27-42 hours) thus indicating the suitability of cryopreserved SHED for future therapeutic uses (Figure 3.3 A). Besides, the cumulative population doubling (Figure 3.3 B) and cumulative cell number (Figure 3.3 C) indicated that it is possible to get adequate cells for transplantation by passage 2 or on day 34 post-isolation.

3.5 Conclusion

SHED has large donor pool and can be isolated easily. It shares the characteristics of MSCs and have similar/better growth kinetics compared to MSCs obtained from other sources. Moreover, SHED could help to minimize age related loss of stemness as it is isolated from young children. Hence, SHED could be considered as a potential source of MSCs for cell-based regenerative therapy.

CHAPTER 4: THE REGENERATIVE POTENTIAL OF *IN VITRO* EXPANDED STEM CELLS FROM HUMAN EXTRACTED DECIDUOUS TEETH IN POOLED HUMAN SERUM

4.1 Introduction

In vitro expansion of mesenchymal stem cells (MSCs) is often critical to yield adequate number of cells (200-400 million) needed for transplantation particularly in regenerative therapy (Haque et al., 2015; Pittenger et al., 1999). During *in vitro* expansion, MSC are likely to exhibit early cellular senescence (Bonab et al., 2006; Estrada et al., 2012) as well as lose chemokine receptors (Honczarenko et al., 2006). Both these limitations have been identified as the major causes for poor engraftment of MSCs upon transplantation (Haque et al., 2015). Rapidly proliferating MSCs maintain their stemness (Saller et al., 2012) and are more efficient for engraftment (Lee et al., 2006). Again, site-specific migration and homing of transplanted MSCs are also vital for the successful engraftment for regenerative therapy (Eggenhofer et al., 2014).

Proliferation potential and cellular morphology can indicate status of cellular aging. On the basis of morphology, MSCs are can be grouped into rapidly self-renewing (RS), flat spindle-shaped (FSS), spindle-shaped (SS), and flattened (FC) cells (Colter et al., 2001; Haasters et al., 2009; Saller et al., 2012). Among these four groups RS cells have the highest regenerative potential, whereas FC cells have the lowest (Colter et al., 2001; Haasters et al., 2009; Saller et al., 2012). The use of *in vitro* expanded MSCs with a higher number of RSs having highest self-renewal potential and stemness (Haasters et al., 2009; Lee et al., 2006) and lower number of FCs having the lowest self-renewable capacity (Colter et al., 2001) might be more efficient in engraftment.

A number of growth factors namely vascular endothelial growth factor A (VEGF-A), platelet derived growth factor (PDGF) BB, fibroblast growth factor-2 (FGF-2),

hepatocyte growth factor (HGF), and epidermal growth factor (EGF) can boost MSC proliferation (Rodrigues et al., 2010). However, use of these growth factors during *in vitro* expansion may cause priming and influence differentiation potential of MSCs (Handorf & Li, 2011; Prasanna et al., 2010). MSCs response to the chemokines through receptors which regulate site-specific migration (Liu et al., 2012a; Ponte et al., 2007; Song et al., 2011). During *in vitro* expansion, MSCs lose their chemokine receptors (Honczarenko et al., 2006; Wynn et al., 2004) thus affecting the regenerative outcomes as they lose their site-specific migration potential.

Human serum (HS) contains a cocktail of paracrine factors. However, their composition may vary depending on the physiological status of the donor. It has been reported that pooled human serum (pHS) from adult AB-blood donors and pooled cord blood serum could maintain the differentiation potential, motility and immunosuppressive properties of MSCs (Kobayashi et al., 2005; Phadnis et al., 2006; Poloni et al., 2009; Tateishi et al., 2008). However, similar studies on pHS using blood irrespective of the donors' blood group are yet to be conducted. In addition, the composition of the paracrine factors of xenogeneic serum such as foetal bovine serum (FBS) also vary from that of HS. Hence, it is expected that MSCs might respond differently when cultured in pHS compared to the FBS supplemented media. Therefore, in this study we aimed to compare the effect of pHS and FBS on the proliferation, morphology and migration of stem cells from human extracted deciduous teeth (SHED) *in vitro*. Indeed, SHED have been investigated as a suitable source of MSCs for potential regenerative therapy because of their accessibility, less invasive techniques required to isolate them, pose no risk to the donor, and have competitive self-renewal capability (Govindasamy et al., 2011a; Wang et al., 2012a). Specific objectives of this study are as follows:

a) To determine the growth kinetics and metabolic activity of SHED when expanded in either FBS or pHS supplemented medium *in vitro*.
b) To determine the morphology of SHED when expanded in either FBS or pHS supplemented medium *in vitro*.
c) To determine the migration pattern of SHED when expanded in either FBS or pHS supplemented medium *in vitro*.

4.2 Materials and Methods

4.2.1 Ethics approval for the collection of human extracted deciduous teeth and blood

Sample collection procedures for the current research was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Reference #DF RD1301/0012[L], for blood collection; DFCO1107/0066[L], for teeth collection). All the samples were obtained following informed written consent from the donors or their guardian.

4.2.2 Preparation of pooled human serum

Blood was collected without any anticoagulant from healthy male donors (n=6) aged 21-35 years (Table 4.1). Exclusion criteria includes: smoking, alcohol consumption, drug and/narcotics addiction, inflammatory diseases either chronic or diagnosed within past 4 weeks of blood collection, major surgical treatment in the last 1 year, and immunotherapy. 20 ml of blood from each donor was coagulated separately in room temperature in sterile 50 ml centrifuge tube (BD Bioscience). Coagulated blood was centrifuged at 400×g for 15 minutes, and the crude serum was transferred into another sterile 50 ml centrifuge tube (BD Bioscience) for a second round of centrifugation at 1800×g for 15 minutes to remove cell debris or insoluble particles. The final serum

supernatants were heat treated at 57±2°C for 30 minutes as well to prepare complement inactivated human serum (HS) (Figure 4.1). Individual heat treated sera (n=6) was pooled to prepare pHS. Following sterilization by filtration through a 0.2 µm membrane filter (Thermo Scientific) pHS was stored at -20^{0}C for further experiments.

Table 4.1: Demographic profile of the blood donors (n=6)

Sl no.	Blood group	Age (year)	Height (cm)	Weight (Kg)
1	A (+)ve	32	164	76
2	B (+)ve	29	175	91
3	B (+)ve	25	174	80
4	O (+)ve	28	162	79
5	B (+)ve	30	167	68
6	O (+)ve	28	163	69

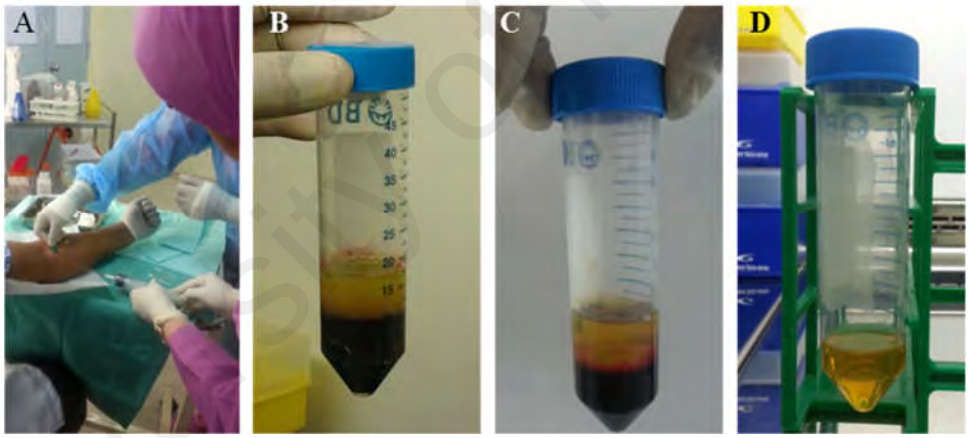

Figure 4.1: Serum preparation from whole blood. A) collection of whole blood in syringe, B) separation of serum and blood cells (before centrifugation), C) crude serum on top of the coagulated blood (after centrifugation at 400g), D) processed serum that used to prepare pooled human serum (pHS)

4.2.3 *In vitro* maintenance of stem cells from human extracted deciduous teeth

Initial isolation and expansion of SHED (n=3) were done in 10% FBS supplemented KnockOut™ DMEM (Gibco) until passage 3. Subsequent cultures (from passage 4-7) were maintained in KnockOut™ DMEM (Gibco) supplemented with either 10% FBS or 10% pHS. To minimize xeno-contamination animal derived component free TrypLE™ express (Gibco) was used as cell dissociation reagent.

4.2.4 Effect of foetal bovine serum and pooled human serum on proliferation of stem cells from human extracted deciduous teeth

Live cell counts of SHED (n=3) from passage 5 to passage 7 were taken in consideration to determine the effect of pHS and FBS supplemented media on proliferation. Cells were seeded in 6-well plates at a density of 3000cells/cm^2. After reaching 70-80% confluency cells were dissociated with TrypLE^{TM} express (Gibco) and number of live cells was determined using trypan blue (Gibco) dye exclusion method.

Population doubling time (PDT) in hours was derived using the following formula:

$$T2 = \frac{t}{\log_2 (\frac{N}{N_0} + 1)}$$

where, N_0 is the number of cells at the beginning of observation, N is the increase in the number of cells during the period of time of the length t, and T2 is the doubling time (Korzyńska & Zychowicz, 2008).

Population doubling (PD) at each passage (passage 5-7) was calculated from the cell count by using the following equation:

$$X = \frac{\log_{10} (N_H) - \log_{10} (N_I)}{\log_{10} (2)}$$

where X = population doublings, N_I = inoculum number, and N_H = cell harvest number. To get the cumulative population doubling (CPD), the PD increase at current passage was added to the PD of previous passages (Cristofalo et al., 1998; Li et al., 2015).

Cumulative cell number was calculated by multiplying the initial seeding cell number of 100,000 cells at passage 5 by the fold expansion at each passage till passage 7 (Dolley-Sonneville et al., 2013).

4.2.5 Effect of foetal bovine serum and pooled human serum on metabolic activity of stem cells from human extracted deciduous teeth

SHED (n=3) yielded at passage 5 were seeded in 96-well plates containing FBS and pHS supplemented medium at a density between 1×10^5 cells/well to 6225 cells/well with two dilution factor. After 24 hours of incubation at 37 °C in 95% humidified air and 5% CO_2, the effect of these media on the metabolic activity of SHED was evaluated using PrestoBlue® cell viability reagent (Invitrogen, Fredrick, MD, USA). At 24 hours of incubation all the wells were washed twice with DPBS (Gibco). 100 µl of KnockOut™ DMEM medium (Gibco) along with 10% PrestoBlue® (Invitrogen) reagent v/v was added to each well and the plates were further incubated for 2 hours. Absorbance was measured at 570 nm with reference wavelength set at 600 nm by microplate reader (Infinite 200 PRO, Tecan, Switzerland). The absorbance values were converted to the corrected absorbance of PrestoBlue® reagent. PrestoBlue® (Invitrogen) is a resazurin based dye. In live cells, non-fluorescent resazurin is reduced to strong fluorescent resorufin by accepting electrons from NADPH, FADH, FMNH, NADH and cytochromes. Thus, the greater the corrected absorbance of PrestoBlue® (Invitrogen) reagent, the higher the number of metabolically active live cells.

4.2.6 Effect of foetal bovine serum and pooled human serum on the morphology of stem cells from human extracted deciduous teeth

Photomicrographs of SHED (n=3) at passage 5 on day 2 were taken using an Inverted microscope (Primo Vert, Carl Zeiss) and ImageJ were used for the morphological study. In order to minimize the error and bias, all the cells present in one photomicrograph were counted. Clumped cells or cells without clear edge were excluded. After manually marking the surroundings of each cell's area, length and maximum Ferret's diameter were measured using ImageJ software. The distance

between two parallel lines tangent on either side of the object is known as Feret's diameter (Haasters et al., 2009). Aspect ratio of each cell was determined by dividing Feret's diameter by the length of the cell. Data was visualized using dot plot graph, showing area vs. aspect ratio.

4.2.7 Effect of foetal bovine serum and pooled human serum on migration of stem cells from human extracted deciduous teeth

SHED (n=3) yielded at passage 5 were seeded in 24-well plates containing FBS and pHS supplemented medium and incubated at 37 °C in 95% humidified air, 5% CO_2 until reached 70-80% confluence. Upon reaching confluency a scratch was made in each well using a 200µl pipette tip. Photomicrographs of the scratches were taken from beginning to 48 hours at every 12 hours interval under an inverted microscope (CKX41, Olympus, Centre Valley, USA). The average gap width of three different points along the scratch was measured. Both gap width and the surface area of the gap were computed using Infinity Analyse (Lumenera Corporation, Ottawa, ON, Canada). Closure of the gap width and surface area presented as the percentile of closure of the gap or area at 0 hour ±SD.

4.2.8 Cytokine and growth factors analysis of human serum

Luminex-based ProcartaPlex human cytokine/chemokine 10plex immunoassay kit from e-Bioscience (affymetrix, e-Bioscience, Vienna, Austria) was used to analyse the presence of 10 selected paracrine factors in FBS and each HS (n=6) used to prepare pHS (Table 4.2). These paracrine factors were selected on the basis of their involvement in regulating proliferation, migration and stemness of MSCs (Ahn et al., 2009; Ball et al., 2007; Bianchi et al., 2003; Fierro et al., 2007; Forte et al., 2006; He et al., 2010; Hu et al., 2013; Jiang et al., 2002; Kawada et al., 2004; Kolf et al., 2007; Krausgrill et al.,

2009; Lennartsson & Rönnstrand, 2012; Metcalf, 2003; Murakami et al., 2013; Pan et al., 2013; Pons et al., 2008; Rojas et al., 2005; Salcedo et al., 1999; Shi et al., 2006; Son et al., 2006; Sulpice et al., 2009; Tamama et al., 2006; Tamama et al., 2010; Yanada et al., 2006; Yu et al., 2015).

Table 4.2: List of selected 10 paracrine factors that were analysed using ProcartaPlex human cytokine/chemokine 10plex immunoassay kit

Name of the paracrine factor GenBank ID	**Function (References)**
Epidermal growth factor (EGF) [NM_001178130]	Regulates proliferation of MSCs isolated from different origins while maintaining their regenerative potential (Hu et al., 2013; Tamama et al., 2006; Tamama et al., 2010).
Fibroblast growth factor 2 (FGF-2) NM_002006	Stimulates the *in vitro* expansion of human BM-MSCs by activation of JNK signalling (Ahn et al., 2009). Slows down the aging process of MSCs by decreasing the gradual loss of telomere sequences (Bianchi et al., 2003; Yanada et al., 2006). Cytoprotective role of FGFs have also been acknowledged by researchers (Werner & Grose, 2003).
Granulocyte colony stimulating factor (G-CSF) NM_000759	Promotes cellular proliferation and migration, and prevents apoptosis (Murakami et al., 2013). Mobilizes HSC and MSCs from bone marrow (Kawada et al., 2004). Improves chemotactic property of MSCs *in vitro* (Murakami et al., 2013).
Granulocyte macrophage colony stimulating factor (GM-CSF) NM_000758	Acts as chemoattractant and induces mobilization of progenitors in the circulation (Rojas et al., 2005).
Hepatocyte growth factor (HGF) NM_001010932	Promotes proliferation, and survival of various cell types (Forte et al., 2006). Induces migration and site-specific homing of various cell types including MSCs from different origins (Son et al., 2006).
Leukaemia inhibitory factor (LIF) NM_002309	Helps to maintain self-renewal and multi-differentiation potential of various stem cells including MSCs (Kolf et al., 2007; Metcalf, 2003).
Platelet derived growth factor BB (PDGF-BB) NM_033016	Induces both expansion and migration of MSCs (Fierro et al., 2007; Tamama et al., 2006). Helps survival of MSCs as well (Krausgrill et al., 2009).
Stem cell factor (SCF) NM_000899	Regulates the migration, differentiation and proliferation of several cell types (Lennartsson & Rönnstrand, 2012). Induces the migration and homing of MSCs (Pan et al., 2013).
Stromal cell-derived factor-1α (SDF-1α) NM_199168	help site-specific migration and homing of MSCs through its receptor CXCR4 (He et al., 2010; Yu et al., 2015).
Vascular endothelial growth factor (VEGF) NM_00117162	Increases proliferation and survival MSCs (Pons et al., 2008).

4.2.9 Molecular network analysis

The corresponding Entrez Gene IDs of the 10 paracrine factors (Table 4.2) were imported into the core analysis tool of Ingenuity Pathway Analysis (IPA) (Ingenuity systems; www.ingeniuty.com) to identify their role in regulating biological functions and pathways. When $p<0.05$, the association was considered significant.

4.2.10 Data analysis

Data were analysed using SPSS version 22. The significant level was set at $p<0.05$.

4.3 Results

4.3.1 Proliferation of stem cells from human extracted deciduous teeth

Expansion of SHED (n=3; from passage 5 to passage 7) in pHS supplemented medium showed significanly higher proliferation ($p<0.05$) compared to FBS supplemented medium (Figure 4.2 A, B). Cumulative cell number showed that 1×10^6 viable SHED seeded at passage 4 could yield approximately 200×10^6 viable SHED at passage 7 in pHS supplemented medium. Meanwhile, FBS supplemented medium could only yield 65×10^6. The cumulative number of viable SHED from passage 5 to passage 7 in the pHS supplemented medium found to be significantly higher compared to that in FBS supplemented medium ($p<0.01$) (Figure 4.2C).

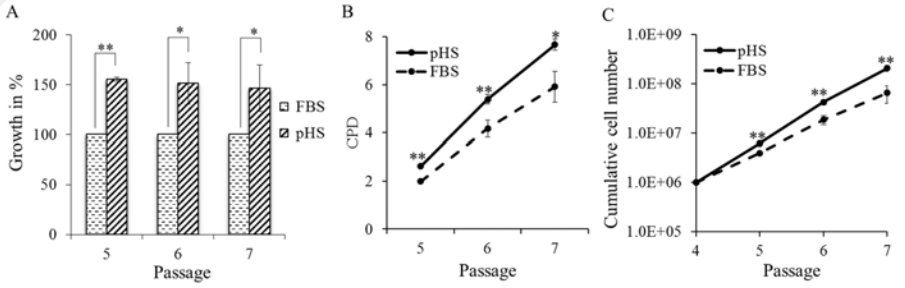

Figure 4.2: Effect of FBS and pHS on the proliferation of SHED. (A) Comparative yield of SHED in FBS and pHS supplemented media (B) Cumulative population doubling (CPD) of SHED cultured in FBS and pHS supplemented media. (C) Cumulative cell number of SHED cultured in FBS and pHS supplemented media. (n=3, **= $p<0.01$, *=$p<0.05$)

4.3.2 Metabolic activity of stem cells from human extracted deciduous teeth

While the seeding density was equal or above 50000 cell/per well of 96-well plates, no significant difference was observed in the metabolic activity of SHED (n=3) at 24 hours of incubation when maintained in FBS and pHS supplemented media. However, the metabolic activity of the SHED maintained in pHS supplemented medium was significantly higher than that of the cells maintained in FBS supplemented medium when SHED were seeded at lower density (≤25000 cells/well of 96-well plate) (Figure 4.3).

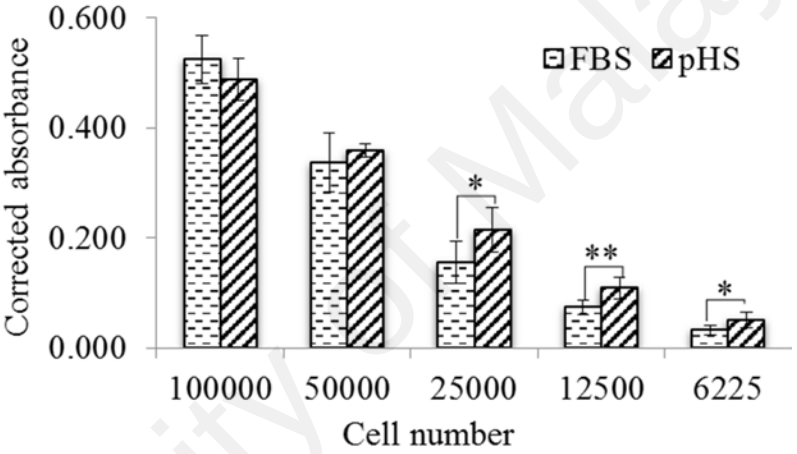

Figure 4.3: Effect of FBS and pHS on the metabolic activity of SHED at 24 hours of incubation. SHED expanded in pHS supplemented medium have shown significantly higher metabolic activity over the cells maintained in FBS supplemented medium while seeding cell density was equal or below 25000 cells/well of 96-well plate. No significant difference was seen between the SHED maintained in FBS and pHS supplemented media while seeding density was equal or higher than 50000 cells/well of 96-well plate. (n=3, **= $p<0.01$, *=$p<0.05$)

4.3.3 Morphology of stem cells from human extracted deciduous teeth

A total of 337 SHED (116, 116, and 105 SHED from Sample 1, Sample 2 and Sample 3 respectively) in FBS media and 343 SHED (144, 99, 100 SHED from Sample 1, Sample 2 and Sample 3 respectively) in pHS media were included for the morphological analysis. On the basis of the morphology SHED (n=3) were categorized into four subpopulations: RS, FSS, SS, and FC (Figure 4.4A-D). Number of FCs was significantly lower in pHS supplemented media (3%) compared to that in FBS (7%)

media ($p<0.05$). However, no significant difference was observed between the proportion of RS cells in FBS (64%) and pHS (74%) media (Figure 4.4E).

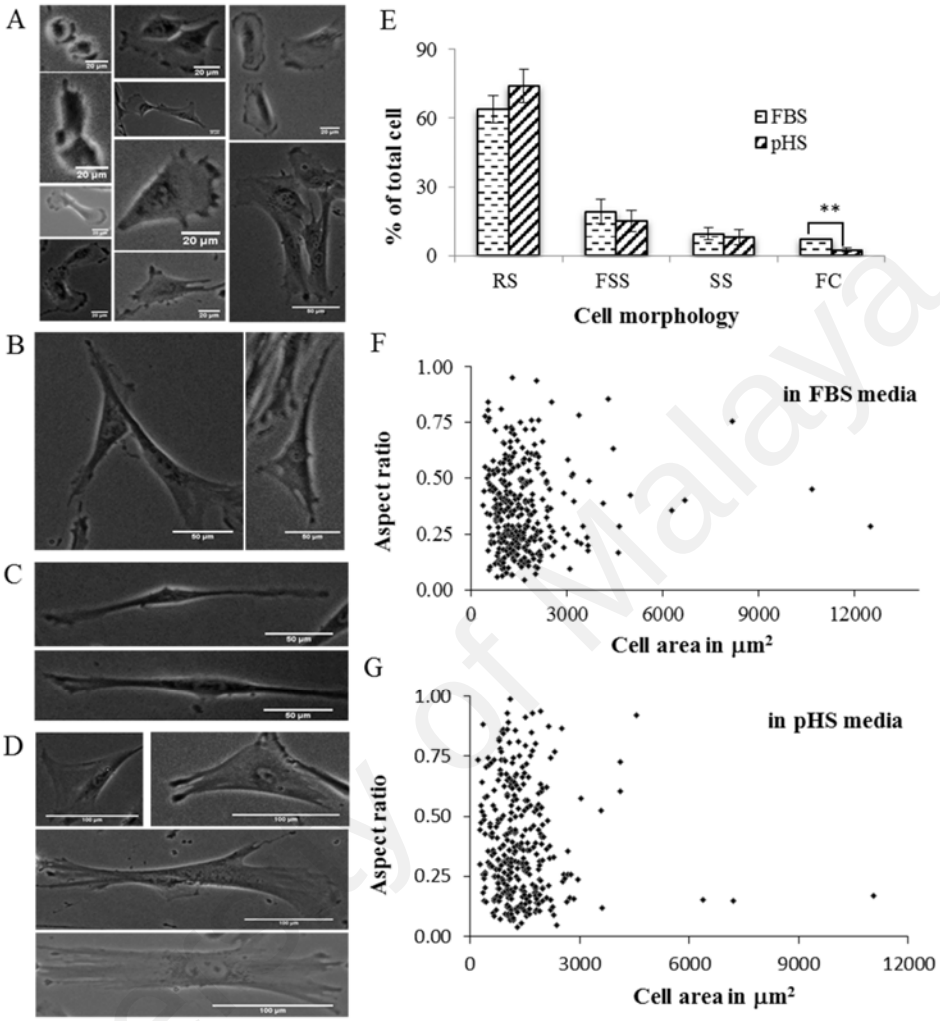

Figure 4.4: Effect of FBS and pHS on the size and morphology of SHED. (A) RS cells subpopulation of SHED with different morphology, (B) Triangular spindle shaped morphology of FSS cells, (C) elongated and spindle shaped morphology of SS cells, (D) large and flattened morphology of FC cells. (E) Difference between the subpopulation of SHED cultured in FBS and pHS media. (F) Dot plot graph shows the morphometric results of SHED in FBS medium as area vs. aspect ratio. (G) Dot plot graph shows the morphometric results of SHED in pHS medium as area vs. aspect retio. (n=3; **= $p<0.01$, *=$p<0.05$; photomicrographs A-D were taken using inverted microscope, Primo Vert, Carl Zeiss). Scale bars: A- 20 µm; B, C- 50 µm; D-100 µm

Cell surface area and aspect ratio of SHED (n=3) cultured in FBS and pHS media were measured as well and data presented in dot plots (Figure 4.4F,G). Surface area of FC subpopulations were in the range of 3018-12499 μm^2. Surface areas of the other

three subpopulations were recorded in the range of, RS: 202-2899 μm^2, SS: 566-2721 μm^2, and FSS: 807-2939 μm^2. In the FBS medium, the number of FC was significantly higher while the number of RS was markedly lower compared to those in pHS medium, surface area(±SE) per cell was significantly higher (p=0.023) in FBS medium (1639±63.66 μm^2) compared to cells in pHS medium (1354±51.69 μm^2).

4.3.4 Migration of stem cells from human extracted deciduous teeth

The width and surface area of the gap was obtained from the scratch assay to determine the effect of FBS and pHS on the migration of SHED (n=3). No significant difference in the closure of gap width and surface area was observed between the SHED cultured either in FBS and pHS supplemented media (Figure 4.5 A, B). However, migration of cells in pHS medium appeared to be more directional, whereas in FBS supplemented medium migration was disorganized and not as structured (Figure 4.5 C).

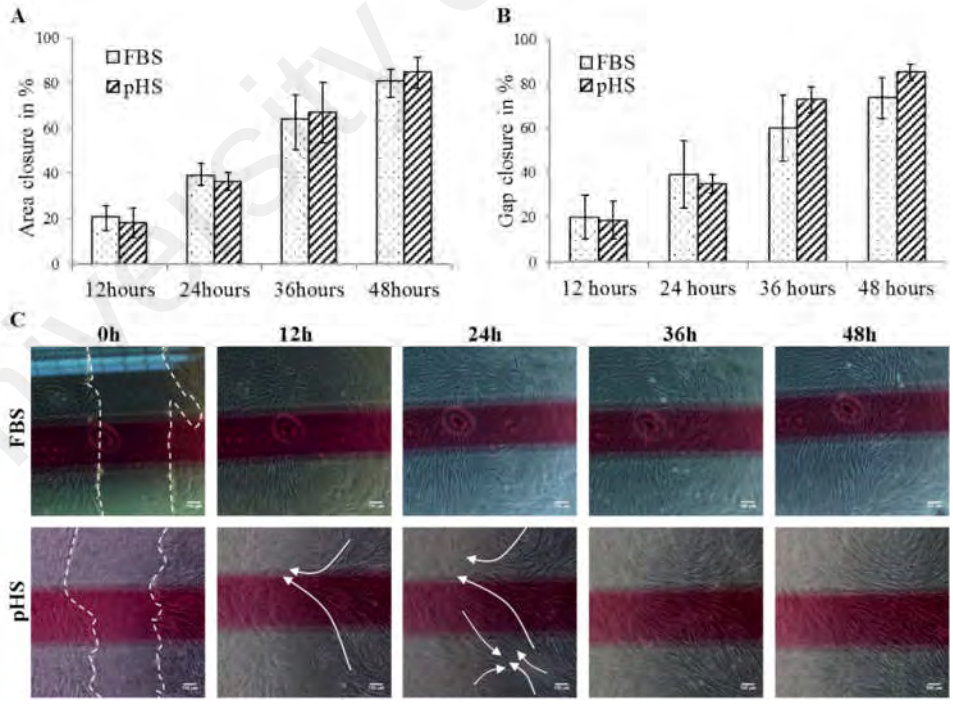

Figure 4.5: Migration of SHED cultured in FBS and pHS supplemented media. (A) Closure of area gap by SHEDs cultured in FBS and pHS supplemented media. (B) Closure of width by SHEDs cultured in FBS and pHS supplemented media. (C) Representative figures of scratch assay. (n=3, Photomicrographs C were taken using inverted microscope, Olympus). Scale bars: C- 100 µm

4.3.5 Presence of selected paracrine factors

Presence of selected 10 paracrine factors and growth factors was detected (Table 4.3) in the HS (n=6) that were used to prepare pHS. None of these paracrine factors were detected in FBS.

Table 4.3: Amount of selected paracrine factors in human sera (n=6)

Names	Amounts in pg/ml							
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Range	Average
EGF	55.7	20.9	14.0	40.6	81.1	32.0	14.0-81.1	40.7
FGF-2	0.0	0.0	0.0	0.0	56.8	0.0	0.0-56.8	9.5
G-CSF	39.7	0.0	44.2	0.0	241.1	50.0	0.0-241.1	62.5
GM-CSF	11.5	0.0	18.0	136.4	4.7	21.2	0.0-136	32.0
HGF	42.2	135.2	64.6	44.2	254.4	19.4	19.4-254.4	93.4
LIF	0.0	0.0	0.0	1.2	9.3	0.0	0.0-9.3	1.7
PDGF-BB	507.1	265.1	167.2	284.5	127.8	107.0	107.0-507.1	243.1
SCF	18.0	0.0	11.7	0.0	80.4	3.5	0.0-80.4	18.9
SDF-1A	227.9	140.6	153.7	91.9	195.3	0.0	0.0-227.9	134.9
VEGF-A	464.7	43.6	93.0	102.5	726.3	162.1	43.6-726.3	265.4

4.3.6 Biological functions regulated by the analysed paracrine factors

Based on the corresponding Entrez Gene IDs of the 10 analysed paracrine factors and growth factors (Table 4.2), IPA predicted their involvement in maintaining biological functions and pathways. Involvement of the analysed paracrine factors in regulating biological functions related to the proliferation, viability, migration and morphology of cells shown in Figure 4.6.

Rank	Functions	p-Value	Paracrine factors involved									
			G-CSF	GM-CSF	EGF	FGF-2	HGF	LIF	PDGF-BB	SCF	SDF-1A	VEGF-A
1	chemotaxis of cells	7.88E-16	-	+	+	+	+	+	+	+	+	+
2	quantity of cells	1.91E-15	+	+	+	+	+	+	-	+	+	+
3	synthesis of DNA	2.46E-14	-	+	+	+	+	+	-	+	+	+
4	expansion of cells	3.49E-13	-	+	+	+	-	+	-	+	+	-
5	cell cycle progression	7.57E-13	+	+	+	+	+	+	-	+	+	+
6	proliferation of stem cells	6.10E-12	-	-	+	+	-	+	+	-	+	-
7	cell movement	1.19E-11	+	+	+	+	+	+	+	+	+	+
8	morphology of cells	1.52E-11	-	-	+	+	+	+	+	+	+	+
9	apoptosis	1.67E-10	+	+	+	+	+	+	+	+	+	+
10	cell viability	1.84E-10	+	+	+	+	+	+	-	+	+	+

Figure 4.6: Involvement of the analysed 10 paracrine factors in regulating the biological function related to cell proliferation, viability, migration and morphology. Biological functions were predicted by Ingenuity Pathway Analysis (Ingenuity systems; www.ingeniuty.com; +, involved; -, not involved)

IPA also predicted the pathways that could be regulated by the analysed paracrine factors. Form those pathways, it has been found that 'Ephrin Receptor Signalling Pathway' might have the influence on the higher proliferation and more directional migration of SHED cultured in pHS medium (Figure 4.7).

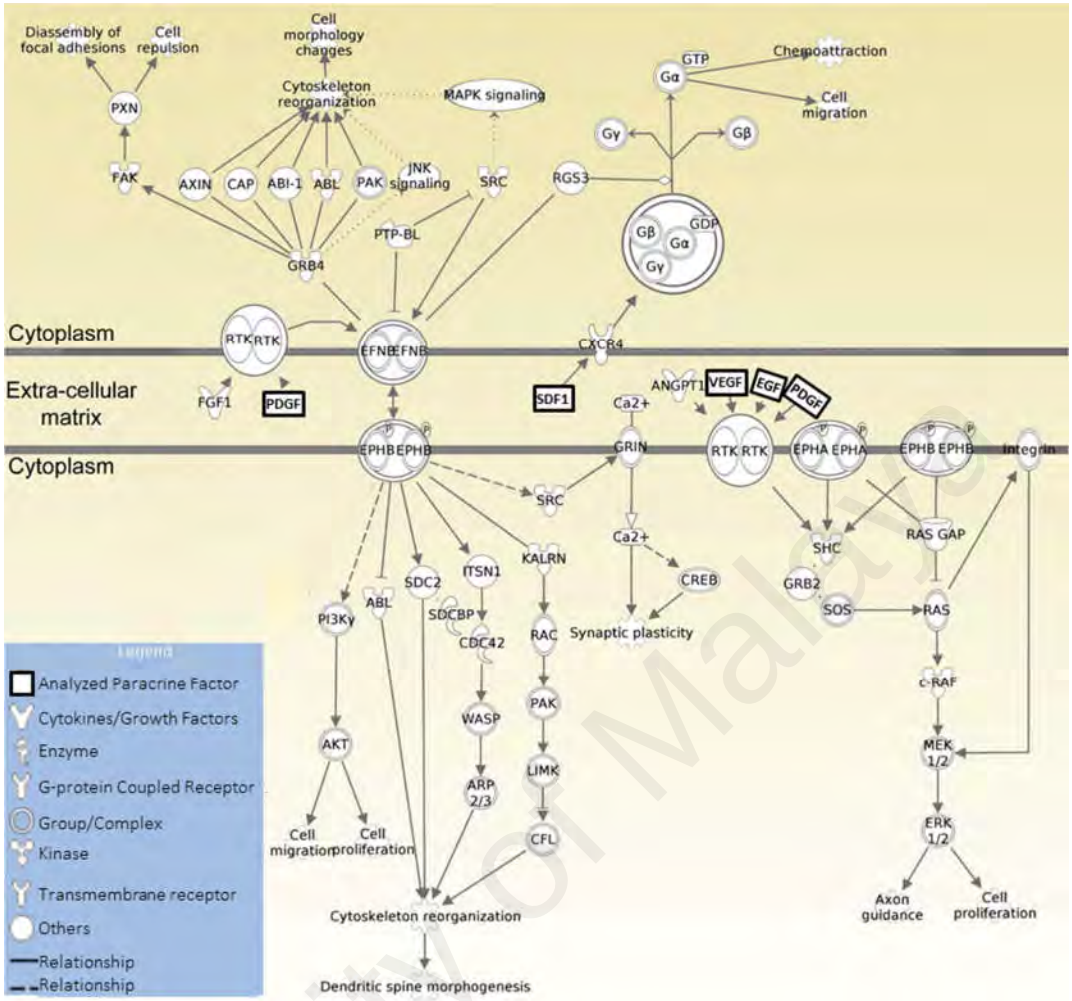

Figure 4.7: Ephrin Receptor signalling Pathway. Paracrine factors analysed in the current study (EGF, PDGF-BB, SDF-1A and VEGF, shown in black rectangle) which were present in the pHS were predicted to regulate the proliferation, viability, migration and morphology of SHED through Ephrin Receptor signalling Pathway (Ingenuity systems; www.ingeniuty.com)

4.4 Discussion

In regenerative therapy, a significantly higher number of transplanted stem cells is critical as the initial isolated number is far less than that is required (Haque et al., 2015; Pittenger et al., 1999). Hence, an *in vitro* expansion of the isolated target stem cells prior to transplantation (Haque et al., 2015; Pittenger et al., 1999) comprises the initial step in regenerative therapy. Routinely, FBS supplemented media are being used for such *in vitro* expansion. However, a number of factors such as early cellular senescence, and loss of migratory potential are common during the expansion of the stem cells

(Estrada et al., 2012; Honczarenko et al., 2006). Platelet lysate and ABO blood group specific serum supplement has been used as an alternative to the FBS supplement during *in vitro* expansion (Kobayashi et al., 2005; Phadnis et al., 2006; Poloni et al., 2009; Tateishi et al., 2008). The reported results have been shown to be encouraging; however, due to blood group constrain, it narrows down the number of donors. As a better alternative, we proposed pHS irrespective of the donors' blood group be used as serum supplement during *in vitro* expansion of stem cells namely, SHED.

Several techniques such as hypoxic culture conditions (1-2% oxygen), and purified cytokines or growth factor cocktail supplement have been attempted (Bianchi et al., 2003; Estrada et al., 2012; Saller et al., 2012) to minimize aging and loss of migratory properties of MSCs during *in vitro* expansion that in turn could compromise its regenerative potential (Bonab et al., 2006). Hypoxic culture conditions provide better support for proliferation yet the final outcome could be compromised due to the presence of xenoantigen in FBS supplemented media (Haque et al., 2015; Heiskanen et al., 2006; Komoda et al., 2010). It has also been reported that paracrine factors instead of FBS resulted in priming of MSCs in culture that might regulate the stemness and regenerative potential (Handorf & Li, 2011; Prasanna et al., 2010).

To address the issue of aging and inefficient migration during engraftment, we compared between pHS and FBS. As expected, significantly higher proliferation of SHED was observed with pHS supplement compared to FBS supplement (Figure 4.2). When cells were seeded at a density of ≤25000 cells/well of a 96-well plate, significantly higher number of viable cells was observed in pHS supplemented media compared to cells in FBS supplemented media after 24 hours of incubation. However, no significant difference was observed when the seeding density was ≥50000 cells/well of 96-well plate (Figure 4.3). It could be attributed to the confluency of SHED at this

density. Notably, proliferation and morphology of MSCs are considered as the marker of aging (Colter et al., 2001; Haasters et al., 2009; Saller et al., 2012; Sethe et al., 2006).

Among the four different MSCs subpopulations, FC and RS subpopulations showed the lowest and highest self-renewal capability respectively (Docheva et al., 2008; Prockop et al., 2001). Previous studies reported that FC cell subpopulations from bone marrow (BM) derived MSCs showed loss of differentiation potential as they were differentiated only to osteogenic progenitors or entered early phase of senescence (Colter et al., 2001; Matsuoka et al., 2013). According to Saller et a., (2012), in FBS supplemented media, BM derived MSCs produced significantly higher number of RS cells in hypoxic condition (77%) compared to normoxic condition (67%) and lower number FC cells in hypoxia (1%) compared to that in normoxia (10%) (Saller et al., 2012).

In this study, the cellular morphology of the cell subpopulations showed that pHS supplement maintains a higher morphological homogeneity in normoxic condition. During sub-culturing, the percentage of FC subpopulation was significantly lower while the percentage of RS subpopulation was higher in pHS medium (Figure 4.4E). It might be because of the presence of paracrine factors and the biological functions regulated by them in the pHS media (Figure 4.6). Furthermore, higher number of RS subpopulation and lower number of FC subpopulation in pHS media might be the cause behind the higher proliferation (Figure 4.2) of SHED in this medium. Notably, higher self-renewal capability and plasticity, cell population with higher number of RS cells is more preferred in regenerative therapy (Saller et al., 2012). Higher proliferation and homogeneity of SHED in pHS medium denotes its suitability for expansion of MSCs before transplantation. However, further experiments are required to compare the

difference between pHS and FBS on the morphological homogeneity in hypoxic condition.

When rate of *in vitro* migration was assessed using the scratch assay, use of either FBS or pHS supplement did not show any significant difference (Figure 4.5 A, B). However, a more directional or orderly migration was observed in pHS supplemented medium (Figure 4.5C). Average cell surface area±SE in FBS medium (1639±63.66) was significantly higher ($p= 0.023$) compared to that in pHS medium (1354±51.69). The significant difference in the cell surface area might be related to the insignificant difference in scratch assay using SHED expanded in FBS and pHS media.

Since proliferation, morphology and migration of SHED were found to be more favourable in pHS supplement media, we compared the presence of 10 selected paracrine factors responsible for these biological actions in the serum used to prepare pHS and FBS. Individual human sera (n=6) that were used to prepare pHS was analysed. The analysis showed the presence of all the 10 selected paracrine factors in individual human sera, while in FBS none of them were detected (Table 4.3). Earlier studies reported the proliferative effect of EGF, FGF-2, G-CSF, GM-CSF, HGF, LIF, PDGF-BB and VEGF on MSCs (Table 4.2). While the role of FGF-2, G-CSF, GM-CSF, HGF, SCF, PDGF-BB and SDF-1A were reported to enhance the migration of MSCs (Table 4.2). Thus, the presence of paracrine factors in the pHS could be linked to the differences in biological functions such as proliferation, diversity in morphology and migration of SHED, which in turn are important for regenerative therapy. Besides paracrine factors, extracellular matrix protein such as fibronectin, semaphorins and netrin-1, also facilitate the migration of cells. By measuring the presence of the extracellular matrix proteins along with the paracrine factors in the different media supplement or cell culture supernatant would help to draw a more conclusive deduction.

Biological functions and possible corresponding regulatory pathways were analysed by IPA in relation to the involvement of the selected paracrine factors. The predicted results supported the influential role of these paracrine factors on the proliferation, viability, homogeneity and migration of SHED (Figure 4.6). Among the IPA predicted pathways, Ephrin receptor signalling pathway was found to be involved in the regulation of proliferation, morphology and directional migration of SHED (Figure 4.7). Paracrine factors namely EGF, PDGFBB, SDF-1A and VEGF present in the pHS are reported to be involved in Ephrin receptor signalling pathway (Arvanitis & Davy, 2008; Boyd et al., 2014). This predicted pathway also supports our hypothesis that the presence of paracrine factors in pHS might be the cause behind the higher proliferation, homogeneity and migration of SHED.

4.5 Conclusion

Proliferation of SHED in pHS were significantly higher compare to that in FBS. Presence of higher number of RS subpopulation and lower number of FC subpopulation were observed in SHED expanded in pHS media compared to that FBS media. Moreover, the pattern of migration of SHED in pHS media was more organized and directional. All these results showed the suitability of pHS for the expansion of SHED to be used for therapeutic purposes.

CHAPTER 5: SURVIVAL AND IMMUNOMODULATION OF STEM CELLS FROM HUMAN EXTRACTED DECIDUOUS TEETH EXPANDED IN POOLED HUMAN AND FOETAL BOVINE SERA

5.1 Introduction

Immunomodulatory properties of MSCs make them one of the most popular source of stem cells and permit the use of MSCs from both autologous and allogeneic sources in regenerative therapy (Gebler et al., 2012; Klyushnenkova et al., 2005; Molina et al., 2015; Nauta & Fibbe, 2007). Numerous clinical trials have reported that MSCs are safe for therapeutic use (Baron et al., 2010a; Connick et al., 2012; Connick et al., 2011; El-Kheir et al., 2013; Gupta et al., 2013; Hare et al., 2012; Hare et al., 2009; Lee et al., 2012b; Lee et al., 2013; Otsuru et al., 2012; Peng et al., 2011; Reinders et al., 2013; Tan et al., 2012), however, lack of long term regenerative benefits has been considered as its major drawback (Malliaras & Marban, 2011; Volarevic et al., 2011). Before transplantation MSCs must be expanded *in vitro*. Often MSCs are expanded in Neu5Gc xenoantigen containing FBS supplemented media (Heiskanen et al., 2006; Komoda et al., 2010) which could contaminate the MSCs. Despite having immunomodulatory properties, hyper-immunogenicity to xeno-contaminated MSCs regardless of their source (autologous or allogeneic) has also been reported in both *in vivo* and *in vitro* studies (Komoda et al., 2010; Li & Lin, 2012).

To address the issue of xeno-contamination, chemically defined xeno-free medium supplemented with several recombinant human proteins has been developed (Corotchi et al., 2013; Patrikoski et al., 2013; Simoes et al., 2013). However, purified recombinant proteins of xeno-free medium has been reported to cause priming and reduce regenerative potential of MSCs during their *in vitro* expansion (Handorf & Li, 2011; Prasanna et al., 2010). Researchers have also proposed human serum, plasma, and

platelet lysate as possible replacement of FBS and recombinant proteins (Aldahmash et al., 2011; Jonsdottir-Buch et al., 2013; Lin et al., 2005; Shahdadfar et al., 2005) that could reduce xeno-contamination during *in vitro* expansion of MSCs (Komoda et al., 2010). In recent years, the potential of pooled allogeneic serum from adult AB-blood donors and pooled cord blood serum in maintaining regenerative properties of MSCs have been reported (Cooper et al., 2010; Kobayashi et al., 2005; Le Blanc et al., 2007; Phadnis et al., 2006; Poloni et al., 2009; Tateishi et al., 2008; Turnovcova et al., 2009). Collection of blood irrespective of donors' blood group creates a larger donor pool compared to when the collection of blood is confined to a particular group. Moreover, in the previous chapter we have shown that pooled human serum (pHS) prepared from irrespective of blood groups supported higher proliferation, viability and migration of SHED. Hence, in this study we used pHS to expand SHED to evaluate the effect of FBS and pHS on their maintenance of immunomodulatory properties.

Studies on the immunomodulatory properties of MSCs and its vesicles were conducted in FBS or pooled AB serum supplemented media (Bruno et al., 2016; Di Trapani et al., 2016; Fayyad-Kazan et al., 2016; Hsu et al., 2015; Komoda et al., 2010; Yildirim et al., 2016). As the composition of FBS and pooled AB-serum are different from the recipients' serum composition, the immunomodulatory data reported could be misinterpreted. To overcome this issue, in this study we aimed to analyse the immunomodulatory properties of SHED in the media supplemented with AuHS of corresponding monocytes/macrophages or lymphocytes. The objectives of this study are to;

a) determine the effect of complement on SHED expanded in FBS and pHS supplemented media.

b) determine the effect of monocytes on SHED expanded in FBS and pHS supplemented media.

c) determine the effect of lymphocytes on SHED expanded in FBS and pHS supplemented media.

5.2 Materials and Methods

5.2.1 Ethics approval for the collection of human extracted deciduous teeth and blood

In this study, all the samples were obtained following informed written consent from the guardian of the young donors and adult donors. Sample collection procedure for the current research was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Reference #DF RD1301/0012[L], for blood collection; DF CO1107/0066[L], for teeth extraction). Blood was collected by three trained health nurses from Department of Oro-Maxillofacial Surgical and Medical Sciences, Faculty of Dentistry, University of Malaya. Clinically sound intact deciduous molars from healthy children (n=3, 5-9 years) which were indicated for extraction were collected from the Department of Paediatric Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya.

5.2.2 Exclusion criteria

Blood was collected from healthy male donors aged 21-35 years. Exclusion criteria includes: smoking habits, alcohol consumption, drug and/narcotics addiction, diagnosis of any inflammatory diseases either chronic or at least in the last four weeks, major surgical treatment in the last one year, and immunotherapy.

5.2.3 Preparation of pooled human serum and serum with or without complement

To separate serum, 20 ml of blood was collected from each donor, transferred into a 50 ml sterile centrifuge tube (BD Bioscience) containing no anticoagulant and allowed to stand at room temperature for an hour to facilitate coagulation. The coagulated blood was centrifuged at 400×g for 15 minutes followed by a second round of centrifugation at 1800×g for 15 minutes. The final serum supernatant was aliquoted into two equal parts. One part of the serum was left untreated to keep the complement intact, while the other part was heat treated at 57±2 °C for 30 minutes to obtain a complement inactivated human serum. Individual heat treated sera (n=6) were combined to form the pooled human serum (pHS). Heat treated or untreated sera were sterilized by filtration through a 0.2 μm membrane filter (Thermo Scientific).

5.2.4 Isolation of peripheral blood mononuclear cells

To isolate PBMCs, blood (n=4) was collected in green top vacutainer containing sodium heparin (GmbH, Hamburg, Germany). Sodium heparinised 10 ml blood was transferred into a 50 ml centrifuge tube (BD Bioscience) and diluted with equal volume of DPBS without calcium and magnesium (Gibco). Then the diluted blood was transferred into another 50 ml centrifuge tube (BD Bioscience) containing 15 ml Ficoll-Paque Plus (GE Healthcare, Upsala, Sweden) and centrifuged at 400×g for 30 minutes with brake off. Buffy coat containing PBMCs was collected and washed twice with DPBS by centrifugation at 200×g for 10 minutes. After discarding the supernatant, the pellet was mixed with basal medium. Cell number and viability was counted using trypan blue (Gibco) dye exclusion method.

5.2.4.1 Separation of monocytes/macrophages and lymphocytes from peripheral blood mononuclear cells

PBMCs were seeded in KnockOut™ DMEM (Gibco) supplemented with 10% (v/v) AuHS, 2 mM Glutamax (Gibco), and 50 units/ml Penicillin-Streptomycin (Gibco) at a density of 5.5×10^5 cells/ml and incubated in 5% CO_2 humidified incubator at 37°C. Following incubation using pipette non-adherent lymphocytes were collected very carefully into 15 ml centrifuge tubes (BD Bioscience). Adherent monocytes/macrophages were washed gently twice using DPBS (Gibco) to minimize lymphocyte contamination and fed with autologous human serum (AuHS) supplemented KnockOut™ DMEM (Gibco) for further uses.

Lymphocytes collected were centrifuged at 200×g for 10 minutes. Supernatants were discarded and the pellets were resuspended in AuHS supplemented KnockOut™ DMEM (Gibco) for further experiments.

5.2.5 *In vitro* maintenance of stem cells from human extracted deciduous teeth

Initial isolation and expansion of SHED (n=3) were maintained in 10% FBS supplemented KnockOut™ DMEM (Gibco) until passage 3. Subsequent cultures (from passage 4-7) were maintained in KnockOut™ DMEM (Gibco) supplemented with either 10% FBS or 10% pHS. In order to minimize xeno-contamination, animal derived component free TrypLE™ express (Gibco) was used as cell dissociation reagent.

5.2.6 Effect of complement on stem cells from human extracted deciduous teeth expanded in pooled human serum and foetal bovine serum media

SHED maintained in FBS and pHS supplemented medium (FBS-SHED and pHS-SHED respectively) were seeded (passage 7) at a density between 1×10^5 cells/well to 3112 cells/well with two dilution factor in 96-well plates containing KnockOut™

DMEM (Gibco) medium supplemented with 40% freshly prepared human serum (n=3). Complement inactivated human sera (n=3) from the same donors were used to supplement media as control. After 12 hours of incubation at 37 ℃ in 95% humidified air, and 5% CO_2, the effect of complement on the survival of SHED was analysed using PrestoBlue® cell viability reagent (Invitrogen). In brief, after 12 hours of incubation all the media were discarded and the wells were washed twice with DPBS (Gibco). KnockOut™ DMEM medium (Gibco) along with 10 % PrestoBlue® (Invitrogen) reagent (v/v) was added to each well and the plates were further incubated for 2 hours. Absorbance was measured at 570 nm with reference wavelength set at 600 nm by microplate reader (Tecan). The absorbance values were converted to the corrected absorbance of PrestoBlue® (Invitrogen) reagent.

5.2.7 Immunomodulatory effect of stem cells from human extracted deciduous teeth

At passage 5, confluent FBS-SHED and pHS-SHED were dissociated and co-cultured with either monocytes/macrophages or lymphocytes in 24-well plates at 1:10 and 1:50 ratio respectively. The culture media was supplemented with freshly prepared 10% untreated human serum of the corresponding monocytes/macrophages or lymphocytes. Culture of monocytes/macrophages, lymphocytes and SHED were maintained individually in the same media as control. After 24 and 120 hours of incubation, supernatants were collected and centrifuged at 200×g for 10 minutes. Following centrifugation supernatants were aliquoted into two equal parts and stored in -80°C for cytotoxicity assay and immunoassay.

5.2.7.1 Cytotoxicity assay

Lactate dehydrogenase (LDH) assay kit (Sigma) was used to analyse the cytotoxic effect of monocytes/macrophages and lymphocytes to SHED. LDH is an enzyme that is found in all living cells and involved in the catalyses of the conversion of lactate into pyruvate to generate nicotinamide adenine dinucleotide (NADH). LDH is released during cell or tissue damage, thus it is considered as a marker of cellular injury or cytotoxicity. LDH activity is presented as milliunit/ml (nmole/min/ml). One unit of LDH activity is the amount of enzyme that generate 1 μmol of NADH per minute at 37° C. Absorbance measurement at 450 nm is used to measure the presence of NADH in the samples.

5.2.7.2 Immunoassay

Supernatants collected from the co-cultures after 24 and 120 hours of incubation were used to measure the amount of selected paracrine factors in them by using Luminex-based ProcartaPlex human cytokine/chemokine 16plex immunoassay kit from e-Bioscience (affymetrix, e-Bioscience, Vienna, Austria). Paracrine factors were selected on their involvement in cell survival and regulation of immune functions (Table 5.1)

Table 5.1: Selected paracrine factors analysed in the current research

Name of the paracrine factor	Function (References)
Fibroblast growth factor 2 (FGF-2)	✓ Shows broad spectrum of mitogenic effect (Salcedo et al., 1999; Werner & Grose, 2003) ✓ Stimulates the *in vitro* expansion of human BM-MSCs by activation of JNK signaling (Ahn et al., 2009). ✓ Slows down the aging process of MSCs by decreasing the gradual loss of telomere sequences (Bianchi et al., 2003; Yanada et al., 2006). ✓ Cytoprotective role of FGFs have also been acknowledged by researchers (Werner & Grose, 2003). ✓ Increase expression of CXCR4 on human endothelial cells and help angiogenesis (Salcedo et al., 1999)

Table 5.1 continued

Name of the paracrine factor	Function (References)
Granulocyte colony stimulating factor (G-CSF)	✓ Promotes cellular proliferation and migration, and prevents apoptosis (Murakami et al., 2013). ✓ Mobilizes HSC and MSCs from bone marrow (Kawada et al., 2004). ✓ Improves chemotactic property of MSCs *in vitro* (Murakami et al., 2013).
Granulocyte-macrophage colony stimulating factor (GM-CSF)	✓ regulates the proliferation and differentiation of hematopoietic progenitor cells (Shi et al., 2006) ✓ Acts as chemoattractant and induces mobilization of progenitors in the circulation (Rojas et al., 2005).
Hepatocyte growth factor (HGF)	✓ Promotes proliferation, and survival of various cell types (Forte et al., 2006). ✓ Induces migration and site specific homing of various cell types including MSCs from different origins (Son et al., 2006).
Leukaemia inhibitory factor (LIF)	✓ Helps to maintains self-renewal and multidifferentiation potential of various stem cells including MSCs (Kolf et al., 2007; Metcalf, 2003).
Macrophage colony stimulating factor (M-CSF)	✓ Supports proliferation, differentiation and survival of monocytes, macrophages and bone marrow progenitor cells (Stanley et al., 1997).
Platelet derived growth factor BB (PDGF-BB)	Induces both expansion and migration of MSCs (Fierro et al., 2007; Tamama et al., 2006). Helps survival of MSCs as well (Krausgrill et al., 2009).
Stem cell factor (SCF), KIT ligand	✓ Regulates the migration, differentiation and proliferation of several cell types including HSC (Lennartsson & Rönnstrand, 2012). ✓ Induces the migration and homing of MSCs (Pan et al., 2013).
Stromal cell-derived factor-1a (SDF-1A)	✓ help site specific migration and homing of MSCs through its receptor CXCR4 (He et al., 2010; Yu et al., 2015).
Tumour necrosis factor alpha (TNF-α)	✓ Induces tumour cell apoptosis, inflammation and immune response (Pfeffer, 2003).
Vascular endothelial growth factor A (VEGF-A)	✓ Increases proliferation and survival MSCs (Pons et al., 2008).
Interferon-gamma (IFN-γ)	✓ Induces antigen processing and presentation (Schroder et al., 2004). ✓ Inhibit proliferation and induce apoptosis (Schroder et al., 2004). ✓ Induce immunomodulation and leukocyte trafficking (Schroder et al., 2004).
Interleukin 4 (IL-4)	✓ Induces adaptive immune response by activating B cells and stimulating proliferation of T cells (Choi & Reiser, 1998). ✓ Induces differentiation of T-lymphocytes to T helper cell 2 (T_{H2}) (Choi & Reiser, 1998).
Interleukin 6 (IL-6)	✓ Stimulates the production of acute phase proteins (Fattori et al., 1994). ✓ Favours chronic inflammatory responses by stimulating T- and B-lymphocytes (Gabay, 2006).
Interleukin 10 (IL-10)	✓ Inhibits T_{H1} cells, natural killer (NK) cells, and macrophages (Couper et al., 2008). ✓ Enhances proliferation, survival and antibody production of B cells (Rousset et al., 1992). ✓ Promotes immunosuppressive functions (Pierson & Liston, 2010).
Interleukin 12 (IL-12p70)	✓ Increases IFN-γ production (Del Vecchio et al., 2007) ✓ Induces T helper 1 differentiation (Del Vecchio et al., 2007) ✓ Promotes proliferation, and cytolytic activity of natural killer and T cells (Del Vecchio et al., 2007)

5.2.8 Molecular network analysis

Up or down regulation of paracrine factors in the FBS-SHED co-culture with monocytes/macrophages or lymphocyte were calculated by comparing with the corresponding co-culture of pHS-SHED, and the values were presented in mean fold changes. The corresponding Enterz Gene IDs of the 16 paracrine factors along with the mean fold changes values were imported into the core analysis tool of Ingenuity Pathway Analysis (IPA) (Ingenuity systems; www.ingeniuty.com) to identify their role in regulating biological functions and pathways. Positive fold change values denote up-regulation, and negative values denote down-regulation. Activation and inhibition of biological functions and signalling pathways were predicted by the 'z-score' calculated by IPA on the basis of the expression pattern of downstream transcriptional regulator in IPA software. On the basis of the expression of analysed paracrine factors IPA predicted activation and inhibition of the cascade of upstream transcriptional regulators as well that help to explain the causes behind the changes in the expression analysed paracrine factors. Positive and negative z-score indicates activation or inhibition o respectively. Z-score greater than +1.96 or smaller than -1.96 were considered significant ($p<0.05$).

5.2.9 Data analysis

Data were analysed using SPSS version 22. The significant level was set at $p <0.05$.

5.3 Results

5.3.1 Effect of complement on stem cells from human extracted deciduous teeth expanded in foetal bovine serum and pooled human serum media

FBS-SHED (n=3) and pHS-SHED (n=3) showed no significant difference in metabolic activity measured by PrestoBlue® (Invitrogen) after 12 hours of incubation in the presence or absence of complement (n=3) (Figure 5.1).

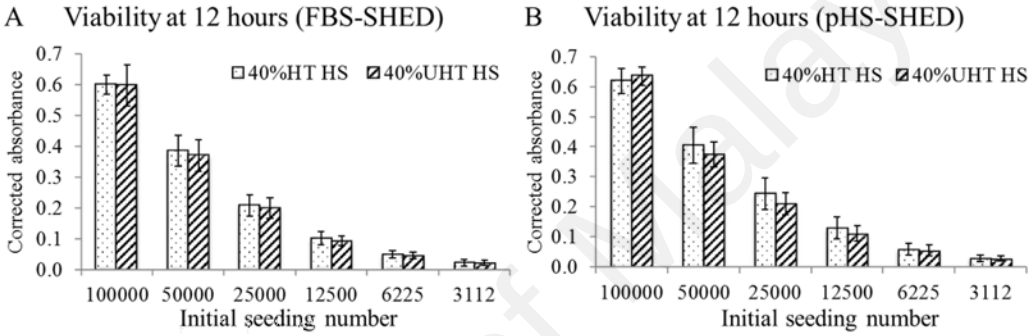

Figure 5.1: Metabolic activity of SHED in the presence of complement. A) Metabolic activity of FBS-SHED (n=3) in the presence or absence of complement (n=3) after 12 hours of incubation. B) Metabolic activity of pHS-SHED (n=3) in the presence or absence of complement (n=3) after 12 hours of incubation. (HT, heat treated; UHT, untreated; HS, human serum)

5.3.2 Cytotoxic effect of immune cells on stem cells from human extracted deciduous teeth

Cytotoxic activity of monocytes/macrophages (n=4) or lymphocytes (n=4) on FBS-SHED (n=3) and pHS-SHED (n=3) measured by LDH after 24 and 120 hours of incubation showed no significant difference (Figure 5.2).

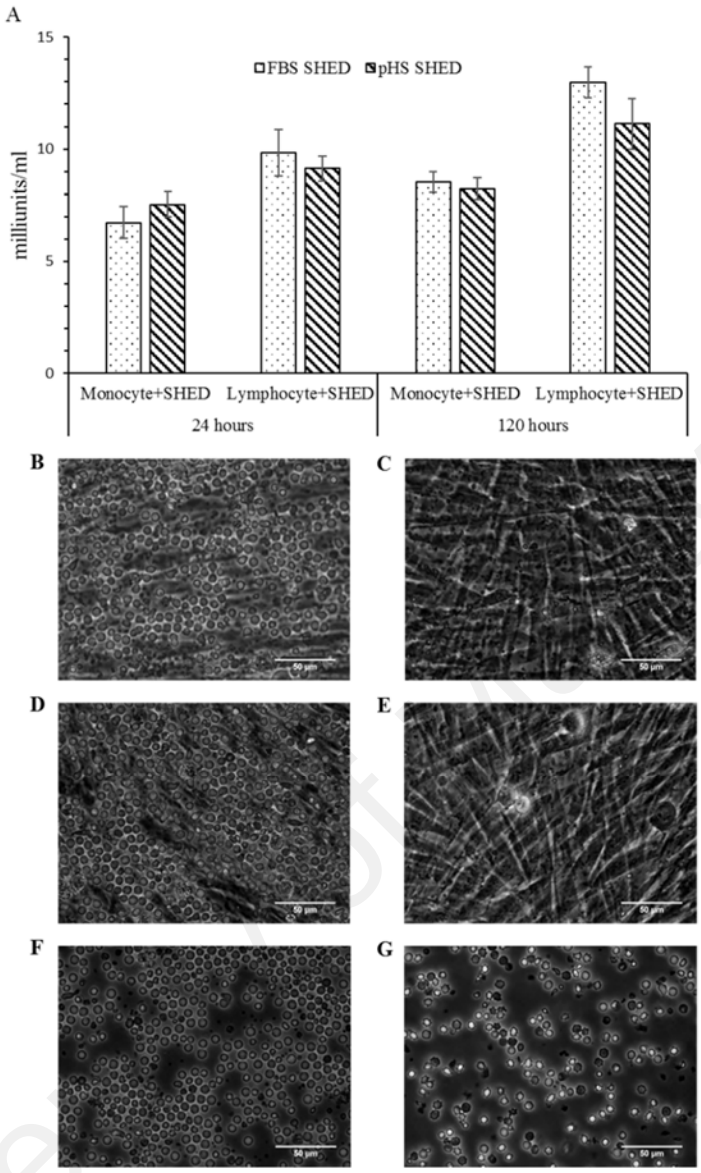

Figure 5.2: Effect of monocytes and lymphocytes on the survival of SHED expanded in FBS and pHS supplemented media analysed using lactate dehydrogenase (LDH) assay. A) FBS-SHED and pHS-SHED showed no significant difference in their survival in the presence or absence of monocytes/macrophages or lymphocytes after 24 and 120 hours of incubation. B) Co-culture of FBS-SHED with lymphocytes, C) Co-culture of FBS-SHED with monocytes/macrophages, D) Co-culture of pHS-SHED with lymphocytes, E) Co-culture of pHS-SHED with monocytes/macrophages, F) Culture of lymphocytes in autologous serum supplemented medium as control, G) Culture of monocytes/macrophages in autologous serum supplemented medium as control. Representative photomicrographs (B-G) were taken after 120 hours of incubation. Scale bars: B-G, 50 µm

5.3.3 Expression of paracrine factors in the co-culture of stem cells from human extracted deciduous teeth and monocytes/macrophage

At 24 hours of incubation in the co-culture of FBS-SHED and monocytes/macrophage, FGF-2, M-CSF, INF-γ and IL-4 were not expressed. While, in the co-culture of pHS-SHED and monocytes/macrophage, TNF-α along with the above mentioned four paracrine factors were not expressed. Among the expressed paracrine factors PDGF-BB was significantly higher in FBS-SHED and monocytes/macrophages co-culture compared to that in pHS-SHED and monocytes/macrophages co-culture. Whereas, the expression of VEFG-A was significantly higher in pHS-SHED and monocytes/macrophages co-culture compared to that in FBS-SHED and monocytes/macrophages co-culture (Figure 5.3).

At 120 hours of incubation in the co-culture of FBS-SHED and monocytes/macrophages, M-CSF and INF-γ were not expressed. Whereas, in the co-culture of pHS-SHED and monocytes/macrophages only the M-CSF was not expressed (Figure 5.3). Among the expressed paracrine factors FGF-2, HGF, PDGF-BB and SDF-1a were significantly higher in FBS-SHED and monocytes/macrophages co-culture compared to that in pHS-SHED and monocytes/macrophages co-culture. While, the expression of INF-γ and IL-6 were significantly higher in pHS-SHED and monocytes/macrophages co-culture compared to that in FBS-SHED and monocytes/macrophages co-culture (Figure 5.3).

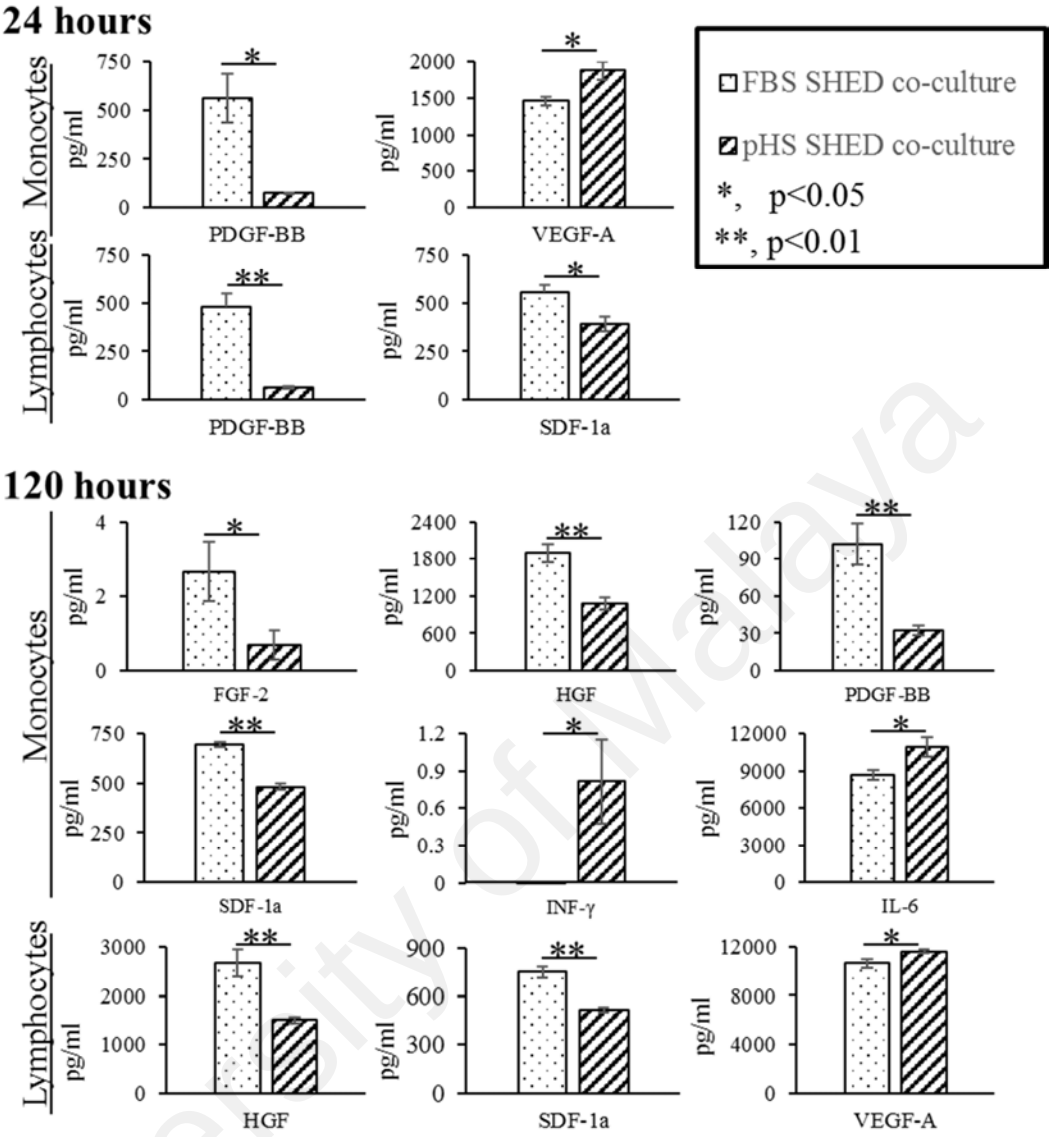

Figure 5.3: Comparative expression of paracrine factors in the co-culture of SHED with either monocytes or lymphocytes. PDGF-BB was significantly higher in FBS-SHED and monocytes/macrophages co-culture compared to that in pHS-SHED and monocytes/macrophages co-culture at 24 hours of incubation. Meanwhile, VEFG-A was significantly higher in pHS-SHED and monocytes/macrophages co-culture compared to that in FBS-SHED and monocytes/macrophages co-culture. At 24 hours, PDGF-BB and SDF=1a were significantly higher in FBS-SHED and lymphocyte co-culture compared to that in pHS-SHED and lymphocyte co-culture. At 120 hours, FGF-2, HGF, PDGF-BB and SDF-1a were significantly higher in FBS-SHED and monocytes/macrophages co-culture compared to that in pHS-SHED and monocytes/macrophages co-culture. Whereas, the expression of INF-γ and IL-6 were significantly higher in pHS-SHED and monocytes/macrophages co-culture compared to that in FBS-SHED and monocytes/macrophages co-culture. Expression of HGF and SDF-1a were significantly higher in FBS-SHED and lymphocyte co-culture compared to that in pHS-SHED and lymphocyte co-culture at 120 hours of incubation. While, the expression of VEGF-A was significantly higher in pHS-SHED and lymphocyte co-culture compared to that in FBS-SHED and lymphocyte co-culture (* = $p<0.05$, ** = $p<0.01$)

5.3.4 Expression of paracrine factors in the co-culture of stem cells from human extracted deciduous teeth and lymphocytes

At 24 hours of incubation in the co-culture of FBS-SHED and lymphocytes, all 16-selected paracrine factor were expressed. In the co-culture of pHS-SHED and lymphocytes, except FGF-2 and MCSF, expression of the rest 14 paracrine factors were detected (Figure 5.3). Among the expressed paracrine factors PDGF-BB and SDF-1a were significantly higher in FBS-SHED and lymphocyte co-culture compared to that in pHS-SHED and lymphocyte co-culture (Figure 5.3).

At 120 hours of incubation in the both co-cultures (FBS-SHED or pHS-SHED with lymphocytes) expression of all the 16 selected paracrine factors were detected (Figure 5.3). Among the expressed paracrine factors HGF and SDF-1a were significantly higher in FBS-SHED and lymphocyte co-culture compared to that in pHS-SHED and lymphocyte co-culture. Meanwhile, the expression of VEGF-A was significantly higher in pHS-SHED and lymphocyte co-culture compared to that in FBS-SHED and lymphocyte co-culture (Figure 5.3).

5.3.5 Expression of paracrine factors in the co-cultures

Changes in the expression of paracrine factors in FBS-SHED co-cultures (with monocytes/macrophages) compared to that in pHS-SHED co-cultures (with monocytes/macrophages) were measured in mean fold change values. Entrez Gene IDs corresponding to the 16 paracrine factors and the fold change values are given in Table 5.2.

Table 5.2: Corresponding Entrez Gene IDs of the selected 16 paracrine factors and their changes in expression (fold change values) in the FBS-SHED co-cultures compared to that in pHS-SHED co-cultures

Cytokines	Gene ID	24 hours		120 hours	
		Cocul^{mono}	Cocul^{lym}	Cocul^{mono}	Cocul^{lym}
FGF-2	NM_002006	.00	∞	3.73	-1.01
HGF	NM_001010932	1.06	1.03	1.76	1.78
LIF	NM_002309	1.02	1.04	-1.03	-1.01
PDGF-BB	NM_033016	7.60	7.62	3.16	1.31
SCF	NM_000899	-2.00	2.66	1.06	1.45
SDF-1a	NM_199168	1.17	1.42	1.44	1.45
VEGF-A	NM_001171628	-1.29	-1.31	1.02	-1.09
G-CSF	NM_000759	-1.40	1.04	1.43	1.00
GM-CSF	NM_000758	-2.34	1.28	-1.08	-1.23
M-CSF	NM_000757	.00	∞	.00	1.14
INF-γ	NM_000619.2	.00	4.64	-∞	-1.22
TNF-α	HQ201306.2	∞	5.45	2.08	1.21
IL-4	NM_000589.3	.00	4.47	-1.16	-1.26
IL-6	NM_000600	-1.13	1.10	-1.26	1.04
IL-10	AY029171.1	1.07	1.53	1.03	1.15
IL-12	NM_000882	2.31	1.03	-1.02	1.15
Cocul^{mono}, co-culture with monocytes/macrophages; Cocul^{lym}, co-culture with lymphocytes					

5.3.6 Paracrine factors in the regulation of biological functions

Corresponding Entrez Gene IDs of the 16 paracrine factors and mean fold change values of paracrine factors in FBS-SHED co-cultures compared to that in pHS-SHED co-cultures (Table 5.2) were imported to IPA to predict the increase or decrease of biological functions in FBS-SHED co-cultures compared to pHS-SHED co-cultures. Important biological functions related to proliferation, viability, apoptosis, differentiation, migration and inflammatory response were taken in consideration (Figure 5.4).

Biological Functions (p value)	Cytokines analyzed																Increase (↑) Decrease(↓)			
																	24 hours		120 hours	
	TGF-2	HGF	LIF	PDGF-BB	SCF	SDF-1A	VEGF-A	G-CSF	GM-CSF	M-CSF	INF-γ	TNF-α	IL-4	IL-6	IL-10	IL-12	Cocul^{M}	Cocul^{L}	Cocul^{M}	Cocul^{L}
Proliferation of hematopoietic progenitor cells (4.94E-15)	-	-	-	-	√	√	-	√	√	-	-	√	-	√	√	-	↓	-	-	-
Proliferation of mononuclear leukocytes (1.08E-15)	-	-	-	-	√	√	-	√	√	√	√	√	√	√	√	-	-	↑	-	-
Proliferation of myeloid cells (4.32E-12)	-	-	-	-	√	-	-	√	√	-	-	√	-	-	√	-	↓	-	-	-
Apoptosis of myeloid cells (3.64E-12)	-	-	-	-	√	-	-	√	√	-	-	√	-	√	√	-	↑	-	-	-
Cell viability of leukocytes (2.10E-22)	-	√	-	-	√	√	-	√	√	√	√	√	√	√	√	-	-	↑	-	-
Differentiation of hematopoietic progenitor cells (7.04E-14)	-	-	-	-	√	√	-	√	√	-	-	√	-	√	√	-	↓	-	-	-
Differentiation of leukocytes (1.84E-19)	-	√	√	-	√	-	-	√	√	√	√	√	√	√	√	-	-	↑	-	-
Activation of mononuclear leukocytes (7.53E-12)	-	-	-	-	-	-	-	-	√	√	√	√	√	√	√	-	-	↑	-	-
Adhesion of immune cells (2.50E-19)	-	√	-	-	√	√	√	-	√	√	√	√	√	√	√	-	-	↑	-	-
Binding of cells (5.33E-13)	√	√	-	-	√	√	√	-	-	-	-	√	√	√	√	-	-	-	↑	-
Binding of leukocytes (1.06E-10)	√	-	-	-	√	√	-	-	-	-	-	√	√	-	√	-	-	-	-	↑
Immune response of antigen presenting cells (4.73E-11)	-	√	-	-	-	-	-	-	√	-	√	√	√	-	-	-	-	↑	-	-
Immune response of phagocytes (6.28E-12)	-	√	-	-	-	-	-	√	√	-	√	√	√	-	-	-	-	↑	-	-
Inflammatory response (1.87E-14)	-	-	-	-	√	√	-	-	√	√	√	√	√	√	√	-	-	↑	-	-
Stimulation of leukocytes (2.70E-15)	-	-	-	-	-	-	-	√	√	-	√	√	√	√	√	-	-	↑	-	-
Leukocyte migration (1.99E-18)	-	√	-	-	√	√	-	√	√	√	√	√	√	√	√	-	-	↑	-	-
Migration of hematopoietic progenitor cells (2.13E-14)	-	√	-	-	√	√	-	-	-	-	√	√	√	-	-	-	-	-	↑	↑
Migration of lymphatic system cells (7.63E-14)	√	√	-	-	-	√	-	√	-	-	-	√	√	√	√	-	-	↑	↑	-

Increase (↑) and decrease (↓) were measured by the positive and negative z-scores. z-score > +1.96 or < -1.96 were considered significant (n=6). [√, involved; -, uninvolved/unchanged; mono, co-culture with monocytes/macrophages; Lymp, co-culture with lymphocytes]

Figure 5.4: Predicted activation or inhibition of biological functions. IPA (Ingenuity systems; www.ingeniuty.com) predicted activation or inhibition of biological functions maintained by the 16 analysed paracrine factors in FBS-SHED co-cultured with monocytes/macrophages or lymphocytes compared to pHS-SHED co-cultured with monocytes/macrophages or lymphocytes respectively after 24 and 120 hours of incubation

From the IPA predicted results it is found that after 24 hours of incubation of FBS-SHED and monocytes/macrophages co-culture, the microenvironment significantly ($p<0.05$) decrease proliferation and differentiation of hematopoietic progenitor cells, proliferation of myeloid cells, and increase apoptosis of myeloid cells compared to the microenvironment of corresponding pHS-SHED co-cultures. Whereas, after 120 hours of incubation, significant ($p<0.05$) increase in the binding of cells, migration of hematopoietic progenitor cells and migration of lymphatic system cells were predicted in the microenvironment of FBS-SHED and monocytes/macrophages co-culture compared to pHS-SHED and monocytes/macrophages co-culture (Figure 5.4).

After 120 hours of incubation of FBS-SHED and lymphocytes co-culture, the microenvironment was predicted to significantly increase binding of leucocytes and migration of hematopoietic progenitor cells compared to the corresponding pHS-SHED and lymphocyte co-culture. While, after 24 hours of incubation, the microenvironment of the FBS-SHED and lymphocytes co-culture predicted to significantly increase proliferation of mononuclear leucocytes, cell viability of leucocytes, differentiation of leukocytes, activation of mononuclear leukocytes, adhesion of immune cells, immune response of antigen presenting cells, immune response of phagocytes, inflammatory response, stimulation of leukocytes, leukocytes migration and migration of lymphatic system cells compared to that of pHS-SHED and lymphocyte co-culture (Figure 5.4).

5.3.7 Paracrine factors in the regulation of pathways

IPA also predicted the involvement of the analysed paracrine factors in regulating the signalling pathways. At 24 hours of incubation, on the basis of the combination of paracrine factors IPA predicted the moderate activation of HMBG1 signalling (z score = .447) and the mild inhibition of dendritic cell maturation (z score = -.447) in the FBS-SHED co-culture with monocytes/macrophages compared to that in pHS-SHED co-culture with monocytes/macrophages (Figure 5.5 A). Whereas after 120 hours of incubation, moderate inhibition of HMBG1 signalling (z score = -1.633) and dendritic cell maturation (z score = -1.342) in the FBS-SHED co-culture with monocytes/macrophages compared to that in pHS-SHED co-culture with monocytes/macrophages were predicted (Figure 5.5 B).

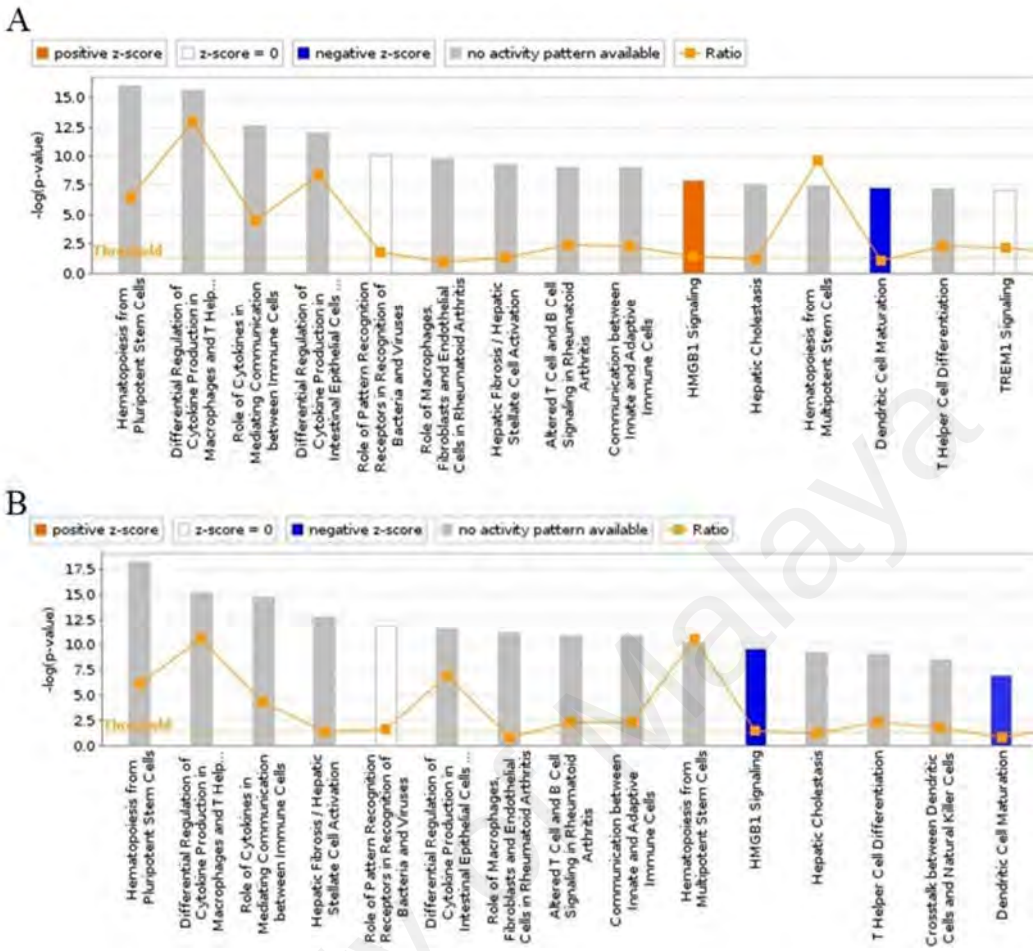

Figure 5.5: Predicted activation and inhibition of signalling pathways in the FBS-SHED co-culture with monocytes/macrophages. Activation or inhibition of the signalling pathways in the FBS-SHED co-culture with monocytes/macrophages compared to that in pHS-SHED co-culture with monocytes/macrophages (A) after 24 hours of incubation and (B) after 120 hours of incubation. Activation and inhibition was predicted by Ingenuity Pathway Analysis (Ingenuity systems; www.ingeniuty.com)

Regulation of the signalling pathways in the co-culture with lymphocytes were also predicted by IPA. After 24 hours of incubation, activation of HMBG1 signalling (z score = 2.646), TREM1 signalling (z score = 2.000) and dendritic cell maturation (z score = 1.342) in the FBS-SHED co-culture compared to that in pHS-SHED were predicted (Figure 5.6 A). Whereas after 120 hours of incubation, mild inhibition of high-mobility group box 1 protein (HMGB1) signalling (z score = -0.378), and mild activation of dendritic cell maturation (z score = 0.447) and triggering receptor

expressed on myeloid cells 1 (TREM1) signalling (z score = 1.000) in the FBS-SHED co-culture compared to that in pHS-SHED co-culture were predicted (Figure 5.6 B).

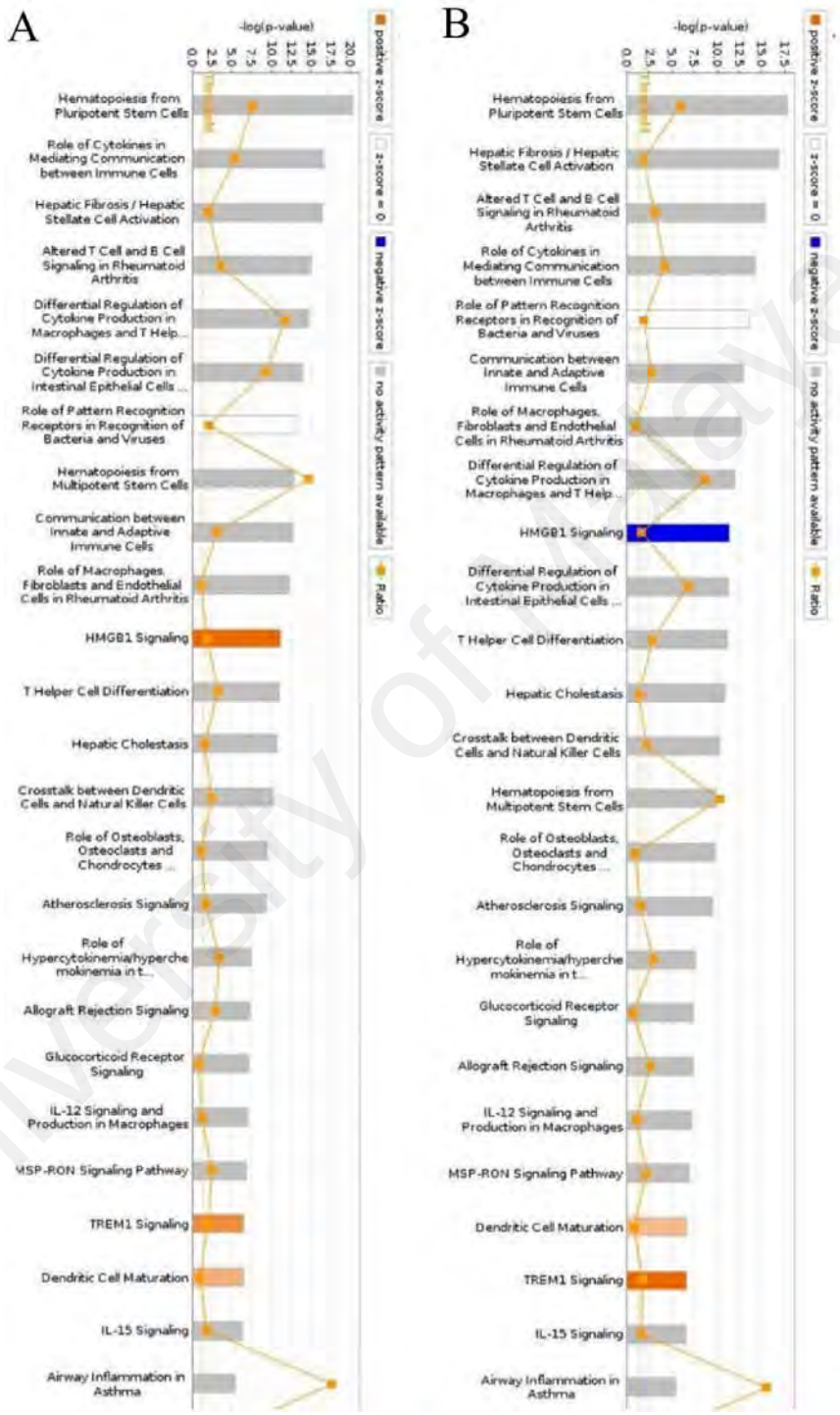

Figure 5.6: Predicted activation and inhibition of signalling pathways in the FBS-SHED co-culture with lymphocytes. Activation or inhibition of the signalling pathways in the FBS-SHED co-culture with lymphocytes compared to that in pHS-SHED co-culture with lymphocytes (A) after 24 hours of incubation and (B) after 120 hours of incubation. Activation and inhibition was predicted by Ingenuity Pathway Analysis (Ingenuity systems; www.ingeniuty.com)

5.3.8 Predicted changes in the expression of upstream regulators

On the basis of the expression of analysed paracrine factors in the supernatants of different co-cultures, IPA predicted the changes in the upstream regulators in the cells of those co-cultures. The results showed the predicted activation of eight upstream regulators and inhibition of three upstream regulators within the cells of FBS-SHED co-culture with lymphocytes after 24 hours of incubation compared to that within the cells of pHS-SHED co-culture with lymphocytes (Figure 5.7).

Upstream Regulator	z-score				Name of the analysed paracrine factors controlled by the upstream regulator
	24 hours		120 hours		
	$Cocul^{Mono}$	$Cocul^{Lym}$	$Cocul^{Mono}$	$Cocul^{Lym}$	
CD28	-	-2.129	-0.569	0.010	GM-CSF, IFN-γ, TNF-α, IL-4, IL-10
HLA-DQ	-	-2.000	0.000	-1.000	GM-CSF, TNF- α, IL-6, IL-10
IL1B	-	2.003	0.950	-0.171	FGF-2, G-CSF, GM-CSF, IFN-γ, TNF-α, VEGF-A, IL-6, IL-10
IL15	-	1.984	-	0.004	GM-CSF, IFN-γ, TNF-α, IL-10
IL18	0.709	2.399	0.709	0.796	GM-CSF, IFN-γ, TNF-α, SDF-1a, IL-4, IL-6, IL-10
IL37	0.763	-2.170	0.025	-0.468	G-CSF, GM-CSF, IFN-γ, TNF-α, IL-6
NFkB	0.647	2.150	0.647	1.347	GM-CSF, IFN-γ, TNF-α, SDF-1a, IL-6, IL-10
TLR2	-	2.219	-0.840	-0.192	GM-CSF, IFN-γ, TNF-α, IL-4, IL-6, IL-10
TLR3	-0.131	2.179	-0.131	0.665	GM-CSF, IFN-γ, TNF-α, IL-6, IL-10
TLR4	0.507	2.521	-0.323	0.955	GM-CSF, IFN-γ, TNF-α, IL-4, IL-6, IL-10, IL-12A
TLR6	-	2.236	-1.000	-0.447	GM-CSF, IFN-γ, TNF-α, IL-4, IL-6

$Cocul^{Mono}$, co-culture with monocytes/macrophages; $Cocul^{Lym}$, co-culture with lymphocytes, -, no activity pattern found (Z-score values greater than +1.96 or smaller than -1.96 considered significant, $p<0.05$)

Figure 5.7: Predicted changes in the expression of upstream regulators. On the basis of the changes in the expression of analysed cytokines in FBS-SHED co-cultures with monocytes/macrophages or lymphocytes compared to that in the corresponding pHS-SHED co-cultures IPA predicted the activation or inhibition of upstream regulator. IPA also identified the analysed paracrine factor those could be controlled by the upstream regulators

5.4 Discussion

In order to yield adequate number of cells for regenerative therapy MSCs are usually expanded in the FBS supplemented media. Several articles reported the presence of Neu5Gc a xenoantigen in the FBS (Heiskanen et al., 2006; Komoda et al., 2010) that might contaminate the MSCs and trigger CDC and ADCC (Ghaderi et al., 2010; Haque et al., 2015; Komoda et al., 2010; Zhu & Hurst, 2002).

In our study, FBS-SHED and pHS-SHED did not show any significant difference in their survival in the presence or absence of complement (Figure 5.1) and the observation concurs with other published results (Komoda et al., 2010; Le Blanc et al., 2007). This could be attributed to the expression of complement regulatory proteins such as CD46, CD55, and CD59 on the cells that make them resistant to CDC (Komoda et al., 2010). Secretion of complement inhibiting factor H from MSCs has also been reported and this could also support the survival of SHED cultured in FBS supplemented media in the presence of complement (Tu et al., 2010). In this study, any cell type other than MSCs were not used as control. Use of non-MSC cells to study the effect of complement could help to support the existing research data.

Cell-mediated cytotoxicity study also showed no significant difference between the survival of FBS-SHED and pHS-SHED in the presence of monocytes/macrophages or lymphocytes (Figure 5.2 A). Studies reported that MSCs prevent expression of co-stimulatory molecules such as CD40, CD80, CD83 and CD86 and induce expression of inhibitory molecules such as B7-H1, B7-H4 and HLA-G. These properties help MSCs to survive in allogenic condition by inhibiting the proliferation and function of cytotoxic T cells, NK cells and B cells, and preventing differentiation of monocytes into antigen-presenting DCs (Francois et al., 2012; Gebler et al., 2012; Plock et al., 2013). These are might be the reasons behind the survival of FBS-SHED and pHS-SHED in the presence of immune cells.

Cell microenvironment also play an important role in the survival of MSCs. Studies reported that high levels of IFN-γ and TNF-α help MSCs to become immunosuppressive to the cells of both innate and adaptive immunity (Di Trapani et al., 2016; Krampera et al., 2006; Lee et al., 2015). IFN-γ in the presence of TNF-α or IL-1α induce the expression of intracellular adhesion molecule 1 (ICAM1) and vascular cell

adhesion molecule 1 (VCAM1) that help MSCs to show immunosuppression (Ren et al., 2010). Induced immunosuppressive function of MSCs by IFN-γ and concomitant presence of any of the TNF-α, IL-1α or IL-1β has also been reported (Ren et al., 2008). IFN-γ induce the expression of IDO from MSCs (Yoo et al., 2009) that is known for the inhibition of NK cell and T-cell proliferation (Spaggiari et al., 2008; Tan & Bharath, 2009). In our study, the presence of IFN-γ and TNF-α were detected in the co-cultures (Figure 5.3). Presence of these paracrine factors in the co-culture microenvironment might help both FBS-SHED and pHS-SHED to survive in the presence of monocytes/macrophages and lymphocytes.

Though the both FBS-SHED and pHS-SHED survived in the presence of complement (Figure 5.1) and immune cells (Figure 5.2), the number of dead cells were marginally higher in the FBS-SHED and lymphocytes co-cultures after 24 and 120 hours of incubation compared to that in the corresponding co-cultures of pHS-SHED and lymphocytes (Figure 5.2 A). We imported the values of changes in the expression of 16 analysed cytokines to IPA to determine the status of the microenvironment of co-cultures (Table 5.2).

After 24 hours of incubation, IPA predicted significant increase inflammation related biological functions for the microenvironment of the FBS-SHED and lymphocyte co-culture compared to the that of pHS-SHED and lymphocytes (Figure 5.4). This observation supported by the predicted the significant activation of HMGB1 and TREM1 signalling pathways in the FBS-SHED and lymphocytes co-cultures after 24 hours of incubation compared to that in the pHS-SHED and lymphocytes co-cultures after 24 hours of incubation (Figure 5.6). The role of HMGB1 is controversial. Several researchers reported that HMGB1 promotes inflammation, tissue destruction, and the activation of immunity (Bianchi, 2009; Lotze & Tracey, 2005; Rovere-Querini et al.,

2004). In contrast, the role of HMGB1 in regulation of inflammation and tissue regeneration have also been reported (Bianchi, 2009; Campana et al., 2014). On the other hand, TREM1 work as an inducer of inflammation by itself or by inducing the expression of toll like receptors (TLRs) (Arts et al., 2013). It is noteworthy to mention that IPA predicted the activation of TLRs in the FBS-SHED and lymphocytes co-cultures after 24 hours of incubation as well (Figure 5.7).

5.5 Conclusion

The effect of complement and cell-mediated cytotoxicity assays showed that SHED maintain their immunomodulatory properties when cultured in FBS and pHS. This finding further support the previously published results on the immunomodulatory properties of MSCs expanded in FBS supplemented media *in vitro*. However, the immunoassay of 16 paracrine factors and prediction using IPA indicated the possibility of pHS over FBS as a media supplement for expansion of SHED.

CHAPTER 6: EFFECT OF FOETAL BOVINE SERUM AND AUTOLOGOUS HUMAN SERUM FOR THE PRODUCTION OF SECRETOME WITH MORE REGENERATIVE CYTOKINE

6.1 Introduction

The growing evidence on the role of paracrine factors in regeneration of affected organs has led to the introduction of cell culture supernatants and secretomes as a new therapeutic tool of regenerative medicine. The regenerative potential of the secretomes of stem and progenitor cells were reported in the treatments of neuronal disorders (Pires et al., 2014), vascular diseases (Dao et al., 2013), and cutaneous wounds (Yew et al., 2011). Like other adult stem cells secretomes (Madrigal et al., 2014), regenerative potential of the secretome of freshly isolated healthy peripheral blood mononuclear cells (PBMCs) (Hoetzenecker et al., 2013; Mildner et al., 2013) and apoptosis induced PBMCs (Altmann et al., 2014; Hoetzenecker et al., 2012; Lichtenauer et al., 2011) have also been reported.

For decades, foetal bovine serum (FBS) have been used as *in vitro* cell culture media supplement, this is because it provides proteins, growth factors, hormones, lipids, vitamins, attachment factors and other important trace elements that are needed for the survival and proliferation of cells in culture (Gstraunthaler, 2003). Composition of FBS varies from lot-to-lot, and xenoantigen such as Neu5GC present in the FBS has the potential to trigger immune response (Lindroos et al., 2009). As an alternative, serum-free media are being used as cell culture media and to produce secretomes (Altmann et al., 2014; Hoetzenecker et al., 2012; Hoetzenecker et al., 2013; Lichtenauer et al., 2011; Mildner et al., 2013) that are supplemented with purified or recombinant protein. The supplement is required in order to maintain safety, reproducibility and consistency of cells in culture (Haque et al., 2015; Mujaj et al., 2010). These recombinant or purified

proteins potentially regulate the secretomes composition by modulating the autocrine and paracrine signalling pathways (Mirshahi et al., 2000).

As an alternative to either FBS or serum-free media supplemented with purified protein, the present study analysed the benefits of using autologous human serum (AuHS) on the regenerative cytokine composition of secretomes and their respective biological functions. In other words, the main aim of the current study is to demonstrate how autologous serum supplement would differ from the bovine serum supplement on the secretomes cytokine composition. To achieve the aim of this study the specific objectives are as follows:

a) To determine the viability of PBMCs cultured in FBS or AuHS supplemented media.
b) To determine the differentiation of PBMCs cultured in FBS or AuHS supplemented media.
c) To determine the cytokine composition of the supernatant from PBMCs cultured in FBS or AuHS supplemented media.

6.2 Materials and Methods

6.2.1 Ethics statement

All samples were obtained following informed written consent from the donors. Sample collection procedure was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya [Reference #DF RD1301/0012(L)].

6.2.2 Preparation of serum and isolation of peripheral blood mononuclear cells

6.2.2.1 Collection of blood

Blood was collected from healthy male donors aged 21-35 years (n=7). Exclusion criteria includes: smoking habits, alcohol consumption, drug and/narcotics addiction, diagnosis of any inflammatory diseases either chronic or at least in the last four weeks, major surgical treatment in the last one year, and immune therapy. 50 ml of blood was collected from each donor in two steps: (i) 20 ml without any anticoagulant for serum preparation, and (ii) 30 ml in vacutainer containing sodium heparin (GmbH, Hamburg, Germany) for PBMCs isolation.

6.2.2.2 Serum preparation

To prepare the serum, 20 ml blood was transferred into a 50 ml centrifuge tube (BD Bioscience) and was allowed to stand at room temperature for an hour to facilitate coagulation. Then the tube containing coagulated blood was centrifuged at 400×g for 15 minutes and the crude serum was transferred into another sterile centrifuge tube for second round of centrifugation at 1800×g for 15 minutes to remove cell debris or insoluble particles. The final serum supernatant was heat treated at 57±2 °C for 30 minutes. The heat treated serum which has been complement inactivated was used as AuHS supplement to culture PBMCs.

6.2.2.3 Peripheral blood mononuclear cells isolation

To isolate PBMCs (n=7), 10 ml blood was transferred into a 50 ml centrifuge tubes (BD Bioscience) and diluted with equal volume of Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (Gibco, Grand Island, NY, USA). Then

the diluted blood was added into another 50 ml centrifuge tube (BD Bioscience) containing 15 ml Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and centrifuged at 400×g for 30 minutes with brake off. Buffy coat (Figure 6.1) containing PBMCs was collected and washed twice with DPBS by centrifugation at 200×g for 10 minutes. After discarding the supernatant, the pellet was mixed with basal medium. Cell number was counted using trypan blue (Gibco) dye exclusion method.

Figure 6.1: Buffy coat preparation by using density gradient centrifugation

6.2.3 Media preparation and culture

Isolated PBMCs (n=7) were cultured either in basal medium, RPMI-1640 with L-glutamine (Gibco) and 50 units/ml Penicillin-Streptomycin (Gibco); basal medium with 10% (v/v) FBS (Gibco) supplement or basal medium with 10% (v/v) freshly prepared AuHS. PBMCs were seeded in 12-well plates at a density of 5.5×10^5 cells/ml in different media. Plates were stored at 37°C and 5% CO_2 in humidified chambers, and incubated for 96 hours.

6.2.4 Cell viability assay

PBMCs (n=7) were cultured for 96 hours and the number of live cells were counted using trypan blue (Gibco) dye exclusion method at every 24 hours intervals. Cell viability was also evaluated using PrestoBlue® (Invitrogen) at the same interval.

Number of live cells was recorded as percentile of mean increase or decrease of initial seeding number (percentage of initial seeding – 100%) ± SE. Cell viability was recorded as percentile of mean corrected absorbance (% of corrected absorbance of initially seeded cells) ± SE, while the corrected absorbance of initially seeded cells was considered 100%.

6.2.5 Preparation of cytospin slides of PBMCs

After 24 and 96 hours of incubation, PBMCs (n=3) were harvested from previously mentioned three media, washed twice using DPBS (Gibco) and the pellets were resuspended in 10% FBS (Gibco) containing DPBS (Gibco). Final density of the cells was maintained at 5×10^5 cells/ml. Microscope slides of suspended PBMCs were prepared using poly-L-lysine coated slides (Thermo Scientific Shandon, Cheshire, UK) and cytocentrifuge (Thermo Scientific Shandon). Then the slides were dried overnight in a desiccator followed by fixation in ice-cold absolute acetone (Sigma-Aldrich, Steinheim, Germany) for 5 minutes and dried at room temperature (24±2 °C) for 10-15 minutes.

6.2.6 Differential count of PBMCs

In brief, the acetone fixed air-dried slides were then stained with 5% Giemsa stain (Sigma-Aldrich) containing double distilled water for 20 minutes for differential count of PBMCs. Then the slides were washed by dipping them briefly in buffered water and left in vertical position for air dry in room temperature. Following drying photomicrographs were taken and differential count of PBMCs (myeloid and lymphoid cells) was done on the basis of their morphology. Differential counts of PBMCs (n=3) harvested from three different media at 24 and 96 hours post incubation were presented as mean % of total cells ± SE.

6.2.7 Paracrine factors profiling

Culture supernatants were harvested at 24 hours and 96 hours after initial incubation for paracrine factors profiling. Luminex-based ProcartaPlex human cytokine/chemokine 17plex immunoassay kit from e-Bioscience (affymetrix, e-Bioscience, Vienna, Austria) was used to analyse the presence of 17 selected paracrine factors involved in regeneration based on the literature (Table 6.1).

Table 6.1: Paracrine factors analysed in the current research

Name of the paracrine factor	Function (References)
Brain-derived neurotrophic factor (BDNF) [NM_001143816]	✓ Promotes survival of neurons, synaptogenesis and synaptic plasticity (Lu et al., 2013a)
Epidermal growth factor (EGF) [NM_001178130]	✓ Regulates cellular proliferation, differentiation, survival and motility of various cells (Herbst, 2004; Kimura et al., 2013; Wells, 1999)
Fibroblast growth factor 2 (FGF-2) [NM_002006]	✓ Promotes angiogenesis, survival of cells, and wound healing (Aviles et al., 2003; Werner & Grose, 2003) ✓ Stimulates migration and proliferation of endothelial cells (Beenken & Mohammadi, 2009; Salcedo et al., 1999) ✓ Encourages mitogenesis of several cell types (Bianchi et al., 2003; Yanada et al., 2006)
Granulocyte colony stimulating factor (G-CSF) [NM_000759]	✓ Regulates granulopoiesis (Zhang et al., 2009) ✓ Promotes survival, proliferation, activation and maturation of hematopoietic progenitors of neutrophil lineage (Zhang et al., 2009) ✓ Enhances mobilization of HSCs and MSCs (Deng et al., 2011; Kawada et al., 2004)
Granulocyte-macrophage colony stimulating factor (GM-CSF) [NM_000758]	✓ Stimulates proliferation and differentiation of hematopoietic progenitors (Shi et al., 2006) ✓ Mobilizes endogenous progenitor cells and promotes neovascularization (Rojas et al., 2005; Takahashi et al., 1999)
Hepatocyte growth factor (HGF) [NM_001010932]	✓ Mitogenic for epithelial and endothelial cells (Sulpice et al., 2009) ✓ Increases motility and directional migration of cells (Sohni & Verfaillie, 2013; Son et al., 2006) ✓ Promotes angiogenesis; induces kidney and liver regeneration (Galimi et al., 2001; Sulpice et al., 2009) ✓ Accelerates wound healing process (Valente et al., 2016)
Leukemia inhibitory factor (LIF) [NM_002309]	✓ Inhibits proliferation and induces differentiation of macrophage (Moon et al., 2002) ✓ Promotes neuronal survival and differentiation (Moon et al., 2002) ✓ Stimulates glial development (Moon et al., 2002) ✓ Maintains the stem state of MSCs and other stem cells (Jiang et al., 2002; Metcalf, 2003)

Table 6.1 continued

Name of the paracrine factor	Function (References)
Macrophage colony stimulating factor (M-CSF) [NM_000757]	✓ Regulates production, survival and function of monocytes, macrophages and osteoclasts (Grasset et al., 2010)
Platelet-derived growth factor beta (PDGF-BB) [NM_033016]	✓ Induces fibroblast proliferation, collagen production and angiogenesis (Andrae et al., 2008) ✓ Promotes wound healing (Andrae et al., 2008) ✓ Influences periodontal regeneration (Andrae et al., 2008) ✓ Prevents apoptosis of cells following acute myocardial infarction (Krausgrill et al., 2009) ✓ Induces proliferation and migration in MSCs (Fierro et al., 2007; Tamama et al., 2006)
Stem cell factor (SCF), KIT ligand [NM_000899]	✓ Promotes survival, proliferation, differentiation of hematopoietic stem cells and progenitor cells (Broudy, 1997; Lennartsson & Rönnstrand, 2012) ✓ Promote survival of mature cells as well (Broudy, 1997) ✓ Initiates the migration of effector cells (Lennartsson & Rönnstrand, 2012; Smart & Riley, 2008)
Stromal cell-derived factor-1a (SDF-1A) [NM_199168]	✓ Induces migration of neutrophil to site of infection (Murphy et al., 2007) ✓ Promotes mobilization and directed migration of stem cells (Yu et al., 2015) ✓ Influences neurogenesis (Murphy et al., 2007)
Vascular endothelial growth factor A (VEGF-A) [NM_00117162]	✓ Angiogenesis, arteriogenesis, anti-apoptosis and immunoregulation (Sulpice et al., 2009; Wang et al., 2006)
Interleukin 2 (IL-2) [NM_000586]	✓ Regulates proliferation, activation and differentiation of lymphocytes (Liao et al., 2011)
Interleukin 3 (IL-3) [NM_000588]	✓ Promotes proliferation and differentiation of hematopoietic progenitors (Nitsche et al., 2003)
Interleukin 6 (IL-6) [NM_000600]	✓ Promotes angiogenesis, wound healing and cell migration (Yew et al., 2011) ✓ Promotes axon regeneration (Leibinger et al., 2013)
Interleukin 12 (IL-12p70) [NM_000882]	✓ Increases IFN-γ production (Del Vecchio et al., 2007) ✓ Induces T helper 1 differentiation (Del Vecchio et al., 2007) ✓ Promotes proliferation, and cytolytic activity of natural killer and T cells (Del Vecchio et al., 2007)
Interleukin 23 (IL-23) [NM_016584]	✓ Induces autoimmunity (Gaffen et al., 2014) ✓ Induces tissue destruction (Gaffen et al., 2014)

6.2.8 Immunocytochemistry

Cyotspin slides prepared using PBMCs harvested at 24 hours of initial incubation were used for immunocytochemical staining of CXCR-4 and SDF-1. Anti-human monoclonal CXCR-4 antibody raised in mouse (Santa Cruz sc-53534, Texas, USA) and anti-human polyclonal SDF-1 antibody raised in rabbit (Santa Cruz sc-28876) were used as sources of primary antibodies. Fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG raised in goat (Santa Cruz sc-2010) and peridinin chlorophyll protein

complex with cyanin-5.5 (PerCP-Cy5.5) anti-rabbit IgG raised in goat (Santa Cruz sc-45101) were used as secondary antibodies. Acetone (Sigma-Aldrich) fixed air dried slides were treated with dilution buffer [DPBS (Gibco) containing 10% FBS (Gibco)] and incubated in room temperature for 30 minutes to block unspecific binding. Then the slides were treated as follows: washed three times with DPBS (Gibco) and incubated overnight with 100× diluted primary antibodies at 4°C in humidified chamber; washed three times with DPBS; incubated with the 200× diluted secondary antibodies for one hour at room temperature in the dark; washed three times with DPBS; and finally, counter stained with Fluoroshield with DAPI (Sigma) for nuclear staining.

6.2.9 Molecular network analysis

The corresponding Entrez Gene ID along with the up-regulated and down-regulated values of the 17 paracrine factors were imported into the core analysis tool of Ingenuity Pathway Analysis (IPA) (Ingenuity systems; www.ingenuity.com) to predict activation or inhibition of biological functions, and signalling pathways by AuHS and basal medium secretomes compared to FBS secretome. Up- or down-regulation of paracrine factors expression in the AuHS secretome and the basal medium secretome were calculated by comparing with that in the FBS secretome, and the values were presented in mean fold change. If any cytokine was not expressed, the lowest value that could detected by the multiplex analyser (Luminex, Texas, USA) is considered as the value of its expression. Positive fold change values denote up-regulation, and negative values denote down-regulation. Activation and inhibition of biological functions were predicted by the 'z-score' calculated by IPA on the basis of the expression pattern of downstream transcriptional regulator in IPA software. Positive and negative z-score indicates activation or inhibition of biological function respectively. Z-score greater than +1.96 or smaller than -1.96 were considered significant ($p<0.05$).

The corresponding Entrez Gene ID of the 17 paracrine factors imported into the core analysis tool of IPA were also used to calculate significant association between genes and to identify functional networks. IPA utilises Fisher's exact test indicate significant association ($p = 0.05$). IPA also predicts the activation and inhibition of other factors involved in this network through paracrine factors' fold change values.

6.2.10 Data analysis

Except for molecular network analysis, all other data were analysed using ANOVA and Tukey's HSD post-hoc (SPSS version 22). The significant level was set at $p < 0.05$.

6.3 Results

6.3.1 Cell viability

In FBS supplemented medium, the number of live PBMCs at 96 hours was found to decrease significantly compared to that at 24 and 48 hours ($p<0.05$). While in the AuHS supplemented medium, the number of live PBMCs did not show any significant decrease at 96 hours of incubation compared to that at 24 hours, 48 hours or 72 hours. Number of live PBMCs in basal medium gradually decreased and was reduced by 70 % of the initial count at 96 hours of incubation (Figure 6.2A).

In FBS supplemented medium, the cell viability of the PBMCs that was measured using PrestoBlue® (Invitrogen) showed a significant gradual decrease until 96 hours ($p<0.01$) of incubation compared to that of the initially seeded PBMCs. However, the cell viability of PBMCs in AuHS supplemented medium showed no significant change during the 96 hours of incubation. Cell viability of PBMCs in basal medium decreased significantly after 24 hours of incubation ($p<0.01$) and maintained until 96 hours of incubation (Figure 6.2B).

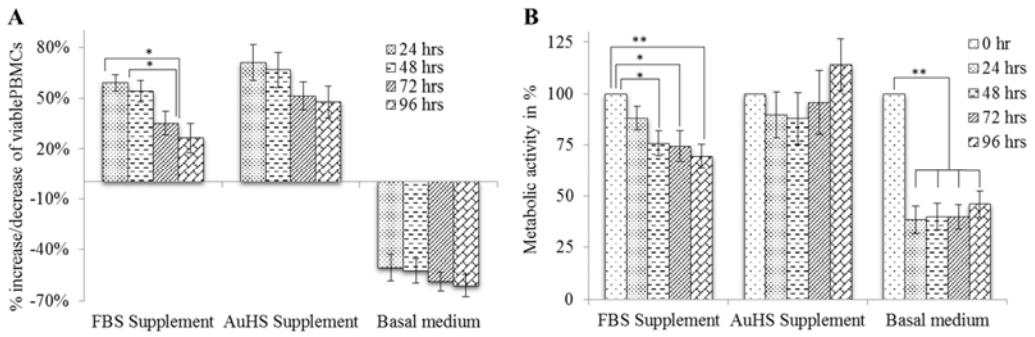

Figure 6.2: Effect of serum supplement on the number of live cells and cell viability of PBMCs. (A) Number of live PBMCs, as detected by the trypan blue Exclusion method, did not decrease significantly in AuHS supplement culture until 96 hours. While, in FBS supplemented medium, the number of live PBMCs at 96 hours decreased significantly compared to that at 24 and 48 hours. In basal medium number of live PBMCs gradually decreased and was reduced by 70 % of the initial count at 96 hours. (B) Cell viability of PBMCs, as measured by PrestoBlue® assay, remained unchanged in AuHS supplement culture up to 96 hours. In FBS supplemented medium, the cell viability of the PBMCs showed a significant gradual decrease until 96 hours compared to that of the initially seeded cells. In basal medium, cell viability decreased significantly after 24 hours and remain same until 96 hours (* = $p<0.05$, ** = $p<0.01$, n=7)

The number of dead PBMCs was also assessed by using trypan blue dye exclusion method. Results showed that percentage of dead cells were significantly higher in basal medium compared to that in AuHS and FBS supplemented media (Figure 6.3). Though there was no significant difference in the percentage of dead cells in AuHS supplemented medium compared to that in FBS supplemented medium, the percentage of dead cells were lower in AuHS supplementation compared to that in FBS supplementation (Figure 6.3).

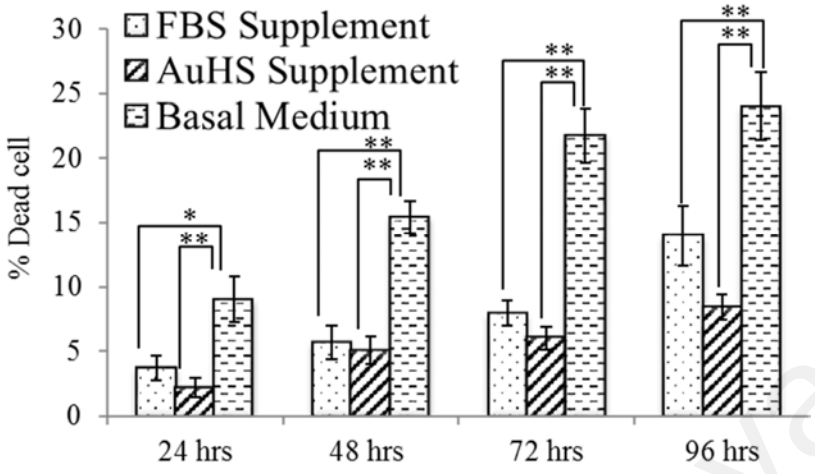

Figure 6.3: Effect of different media supplement on death of PBMCs *in vitro*. Apoptosis was significantly higher in basal medium compared to medium with FBS and AuHS supplementation. There was no significant difference between the percentage of apoptotic PBMCs in culture with FBS and AuHS supplementation. However, percentage of apoptotic PBMCs was lower in AuHS supplemented culture compared to that of FBS supplemented culture. (* = $p<0.05$, ** = $p<0.01$, n=6)

6.3.2 Effect of serum supplement on the differentiation of peripheral blood mononuclear cells

No significant differences in the percentage of lymphocytes and progenitors, and monocytes and granulocytes of total cell were observed among the cells cultured in FBS supplemented medium, AuHS supplemented medium and medium without any supplementation, and between the cells at 24 and 96 hours of incubation (Figure 6.4). However, the percentage of lymphoid and progenitors were slightly higher especially at 96 hours of incubation in PBMCs culture with AuHS supplementation compared to that of FBS supplementation (Figure 6.4).

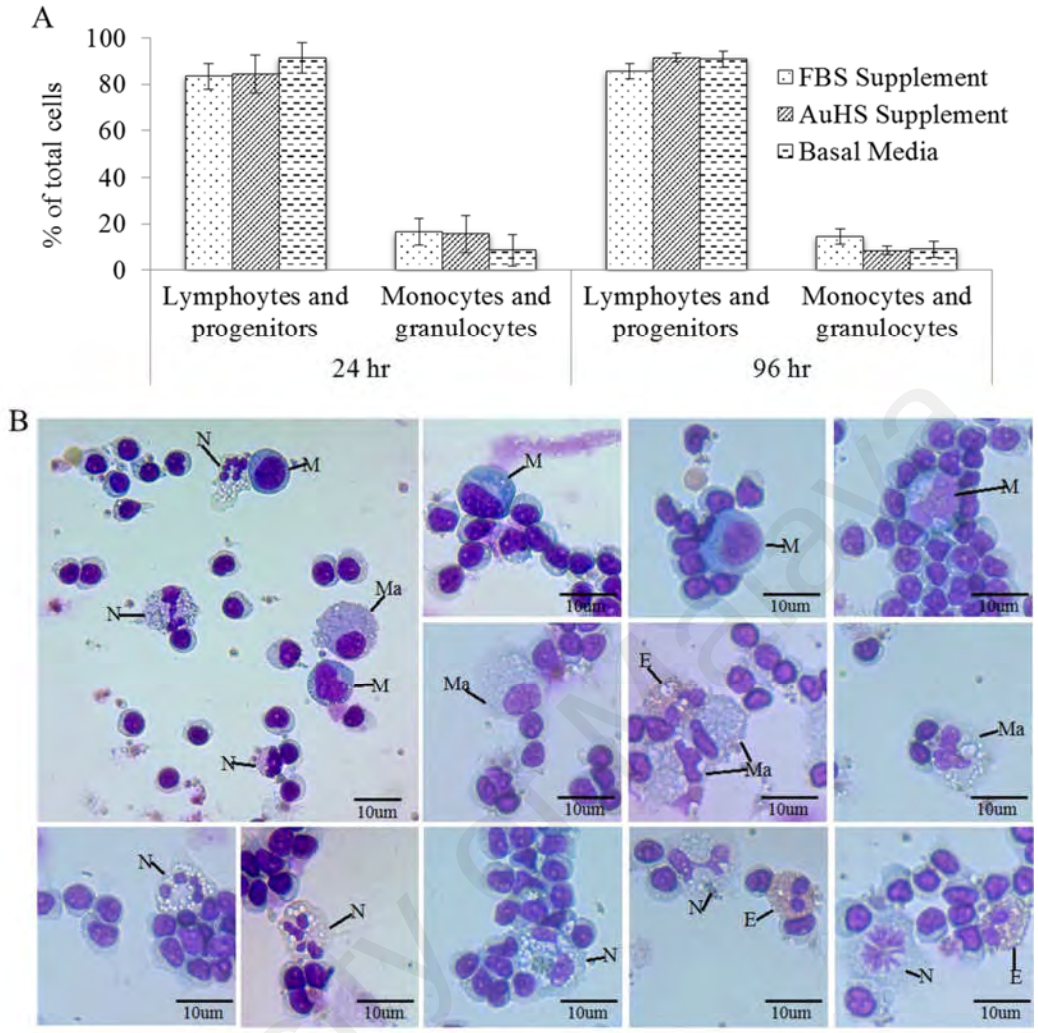

Figure 6.4: Differential count of PBMCs (n=3) at 24 and 96 hours of incubation. (A) No significant difference in the percent of lymphocytes and progenitors, and monocytes and granulocytes of total PBMCs was seen among the cells cultured in media with FBS supplementation, AuHS supplementation and medium without any supplementation; and between the cells at 24 and 96 hours of incubation. However, the percentage of lymphoid and progenitors were slightly higher especially at 96 hours of incubation in PBMCs culture with AuHS supplementation compared to that of FBS supplementation. (B) Cells were counted on the basis of their morphology (M, Monocytes; Ma, Macrophage; N, Neutrophils; E, Eosinophils, all other cells are lymphocytes).

6.3.3 Paracrine factors profile in the secretomes

Seventeen paracrine factors were analysed in the secretomes at the 24th and 96th hour of PBMCs cultures. Relative expression of paracrine factors in different media using the multiplex data was digitized and presented in Figure 6.5. Secretomes of

PBMCs, which were cultured with either AuHS supplement or without any serum supplement (basal medium), were collectively found to contain all 17 paracrine factors. SDF-1A was not detected in any of the secretome of PBMCs cultured with FBS supplement, while other 16 paracrine factors were detected in secretomes of at least 1 donor (Figure 6.5). However, the amount of expressed paracrine factors varied from one individual to another.

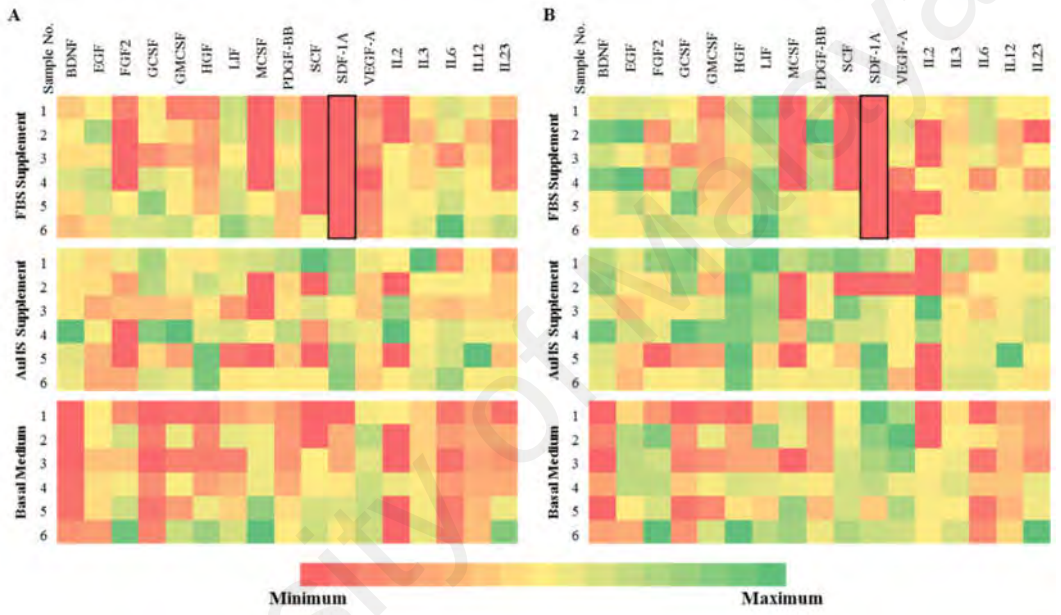

Figure 6.5: Relative expression of paracrine factors in the secretomes of the PBMCs cultured with different serum supplement. Supernatants were collected at 24 hours (A) and 96 hours (B) of incubation. The mean cytokine concentrations (picograms per millilitre) were used to prepare the cytokine portrait. Dark red square indicates that the corresponding paracrine factors were below detection limit of the assay; dark green square indicates the highest expression of the corresponding paracrine factors (n=6). (For detail see Appendix A, B)

6.3.4 Comparative paracrine factors expression in the secretomes

Comparative expressions of the selected paracrine factors in the secretomes collected from three different culture conditions at different incubation time were analysed.

6.3.4.1 AuHS vs. FBS secretome

At 24 hours, significantly higher expressions of HGF ($p<0.05$) and VEGF-A ($p<0.05$) were found in the AuHS secretome compared to the FBS secretome (Figure 6.6). Compared to the FBS secretome, the expression of HGF ($p<0.01$) was significantly higher in the AuHS secretome at 96 hours (Figure 6.6). Whereas the expression of EGF ($p<0.05$) was significantly higher in FBS secretome when compared to AuHS secretome at 24 hours (Figure 6.6). Notably, unlike in AuHS secretome, SDF-1A was not detected in FBS secretome either at 24 or 96 hours (Figure 6.6).

6.3.4.2 AuHS vs. basal medium secretome

At 24 hours, significantly higher expressions of G-CSF ($p<0.01$), BDNF ($p<0.01$) and PDGF-BB ($p<0.05$) were found in the AuHS secretome compared to the basal medium secretome (Figure 6.6). At 96 hours, significantly higher expressions of G-CSF ($p<0.05$), BDNF ($p<0.01$) and HGF ($p<0.01$) were found in the AuHS secretome compared to the basal medium secretome (Figure 6.6). Whereas, only VEGF-A expression was significantly higher in the basal medium secretome when compared to the AuHS secretome at both 24 hours ($p<0.05$) and 96 hours ($p<0.01$) (Figure 6.6).

6.3.4.3 FBS vs. basal medium secretome

At 24 hours, significantly higher expression of G-CSF ($p<0.05$), IL-6 ($p<0.05$) and EGF ($p<0.05$) were detected in the FBS secretome compared to basal medium secretome (Figure 6.6). Compared to basal medium secretome at 96 hours, significantly higher expression of IL-6 ($p<0.05$), BDNF ($p<0.05$) and PDGF-BB ($p<0.01$) were seen in the FBS secretome (Figure 6.6). Significantly higher expression of VEGF-A ($p<0.01$) was observed in basal medium secretome when compared to that of the FBS secretome at both 24 hours and 96 hours (Figure 6.6). Unlike in basal medium secretome, SDF-1A

was not detected in FBS secretome at either 24th or 96th hour of incubation (Figure 6.6).

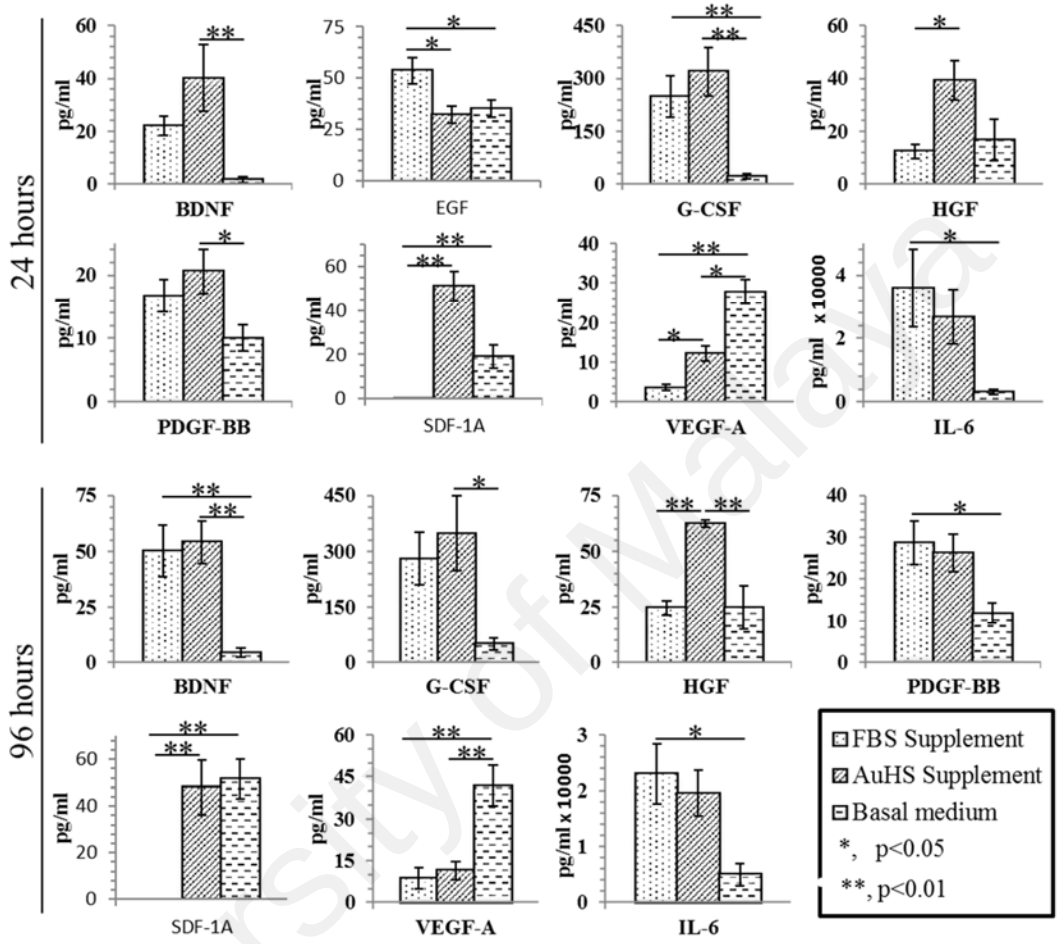

Figure 6.6: Comparative cytokine amount in the secretomes of PBMCs cultured with different serum supplement. Difference in the amount of paracrine factors namely, G-CSF, IL-6, BDNF, EGF, HGF, PDGF-BB, VEGF-A, SDF-1A in the secretomes collected from PBMCs cultured in three different media composition at 24 hours and 96 hours of incubation. (* = $p<0.05$, ** = $p<0.01$, n=6)

6.3.5 Immunocytochemistry

Distinctly markedly higher expression of both SDF-1 and receptor, i.e., CXCR-4 were observed in the PBMCs cultured in AuHS medium compared to that cultured in FBS medium (Figure 6.7). Prominent colocalization of SDF-1 and its receptor CXCR-4 was also observed in the PBMCs cultured in AuHS medium as well (Figure 6.7).

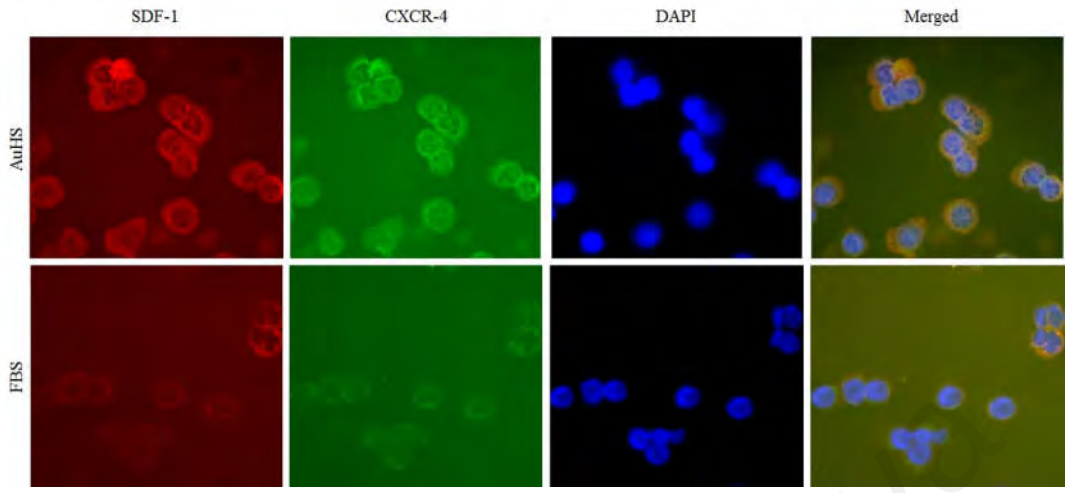

Figure 6.7: Immuno-histochemical staining of CXCR-4 and SDF-1 in PBMCs harvested from AuHS and FBS media at 24 hours of initial incubation. Fluorescent immunostaining of PBMCs cultured in AuHS and FBS supplemented media was done against CXCR-4 and SDF-1 using mouse anti-human monoclonal and rabbit anti-human polyclonal antibodies respectively. Fluorescein isothiocyanate (FITC, green) and peridinin chlorophyll protein complex with cyanin-5.5 (PerCP-Cy5.5, red) conjugated with goat anti-mouse and goat anti-rabbit IgG respectively were used as secondary antibodies. DAPI was used to perform nuclear staining.

6.3.6 Relative up or down regulation of cytokine expression in AuHS and basal medium secretomes compared to FBS secretome

Fold change values showed that after 24 hours of incubation, 13 out of 17 paracrine factors (BDNF, G-CSF, GM-CSF, HGF, LIF, M-CSF, PDGF-BB, SCF, SDF-1A, VEGF-A, IL-2, IL-3, and IL-12p70) expressed more in AuHS secretome, and 9 paracrine factors (FGF-2, HGF, LIF, M-CSF, SCF, SDF-1A, VEGF-A, IL-3, and IL-23) were expressed more in basal medium secretome compared to that in FBS secretome. After 96 hours of incubation, except for IL-6, EGF and PDGF-BB the other 14 paracrine factors were highly expressed in AuHS secretome compared to that in FBS secretome. Expression of 11 paracrine factors (FGF-2, GM-CSF, HGF, LIF, M-CSF, SCF, SDF-1A, VEGF-A, IL-2, IL-3, and IL-23) in basal medium secretome were recorded higher than that in FBS secretome at 96 hours (Table 6.2).

Table 6.2: Relative changes of paracrine factors concentration in the secretomes of PBMCs cultured in AuHS supplemented medium or in basal medium compared to that in FBS supplemented medium

Paracrine factors	24 hours		96 hours	
	AuHS *vs.* FBS	basal medium *vs.* FBS	AuHS *vs.* FBS	basal medium *vs.* FBS
BDNF	1.82	-12.49	1.07	-11.96
EGF	-1.66	-1.53	-1.72	-1.36
FGF-2	-1.02	3.71	1.23	2.55
G-CSF	1.29	-11.32	1.24	-5.73
GM-CSF	1.77	-1.09	2.35	1.32
HGF	3.16	1.34	2.54	1.01
LIF	1.74	1.50	1.73	1.15
M-CSF	1.10	5.28	1.19	4.07
PDGF-BB	1.23	-1.67	-1.09	-2.42
SCF	11.39	6.49	10.49	8.36
SDF-1A	13.46	5.03	12.63	13.58
VEGF-A	3.41	7.79	1.33	4.80
IL-2	3.04	-1.66	6.56	1.73
IL-3	1.71	1.07	1.38	1.38
IL-6	-1.34	-11.75	-1.18	-4.62
IL-12p70	2.52	-2.76	3.36	-1.71
IL-23	-1.31	1.66	1.85	1.98

Positive and negative values denote up- and down-regulation respectively (As SDF-1A was not expressed in FBS secretome, to calculate fold change minimum detection limit of SDF-1A was considered as the value of SDF-1A expression in FBS secretome. n=6).

6.3.7 Biological functions regulated by paracrine factors secreted from human PBMCs

Changes in the expression of paracrine factors (as fold change values, Table 6.2) along with the Entrez Gene IDs corresponding to the 17 paracrine factors (Table 6.1) were imported to IPA to identify the most significant molecular networks and signalling pathways relevant to these paracrine factors, and their role in maintaining biological functions. Ten most important biological functions related to the proliferation, viability, apoptosis, differentiation, and quantity of PBMCs were taken in consideration (Figure 6.8).

Predicted 'z-score' of the sorted functions have shown that AuHS secretomes collected at 24 and 96 hours of incubation may support better proliferation, viability, differentiation. Predicted 'z-score' have also shown that AuHS secretomes inhibit

apoptosis. Significant activation or inhibition of any selected biological functions have not been predicted for basal medium secretome collected at 24 hours compared to that of the FBS secretome. Whereas, at 96 hours of incubation, significant activation of viability of leukocytes and mononuclear cells have been predicted (Figure 6.8).

Biological Functions (p value)	Cytokines analyzed																	Activation (↑) Inhibition(↓)			
																		AuHS		BM	
	BDNF	EGF	FGF2	G-CSF	GM-CSF	HGF	LIF	M-CSF	PDGF-BB	SCF	SDF-1A	VEGF-A	IL-2	IL-3	IL-6	IL-12	IL-23	24 hr	96 hr	24 hr	96 hr
Proliferation of immune cells (1.86E-14)	-	-	-	√	√	-	-	√	-	√	√	-	√	√	√	√	√	↑	↑	-	-
Proliferation of hematopoietic progenitor cells (6.39E-14)	-	-	-	√	√	-	-	-	-	√	√	-	√	√	√	-	-	-	-	-	-
Cell viability of leukocytes (1.50E-19)	-	√	-	√	√	√	-	√	-	√	√	-	√	√	√	-	-	↑	↑	-	↑
Cell viability of mononuclear cells (4.51E-17)	-	√	-	-	√	√	-	√	-	√	√	-	√	-	√	-	-	↑	↑	-	↑
Apoptosis of leukocytes (2.32E-12)	-	-	-	√	√	-	-	√	-	√	√	-	√	√	√	-	-	↓	↓	-	-
Apoptosis of hematopoietic progenitor cells (2.23E-10)	-	-	-	√	√	-	-	-	-	√	-	-	-	√	√	-	-	↓	↓	-	-
Differentiation of leukocytes (4.07E-18)	-	-	-	√	√	√	√	√	-	√	-	-	√	√	√	-	√	-	↑	-	-
Differentiation of hematopoietic progenitor cells (2.83E-14)	-	-	-	√	√	-	-	√	-	√	√	-	√	√	√	-	-	-	-	-	-
Quantity of leukocytes (8.75E-11)	-	-	-	√	√	-	-	-	-	√	-	-	√	√	√	-	-	↑	↑	-	-
Quantity of hematopoietic progenitor cells (8.34E-14)	-	-	-	√	√	-	-	-	-	√	-	-	√	√	-	-	-	↑	↑	-	-

Activation (↑) and inhibition (↓) were measured by the positive and negative z-scores. z-score > +1.96 or < -1.96 were considered significant (n=6). [√, detected; -, unchanged or not detected]

Figure 6.8: Predicted activation or inhibition of biological functions maintained by the 17 analysed paracrine factors in AuHS secretome and basal medium (BM) secretome compared to FBS secretome.

The proliferation, viability, and migration of cells are vital in regenerative therapy. For this, the potential of AuHS secretome in supporting the proliferation, viability, and migration of cells along with inhibition of apoptosis have also been considered. Predicted 'z-scores' of the corresponding biological functions support the superiority of AuHS secretome over FBS secretome and basal medium secretome for regenerative therapy (Table 6.3).

Table 6.3: Predicted activation or inhibition of important biological functions involved in regeneration maintained by the 17 analysed paracrine factors in AuHS secretome and basal medium secretome compared to FBS secretome. Positive value indicates activation and negative value denotes inhibition. Z-score greater than +1.96 or smaller than -1.96 were considered significant (n=6)

Biological functions	**AuHS**		**BM**	
Involved paracrine factors	**24 hours** (z-score)	**96 hours** (z-score)	**24 hours** (z-score)	**96 hours** (z-score)
Cellular proliferation (p=1.56E-15)				
BDNF, M-CSF, GM-CSF, G-CSF, SDF-1A, EGF, FGF-2, HGF, IL-12A, IL-2, IL-23A, IL-3, IL-6, SCF, LIF, PDGF-B, VEGF-A	1.976	2.434	0.380	1.489
Cell viability (p=7.04E-20)				
BDNF, M-CSF, GM-CSF, G-CSF, SDF-1A, EGF, FGF-2, HGF, IL-2, IL-3, IL-6, SCF, LIF, VEGF-A	2.352	2.919	0.766	1.890
Apoptosis (p=2.13E-13)				
M-CSF, GM-CSF, G-CSF, SDF-1A, EGF, FGF-2, HGF, IL-2, IL-3, IL-6, SCF, LIF, PDGF-B, VEGF-A	-1.552	-2.699	-1.334	-1.968
Cells movement (p=8.56E-17)				
BDNF, M-CSF, GM-CSF, G-CSF, SDF-1A, EGF, FGF-2, HGF, IL-2, Il-3, Il,-6, SCF, LIF, PDGF-B, VEGF-A	2.041	2.026	0.576	1.070
Migration of cells (p=1.49E-15)				
BDNF, M-CSF, GM-CSF, G-CSF, SDF-1A, EGF, FGF-2, HGF, IL-2, IL-3, IL-6, SCF, PDGF-B, VEGF-A	1.804	1.785	0.386	0.922

6.3.8 Activation of high-mobility group box 1 protein (HMGB1) signalling pathway

IPA identified a number of signalling pathways that could be influenced by the 17 paracrine factors analysed in the study. Names of the top ten pathways being affected (most to least) by the analysed paracrine factors are listed in Table 6.4. Higher –log p value denotes higher chance of being regulated by the paracrine factors analysed in this study. While the ratio indicates the dividend of presence of the analysed paracrine factors to the total number of paracrine factors involved in the corresponding pathway. IPA also predicts the activation and inhibition of a particular signalling pathway on the basis of the changes in the cytokine expression. By using the fold change values, (Table 6.2) IPA predicted that AuHS secretome collected at 24 and 96 hours have the potential to activate HMGB1 signalling pathway (z=1.633) over that of FBS secretome.

Table 6.4: Top ten signalling pathways identified by IPA that could be regulated by 17 analysed paracrine factors

No.	Pathways	-log p	Ratio
1	Hematopoiesis from Pluripotent Stem Cells	1.96E01	2.05E-01
2	Hematopoiesis from Multipotent Stem Cells	1.57E01	5E-01
3	Role of Cytokines in Mediating Communication between Immune Cells	1.38E01	1.35E-01
4	Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	1.15E01	2.78E-01
5	Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	1.02E01	7.41E-02
6	Hepatic Fibrosis / Hepatic Stellate Cell Activation	9.62E00	3.57E-02
7	HMGB1 Signalling	9.16E00	5.08E-02
8	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	9.13E00	5.04E-02
9	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	8.46E00	2.44E-02
10	Hepatic Cholestasis	8.39E00	3.8E-02

6.3.9 Functional molecular networks of paracrine factors secreted from human PBMCs

Using the Entrez Gene IDs corresponding to the 17 paracrine factors, IPA identified the significant molecular networks relevant to these paracrine factors. Based on the functional molecular network analysis, most significant (p=1.00E-33) network identified as 'cell-to-cell signalling and interaction, cellular growth and proliferation, cellular development' was shown to involve 15 out of the 17 paracrine factors analysed in this study (Figure 6.9).

All the above results support the superiority of AuHS supplemented medium over FBS supplemented medium and basal medium. Notably, in terms of proliferation (Figure 6.2A), viability (Figure 6.2B) and secretome composition AuHS supplement for 24 and 96 hours did not show any significant difference. To compare the difference between them ranked 1 functional network was further analysed (Figure 6.9). Using the Entrez Gene IDs and changes in the expression of paracrine factors in the AuHS secretome collected at 96th hour compared to the secretome collected at 24 hours, IPA also predicted the activation or inhibition of other factors involved in the pathway. From the functional pathway it is found that nuclear factor kappa B (NFκB), extracellular-

signal-regulated kinases (ERK) 1/2, focal adhesion kinase (FAK), AKT, heat shock protein (HSP) 27, high-affinity IgE receptor (Fcer1) and cAMP response element-binding protein (Creb) were predicted to be mildly inhibited in the AuHS secretome collected at 96 hours of incubation compared to AuHS secretome collected at 24 hours of incubation. Strong inhibition of the c-Jun N-terminal kinase (Jnk), P13KP85 and Collagenase type 1 have also been predicted in the AuHS secretome collected at 96th hour. Meanwhile, moderate activation of MEK and Nr1h, and strong activation of MAPK and CD3 have been predicted in the AuHS secretomes collected at 96 hours (Figure 6.9).

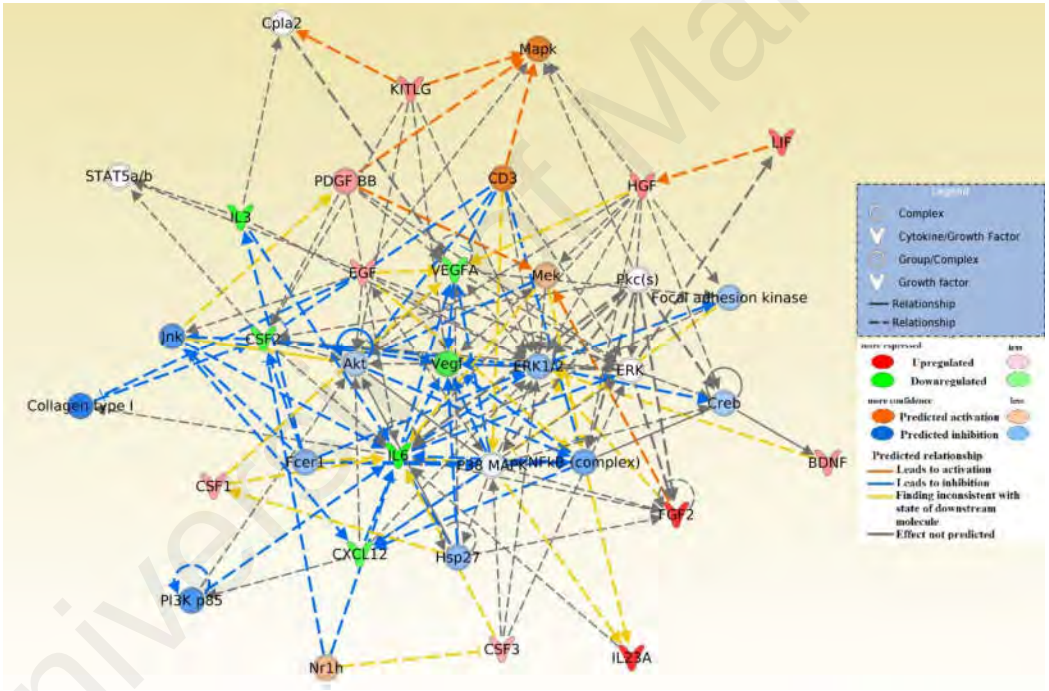

Figure 6.9: Ranked 1 Functional Network illustration to compare between AuHS secretome collected at 24 hours and 96 hours of incubation. Functional pathway predicted mild inhibition of nuclear factor kappa B (NFκB), extracellular-signal-regulated kinases (ERK) 1/2, focal adhesion kinase (FAK), AKT, heat shock protein (HSP) 27, high-affinity IgE receptor (Fcer1) and cAMP response element-binding protein (Creb), and strong inhibition of the c-Jun N-terminal kinase (Jnk), P13KP85 and Collagenase type 1 in the AuHS secretome collected at 96 hours compared to that of 24 hours. Meanwhile, mild activation of MEK and Nr1h, and strong activation of MAPK and CD3 have been predicted in the AuHS secretome collected at 96 hours. Functional pathway was predicted by IPA (Ingenuity systems; www.ingeniuty.com)

6.4 Discussion

Composition of cytokines and other autocrine/paracrine factors in secretomes, vital to maintain the survival and proliferation of cultured cells, largely depends on the initial media composition as well as type of the cultured cells. Animal serum especially FBS has been widely used to supplement media, as it provides proteins, growth factors, hormones, lipids, vitamins, and other important trace elements in culture (Gstraunthaler, 2003). However, FBS can be a vehicle of disease transmission, and can trigger immune response for its Neu5GC antigen (Haque et al., 2015; Lindroos et al., 2009). Serum free media supplemented with recombinant human protein or purified animal serum proteins has been used as an alternative to FBS in cell culture (Haque et al., 2015; Lindroos et al., 2009). A number of purified or recombinant proteins given as supplement in serum free media were shown to affect paracrine secretions that in turn might exert feedback inhibition of the proteins (Mirshahi et al., 2000).

This study investigated the potential of AuHS compared to FBS supplement in producing secretomes while maintaining proliferation and viability of PBMCs *in vitro*. Compared to FBS supplement, AuHS supplement supported *in vitro* PBMCs viability for a longer period of time (Figure 6.2). Secretomes' cytokine profile showed that FBS secretome collected at 24 hours had higher EGF compared to that of AuHS secretome and basal medium secretome. While in AuHS secretome, significantly higher expression of HGF and SDF-1A (at 24 and 96 hours), and VEGF-A (at 24 hours) were detected compared to that of FBS secretome (Figure 6.6). For decades higher expression of EGF is considered as a biomarker of carcinogenesis (Li et al., 2005). On the other hand, both HGF and VEGF-A play important roles in angiogenesis, cell proliferation, anti-apoptosis, chemotaxis, cell growth and differentiation (Galimi et al., 2001; Sulpice et al., 2009; Wang et al., 2006). Again, SDF-1A that is found in AuHS secretome, plays an important role in cell migration, cellular differentiation, cell cycle regulation and

survival (Cheng et al., 2014). Expressions of SDF-1 and its receptor CXCR-4 were further confirmed by double immunostaining (Figure 6.7) and the result confirmed that there was increased expression as well as co-localization of both markers in PBMCs cultured in AuHS compared to that in FBS. Therefore, higher *in vitro* PBMCs viability in AuHS supplemented media might be linked with the secretome composition, i.e., higher amount of HGF, VEGF-A and SDF-1A as well as lower amount of EGF.

Lichtenauer et al. (2011) reported the presence of VEGF, HGF, FGF-2, SDF-1, G-CSF and GM-CSF in the secretomes of both the viable and the induced apoptotic PBMCs secretomes harvested at 24 hours. Consistently, similar paracrine factors were detected in the PBMCs secretome harvested from AuHS supplemented media. In the current study, significantly higher expressions of G-CSF ($p<0.01$) was found in the AuHS secretome compared to the basal medium secretome (Figure 6.6) at 24 hours. At 96 hours, significantly higher expressions of G-CSF ($p<0.05$) and HGF ($p<0.01$) were also found in the AuHS secretome compared to the basal medium secretome (Figure 6.6). Meanwhile, only VEGF-A expression was significantly higher in the basal medium secretome when compared to the AuHS secretome at both 24 hours ($p<0.05$) and 96 hours ($p<0.01$). Hoetzenecker et al. (2013) reported the reduction in the proinflammatory cytokine IL-6 in blood plasma of PBMCs secretome treated animals. In the current study, IL-6 was detected in the secretomes in the order of FBS>AuHS>basal medium.

To elucidate the effect of serum supplement on the differentiation of PBMCs, differential count was done by staining cytospin slides of PBMCs. Apparently, there is no effect of AuHS or FBS supplementation on the differential count of lymphocytes, monocytes, and granulocytes (Figure 6.4). However, at 96 hours of incubation the percentage of lymphoid cells and their progenitors were slightly higher in PBMCs

cultured with AuHS supplementation compared to that of FBS supplementation (Figure 6.4). Besides, percentage of dead PBMCs was also lower in AuHS supplemented culture compared to that of FBS supplemented culture (Figure 6.3). These results complement the notion that AuHS might offer additional advantage to maintain the lymphoid development and differentiation, and inhibit apoptosis of PBMCs, most likely due to the presence of paracrine factors especially SDF-1 in the secretome (Lataillade et al., 2004).

Prediction on the biological function analysis (Figure 6.8) also supports the *in vitro* PBMCs viability and proliferation in AuHS supplemented medium (Figure 6.2). Predicted biological functions also showed the potential of AuHS secretome in supporting the proliferation, viability, and migration of cells along with inhibition of apoptosis (Table 6.3). Therefore, it is expected that AuHS secretome might favour *in vivo* regeneration of organ by increasing proliferation, viability, and migration of stem cells and progenitor cells. Besides, our results also showed that the optimum *in vitro* culture condition for PBMCs could be achieved by autologous serum supplement instead of the bovine serum supplement.

Prediction on activation of signalling pathway showed the AuHS secretome favours activation of HMGB1 signalling pathway more than that of FBS secretome (Table 6.4). HMGB1 plays a key role in maintaining tissue homeostasis. At the same time, it helps to clean damaged and infected tissues, provides protection to non-injured tissues, and accelerates the process of regeneration (Tamai et al., 2011) of tissues such as skeletal muscle (Campana et al., 2014; Dormoy-Raclet et al., 2013), epithelial (Tamai et al., 2011), and spinal cord (Dong et al., 2013). In 2012 Schiraldi et al. reported the role of SDF-1 and CXCR-4 in the HMGB1 mediated inflammatory cell recruitment at the site of damaged tissue (Schiraldi et al., 2012) which is vital for tissue repair and

regeneration. While other studies have reported the expression of CXCR-4 by G-CSF (Saba et al., 2014), SCF and IL-6 (Peled et al., 1999) being induced in hematopoietic stem cells. Expression of these paracrine factors in the AuHS and FBS secretome may be involved in the expression of CXCR-4 in the PBMCs cultured in both AuHS and FBS supplemented media (Figure 6.7). Nonetheless, higher expression of G-CSF and SCF (Table 6.2) in AuHS secretome might be the reason behind the higher expression of CXCR-4 receptor in PBMCs cultured in AuHS medium compared to cells cultured in FBS medium. Moreover, distinctly higher expression of SDF-1 was observed in PBMCs cultured in AuHS medium compared to that in FBS medium (Figure 6.7).

The suitability of AuHS supplemented medium over FBS supplemented medium and basal medium in terms of proliferation (Figure 6.2 A), viability (Figure 6.2 B) and secretome composition (Table 6.2) has been shown. Both the secretomes collected at 24 and 96 hours exhibited equal potential in proliferation, viability and secretome composition when prepared with AuHS supplement.

The functional network analysis, performed by IPA analysis showed a mild inhibition of NFκB, ERK1/2, FAK, AKT, HSP27, Fcer1 and Creb, and a strong inhibition of Jnk, P13KP85 and Collagenase type 1 in the AuHS secretome collected at 96 hours compared to that of 24 hours (Figure 6.9). In other words, these functional networks could be more activated at 24 hours in AuHS supplemented media. Notably, ERK1/2 is involved in liver regeneration (Chen et al., 2013b; Li et al., 2011) and Schwann cell proliferation (Seo et al., 2009); Creb plays a positive role in liver regeneration (Rudnick et al., 2001); AKT and FAC are involved in muscle regeneration (Kim et al., 2011; Quach et al., 2009); PI3K and AKT accelerates axonal regeneration (Zhang et al., 2014), as well as they play important roles in mesenchymal stem cells survival, proliferation, migration, angiogenesis, cytokine production and differentiation

(Chen et al., 2013a). A mild activation of MEK and Nr1h, and a strong activation of MAPK and CD3 were predicted in the AuHS secretome collected at 96 hours. Thus, AuHS secretome collected at 24 hours of incubation might be more advantageous in regenerative therapy compared to that collected at 96 hours. Further investigation on the activation of the predicted signal transduction pathway is required to attest the advantage of using AuHS to propagate stem cells for regenerative therapy.

6.5 Conclusion

This study showed that *in vitro* AuHS supplement favours cell viability of PBMCs up to 96 hours of incubation. AuHS also maintain the differentiation potential of PBMCs *in vitro*. Compared to FBS, AuHS supplement favours regenerative cytokine synthesis as well. For example, SDF-1A, the cytokine responsible for cell migration, cellular differentiation, cell cycle regulation and survival was detected in AuHS secretome but not in FBS secretome. CXCR-4, the SDF-1 receptor was also highly expressed in the cells cultured with AuHS supplementation compared to that in FBS supplementation. In addition, HGF and VEGF-A which are involved in angiogenesis, cell proliferation, inhibition of apoptosis and immunoregulation were up-regulated in the AuHS secretome.

CHAPTER 7: CONCLUSION

We attempted to find out the potential of pHS and AuHS in producing SHED and secretome respectively with more regenerative properties while minimizing the load of xeno-contamination and unusual paracrine effects. Self-renewal, differentiation potential, and site specific migration are vital for the successful cell-based regenerative therapy. Aging and loss of migration potential of MSCs could affect engraftment and eventually the regeneration (Eggenhofer et al., 2014; Estrada et al., 2012; Estrada et al., 2013). Self-renewal potential and cellular morphology are important indicators of cellular aging (Colter et al., 2001; Haasters et al., 2009; Saller et al., 2012). On the other hand, composition of the paracrine factors is the main concern of the secretome. All these issues were taken in consideration in this study.

Within the limitations of this study, pHS and AuHS have been shown to be a suitable alternative for FBS in producing clinical grade MSCs such as SHED and secretome with better regenerative potential. This conclusion was drawn on the basis of the followings:

i. Proliferation of SHED in pHS were significantly higher compared to that in FBS.
ii. A trend of higher numbers of rapidly proliferating cells (RS) and significantly lower number of partially or terminally differentiated cells (FC) were observed in SHED expanded in pHS media compared to that FBS media.
iii. The pattern of migration of SHED in pHS media was more structured and directional.
iv. SHED maintain their immunomodulatory properties when expanded in pHS.
v. Lower inflammatory microenvironment was observed in pHS-SHED and monocytes/macrophages or lymphocytes co-cultures compared to that of

FBS-SHED and monocytes/macrophages or lymphocytes co-cultures, however the experimental data was not statistically significant.

vi. AuHS supplementation favours *in vitro* viability of PBMCs up to 96 hours of incubation.

vii. AuHS maintain the differentiation potential of PBMCs *in vitro*.

viii. Compared to FBS, AuHS supplement favours regenerative cytokine synthesis.

7.1 Study limitations

While analysing the effect of pHS, prepared using blood irrespective of the donors' blood group to determine its suitability in producing clinical grade MSCs from SHED and to evaluate the potential of AuHS in producing secretome from PBMCs, the current study encountered the following limitations:

i. We expanded SHED in pHS at passage 3, due to low cell yield from the primary isolation. As SHED lose homogeneity over time expansion of SHED from passage 1 would have been ideal.

ii. Results on the complement deposition could have been more conclusive to determine the status of viable cells in the presence of complement.

iii. Induced immune activation of the mononuclear cells could provide more precise explanation on the cytotoxicity assay of co-culture.

iv. Other aging markers (e.g., telomere length, beta-galactosidase activity) could be considered to measure the proliferation potential of SHED cultured in pHS and FBS.

v. Only histochemical staining was used to evaluate the differentiation of PBMCs, confirmation with flow cytometry would add value to the data.

vi. Additional logistic support could have been helpful to analyse the cells at hypoxic culture conditions.

7.2 Clinical significance

As discussed in the literature review, researchers have reported that the main shortcoming of cell-based therapy is low number of engrafted MSCs (Malliaras & Marban, 2011; Volarevic et al., 2011). Thus affecting the attainment of the long-term functional benefits of this therapy. Adaptation in recipient's own serum might prove to be beneficial and is not a difficult procedure. 20 ml of peripheral blood can be drawn for adaptation process and at the same time PBMCs could be isolated for secretome production. In a clinical situation, human serum could be easily obtained from the blood bank. We have shown that SHED, a type of MSCs survive in the presence of complement and immune cells when expanded in pHS. Our findings also showed that PBMCs secretomes in AuHS exhibit high regenerative potential. Coupling cell-based therapy with secretome might be beneficial in regeneration process.

MSCs from the dental pulp can be easily isolated and expanded. Dental pulp from deciduous teeth has been shown to be able to differentiate into various cell types (Bento et al., 2013; Su et al., 2016; Vasanthan et al., 2014). The regenerative potential of SHED is mainly attributed to the donor's age. It may be prudent to plan for teeth banking to address individual's or family members' future life threatening conditions.

7.3 Future studies

Suggestions for future studies are as follows:

i. Using hypoxic culture conditions along with the pHS to assess the effect of the combination on the regenerative potential of SHED.

ii. Validating the effect of pHS on the proliferation and morphology of SHED using other available techniques.

iii. Validating the effect of pHS on the migration of SHED using other available techniques (e.g., Boyden chamber assay, Radius™ cell migration assay etc.).

iv. Finding out the role of paracrine factors present in the human serum in regulating "Ephrin receptor signalling pathway".

v. Validating the effect of complement by analysing deposition on SHED.

vi. Conducting *in vivo* studies to determine the regenerative potential of SHED expanded in pHS.

vii. Conducting *in vitro* studies using the PBMCs secretome to determine their regenerative potential (e.g., expression of CXCR4 on the cell surface, proliferation of cells etc.).

viii. Conducting *in vivo* studies using the PBMCs secretome to determine their regenerative potential.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Published Articles

1. Aziz, J., Abu Kassim, N. L., Abu Kasim, N. H., **Haque, N.,** and Rahman, M. T. (2015). Carica papaya induces in vitro thrombopoietic cytokines secretion by mesenchymal stem cells and haematopoietic cells. *BMC Complement Altern Med, 15*, 215.
2. **Haque, N.,** Kasim, N. H., and Rahman, M. T. (2015). Optimization of pre-transplantation conditions to enhance the efficacy of mesenchymal stem cells. *Int J Biol Sci, 11*(3), 324-334.
3. **Haque, N.**, Rahman, M. T., Abu Kasim, N. H., and Alabsi, A. M. (2013). Hypoxic culture conditions as a solution for mesenchymal stem cell based regenerative therapy. *Sci World J, 2013*, 632972.

Papers presented

1. **Haque, N.,** Kasim, N. H., & Rahman, M. T. (2015). Secretome of Peripheral Blood Mononuclear Cells in Autologous Serum Supplemented Medium is Rich in Regenerative Cytokines. ICBHA-2nd GNOBB Conference, Nabab Nawab Ali Chowdhury Senate Bhaban, University of Dhaka, Bangladesh, 09-10 January 2015. Abstract ID 85, pp: 42-44.
2. **Haque, N.,** Kasim, N. H., & Rahman, M. T. (2015). Preparing Mesenchymal Stem Cell for Long Term Regenerative Benefit. ICBHA-2nd GNOBB Conference, Nabab Nawab Ali Chowdhury Senate Bhaban, University of Dhaka, Bangladesh, 09-10 January 2015. Abstract ID 44, pp:7-9.
3. **Haque, N.**, Rahman, M. T., Abu Kasim, N. H., and Alabsi, A. M. (2014). Autologous Human Serum Supports Better Proliferation, Viability and Homogeneity of Peripheral Blood Mononuclear Cells over Fetal Bovine Serum. International Conference on Emerging Trends in Scientific Research, Pearl International Hotel Kuala Lumpur, Malaysia, 15-16 March 2014. Article number ICETSR-352, pp: 411-416.

APPENDIX A

Expression of paracrine factors at 24 hours of incubation of PBMCs in AuHS, FBS and BM media.

Paracrine factor	Media	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
BDNF	AuHS	32.3	37.9	101.5	21.2	20.6	27.4
	FBS	14.2	19.3	38.8	20	87.1	16.9
	BM	7.2	1.7	1.7	<1.33	<1.33	<1.33
EGF	AuHS	22.3	24.8	47.6	23.5	39.1	36.7
	FBS	36.1	58.3	66.2	39.9	76.1	56.8
	BM	17.1	38.2	38.1	30	43.8	43.6
FGF-2	AuHS	4	<1.62	<1.62	5.1	3.5	11.4
	FBS	15	7.8	<1.62	<1.62	<1.62	1.6
	BM	38.2	18.4	9.9	5.3	16.5	2.3
G-CSF	AuHS	296.5	142.2	541	110	397.4	428
	FBS	284.8	490.1	282.6	52.2	188.7	191.8
	BM	39.3	19	46.8	<5.43	26.5	<5.43
GM-CSF	AuHS	132.3	31.7	425.6	49.3	94.9	90.5
	FBS	187	79.8	89.7	37.4	58.6	13.4
	BM	188.5	29	81.2	13.4	110.3	4.7
HGF	AuHS	60.1	62.1	31.3	22	37.6	22
	FBS	24.2	14.9	11.9	8.7	9.8	4.8
	BM	50.7	27.8	10.3	4.2	6	0.9
LIF	AuHS	4.6	0.6	7.4	1.9	6.3	7.1
	FBS	5.3	2.7	2.1	0.5	2.4	3
	BM	7.7	3.9	2.9	1	6.4	3.6
MCSF	AuHS	3.4	<1.98	4.2	<1.98	<1.98	17.3
	FBS	19.3	3.4	<1.98	<1.98	<1.98	<1.98
	BM	54.7	37.8	8.7	8.7	7.9	2
PDGF-BB	AuHS	18	12.6	31.2	15.7	14	31.9
	FBS	12.2	11.6	23.4	16.2	25.2	11.8
	BM	17.4	15.1	9.1	6.2	6.4	6.1
SCF	AuHS	1.1	<0.43	0.4	5.4	<0.43	19.3
	FBS	2.3	<0.43	<0.43	<0.43	<0.43	<0.43
	BM	6.6	5.6	1.6	1.1	<0.43	<0.43
SDF-1A	AuHS	65.3	61.7	38	25.4	55.7	60.8
	FBS	<3.80	<3.80	<3.80	<3.80	<3.80	<3.80
	BM	27.2	37	24.1	13	13.4	<3.80
VEGF-A	AuHS	9.3	10.4	18	8.3	9	17.9
	FBS	4.1	3.1	<0.94	4.1	6	4.1
	BM	29.3	33.3	31	22.6	35	15.6
IL-2	AuHS	3.5	<1.68	24.2	16.2	<1.68	3.5
	FBS	3.8	4.8	3.5	3.5	<1.68	<1.68
	BM	<1.68	<1.68	5.9	<1.68	<1.68	3.5
IL-3	AuHS	41	32.2	26.9	20.1	27.1	101.6
	FBS	37.3	26.3	21.9	20.6	15.8	23.8
	BM	36.3	31.1	25.7	21.3	20.4	20.4
IL-6	AuHS	60240	36907.1	33627.6	8948.3	16405.3	4521.3
	FBS	92287.3	37478.3	26167.3	4025.3	23231.9	32259.9
	BM	3025.4	2085.1	5089.2	973.2	5488.8	1673.9
IL-12	AuHS	4.2	44.2	5.9	2.3	3.4	4.1
	FBS	8.3	2.9	3.0	2.4	1.7	7.1
	BM	1.9	1.8	1.5	1.3	1.5	1.2
IL-23	AuHS	46.4	22.4	51.0	23.9	23.4	11.0
	FBS	151.2	48.7	6.1	4.4	3.4	20.0
	BM	272.6	66.8	17.5	11.8	17.6	2.4

APPENDIX B

Expression of paracrine factors at 96 hours of incubation of PBMCs in AuHS, FBS and BM media.

Paracrine factor	Media	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
BDNF	AuHS	34.3	39.4	89.7	31.4	70.8	58.1
	FBS	25.1	25.5	84	46.1	63.9	33.7
	BM	10	<1.33	<1.33	<1.33	4.8	<1.33
EGF	AuHS	27.8	33.1	58.2	31.4	44.4	52
	FBS	45.5	65.9	104.4	47.7	104	11.1
	BM	22.5	42.8	68.3	69	72.1	37
FGF-2	AuHS	8.9	<1.62	12.2	7.4	7.1	28.3
	FBS	19.9	8.9	2	5.6	2.9	12.6
	BM	41	15.8	21.3	17.9	33.6	2.9
G-CSF	AuHS	174.6	62.3	656.7	158.8	481.7	554.3
	FBS	298.6	562.5	343.9	56.3	243.4	175.4
	BM	56.3	19.2	122.3	37.5	58	<5.43
GM-CSF	AuHS	104	31.3	344.3	133.8	41.1	91.8
	FBS	130.4	58.6	42.6	34.7	34.8	16.7
	BM	161.1	18	90	23.9	116.8	9.6
HGF	AuHS	61.8	66.9	58.1	58.5	67.3	60.4
	FBS	28.9	17	16.3	19.6	30.4	34.7
	BM	68.1	21.5	34.5	10.9	11.9	0.9
LIF	AuHS	5.8	3.7	10.5	11.4	9.7	15.4
	FBS	7.9	4.9	5.7	2.6	4.7	6.8
	BM	9.4	3.8	6.7	2.4	11.7	1.5
MCSF	AuHS	5	<1.98	2	<1.98	<1.98	23.1
	FBS	15.2	10	<1.98	<1.98	<1.98	<1.98
	BM	33.8	36.5	8.8	<1.98	9.9	13.5
PDGF-BB	AuHS	19.8	17.6	40.5	19.1	20.3	39.5
	FBS	18.5	15.6	31	28.8	51.8	26.1
	BM	21	11.2	16.6	7.5	7.8	6.8
SCF	AuHS	2.7	0.8	6.1	15.3	<0.43	16
	FBS	2.8	1.1	<0.43	<0.43	<0.43	<0.43
	BM	10.2	6.2	6.4	5.7	3	1.1
SDF-1A	AuHS	70.6	79.6	38.2	41.3	<3.80	58.3
	FBS	<3.80	<3.80	<3.80	<3.80	<3.80	<3.80
	BM	44.4	18.8	46.3	53.9	64.4	81.7
VEGF-A	AuHS	7.3	12	11.9	11.9	0.9	25.4
	FBS	2.2	15.3	23.1	11.7	<0.94	<0.94
	BM	29	40.7	17.6	53	68.9	42
IL-2	AuHS	<1.68	<1.68	24.4	44.5	<1.68	<1.68
	FBS	3.5	<1.68	3.5	<1.68	<1.68	3.5
	BM	3.5	5.3	5.9	3.5	<1.68	<1.68
IL-3	AuHS	42.8	33.1	25	24.9	15.4	57.1
	FBS	29.2	24.8	25.1	23.1	19.2	21.8
	BM	45.1	26.9	41.4	32.8	27.3	23.8
IL-6	AuHS	32963.8	26947.6	24616.1	9877.7	14692.4	8259.0
	FBS	38448.5	38062.1	21696.5	4873.2	17506.0	17318.9
	BM	3864.6	1762.9	9519.6	1822.5	12419.7	491.8
IL-12	AuHS	4.9	43.3	6.9	4.7	3.1	3.7
	FBS	7.2	3.2	2.8	1.9	1.7	3.0
	BM	2.4	1.7	2.0	1.9	2.0	1.6
IL-23	AuHS	30.0	35.7	120.0	113.3	49.5	135.8
	FBS	117.7	56.9	14.3	37.6	0.2	35.2
	BM	342.0	61.0	76.6	10.5	15.1	14.6

APPENDIX C

Ethics approval for teeth extraction

UNIVERSITI MALAYA

UM.D/PD211/11
11 August 2011

Assoc. Prof. Dr. Noor Hayaty Abu Kasim
Department of Conservative Dentistry
Faculty of Dentistry
University of Malaya

Dear Madam,

ETHICS APPROVAL

It is a pleasure to inform you that your application for medical ethics approval on your research titled 'Isolation, expansion and characterization of various mesenchymal stem cells and its conditioned medium for regenerative therapies' has been granted. Your ethics approval number is **DF CO1107/0066(L).**

Thank you.

Yours Sincerely,

DR. NOR ADINAR BAHARUDDIN
Chairperson
Faculty of Dentistry Medical Ethics Committee

s.k. Dean, Faculty of Dentistry
Head of Conservative Dentistry

NHAK/ish/etik11

Pejabat Dekan, Fakulti Pergigian, Universiti Malaya, 50603 Kuala Lumpur, Malaysia.
Tel: (603) 7967 4812 / 79674800 • Faks: (603) 7956 1607 / 79674809 • Email: dekan_dental@um.edu.my • http://www.um.edu.my

APPENDIX D

Ethics approval for Blood collection

UNIVERSITY OF MALAYA
The Leader in Research & Innovation

MEDICAL ETHICS COMMITTEE
FACULTY OF DENTISTRY
ADDRESS: 50603, KUALA LUMPUR, MALAYSIA
TELEPHONE: 03-79676458 FAXIMILE: 03-79676456

NAME OF ETHICS COMMITTEE/IRB: Medical Ethics Committee, Faculty of Dentistry **ADDRESS**: 50603, Kuala Lumpur	**ETHICS COMMITTEE/IRB REFERENCE NUMBER:** DF RD1301/0012(L)
PROTOCOL NO: **TITLE:** Proliferation, Differentiation and Immunomodulatory Properties of Stem Cells from the Pulp of Human Extracted Deciduous Teeth (SCD) Cultured in Allogeneic Human Serum (aHS) Supplemented Medium	
PRINCIPAL INVESTIGATOR: Prof Dr Noor Hayaty Abu Kasim **TELEPHONE:** **KOMTEL:**	**SPONSOR:**

The following item [√] have been received and reviewed in connection with the above study to be conducted by the above investigator.

[√] Investigator's Checklist — Ver date: 10th JANUARY 2013
[ ] Approval Form for Presentation at Department — Ver date:
[ ] Synopsis Form (For Elective Project only) — Ver date:
[√] Application Form
[√] Study Protocol
[√] Brief CV of Main Investigator
[√] Patient Information Sheet (PIS)
[√] Consent Form
[√] Questionnaire

and have been [√]

[√] Approved
[ ] Conditionally approved (identify item and specify modification below or in accompanying letter)
[ ] Rejected (identify item and specify reasons below or in accompanying letter)

Investigator are required to:

1) follow instructions, guidelines and requirements of the Medical Ethics Committee.
2) report any protocol deviations/violations to Medical Ethics Committee.
3) comply with International Conference on Harmonization – Guidelines for Good Clinical Practice (ICH-GCP) and Declaration of Helsinki
4) note that Medical Ethics Committee may audit the approved study.

Date of approval: 17 January 2013

c.c Dean
Faculty of Dentistry

Head
Department of Conservative Dentistry

Secretary
Medical Ethics Committee
Faculty of Dentistry

.....
DR. RATHNA DEVI VAITHILINGAM
Chairman
Medical Ethics Committee