EFFICACY OF DENTAL PULP STEM CELLS FROM DECIDUOUS TEETH IN TREATING DIABETIC WOUNDS

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FACULTY OF DENTISTRY
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EFFICACY OF DENTAL PULP STEM CELLS FROM DECIDUOUS TEETH IN TREATING DIABETIC WOUNDS

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ABSTRACT

Diabetic foot ulcers (DFU) are one of the common complications in diabetes mellitus (DM). The underlying problems of DFU are delayed wound healing due to hyperglycemia, poor blood circulation, and nerve damage. Mesenchymal stem cells (MSCs) from various sources including dental-derived stem cells have been used in studies in wound healing. The aim of this study is to assess the wound healing ability of human extracted deciduous teeth in both *in vitro* and *in vivo* model in different culture conditions and dosages. The immune gene expression of dental pulp stem cells from three different dental sources, namely human extracted deciduous teeth (SHED), periodontal ligament (PDLSCs), and dental pulp stem cells (DPSCs) were assessed in early and late passage. All three sources exhibited indistinguishable mesenchymal stem cell characteristics. Although SHED has increased immune gene expressions compared to DPSCs and PDLSCs, it is more suitable for clinical usage in its early passages than prolonged passages. This has led to investigations on biological molecules secreted by SHED cultured in low serum culture conditions in two different passages which are passage 2 (P2) and passage 4 (P4). Our findings show that at low serum concentrations, the expression of growth factors and cytokines were higher and had significant differences. The overall expression of genes, namely IL1B, IL2, IL4, IFNG, CXCL5, CD40LG, and CCL7, show increased expression in P2 low serum conditions. The percentage of wound closure in *in vitro* study of wound healing showed enhanced wound closure rate for SHED P2 2% FBS. The further study on enzymatic and non-enymatic antioxidants reported higher Superoxide Dismutase (SOD) and Total Glutathione (GSH) and decreased Malondialdehyde (MDA) and Advanced Oxidation Protein Products (AOPP) level in SHED treated group. The safe dosage for SHED was studied and it showed that there is no damage to liver and kidney in Sprague Dawley

rats after the transplantation of SHED in two different dosages. After that, the wound healing rates on diabetic Sprague Dawley rats' skin were studied on day 5 and 10. The histology of granulation tissue indicates that SHED has high collagen content and a thinner epidermal layer. Immunohistostaining presented an over-expression of Heat-shock protein (Hsp70) marker which indicates higher wound healing rate in SHED treated group. The SOD activity and GSH level was amplified in the SHED-treated group with declining levels of MDA and AOPP. The hydroxyproline accumulation was augmented in the SHED-treated diabetic wound. The diabetic animal wound model was a pre-clinical study and is important in conveying SHED for therapeutic usage. The parameters induced by SHED in treating diabetic wounds are an increase in antioxidants level, reduction of lipid peroxidation and protein oxidation, accumulation of hydroxyproline, augmented expressions of the Hsp70 marker, promotion of the angiogenesis process through expression of wound repair genes, greater collagen deposition, and fewer inflammatory cells.

ABSTRAK

Ulser kaki diabetik (DFU) adalah salah satu daripada komplikasi biasa diabetes melitus (DM). Masalah asas berkaitan DFU adalah kelewatan dalam penyembuhan luka disebabkan oleh hiperglisemia, kekurangan peredaran darah dan kemusnahan saraf. Sel stem mesenchymal (MSCs) dari pelbagai sumber termasuk sel stem daripada kegigian telah digunakan dalam banyak kajian berkaitan penyembuhan luka. Tujuan kajian ini adalah untuk menilai keupayaan gigi susu manusia yang dicabut dalam merawat penyembuhan luka dalam model luka *in vitro* dengan pelbagai kondisi kultur dan seterusnya dalam model *in vivo*. Sel stem dari tiga sumber kegigian yang berbeza iaitu pulpa gigi susu (SHED), ligamen periodontal (PDLSCs), dan gigi kekal (DPSCs) didapati mengekspresi ciri-ciri immunologi pada 'passage' awal dan lewat. Ketiga-tiga sumber mempamerkan karakteristik sel stem mesenchymal. Walaupun SHED telah menunjukkan ekspresi gen imun yang tinggi berbanding DPSCs dan PDLSCs, ia adalah lebih sesuai untuk penggunaan klinikal di passage awal (P2) berbanding dengan passage lewat (P9). Ini telah menjuruskan kepada penyiasatan ke atas molekul biologi yang dirembeskan oleh SHED yang dikultur dalam keadaan serum rendah di passage 2 (P2) dan passage 4 (P4). Kedapatan kami menunjukkan pada konsentrasi serum rendah, ekspresi faktor-faktor pertumbuhan dan sitokin adalah tinggi dan mempunyai perbezaan yang ketara. Ekspresi gen secara keseluruhannya iaitu gen IL1B, IL2, IL4, IFNG, CXCL5, CD40LG dan CCL7 telah menunjukkan peningkatan ekspresi dalam P2 yang dikultur dalam serum rendah. Imej digital *in vitro* penyembuhan luka diabetik melalui cabaran glukosa calaran assay *in vitro* dengan SHED P2 2% FBS telah menunjukkan peningkatan kadar penutupan luka. Tambahan pula, peningkatan aktiviti SOD dan tahap GSH dengan pengurangan pengoksidaan lipid (MDA) dan pengoksidaan protein (AOPP) telah diperhatikan dalam kumpulan dirawat dengan SHED. Kajian ketoksikan

menunjukkan tiada kesan hepatotoksik atau nefrotoksik oleh SHED. Kesan luka diabetik makroskopik menunjukkan penyembuhan luka yang lebih baik dalam kumpulan dirawat dengan SHED pada hari 5 dan 10. Histologi tisu granulasi menunjukkan bahawa SHED mempunyai kandungan kolagen yang tinggi dan lapisan epidermis yang lebih nipis. Pewarna pkh-26 ditandakan pada SHED diperhatikan pada hari 10 selepas pembedahan dilakukan. Kajian 'immunostaining' telah menunjukkan ekspresi Hsp70 protein dalam SHED. Aktiviti SOD dan tahap GSH adalah tinggi dalam kumpulan dirawat dengan SHED dengan penurunan tahap MDA dan AOPP. Pengumpulan 'hydroxyproline' telah menunjukkan peningkatan dalam luka diabetik yang dirawat dengan SHED. Model luka haiwan diabetik adalah satu kajian pra-klinikal dan ia penting dalam menyarankan SHED untuk kegunaan terapeutik. Parameter yang diketengahkan oleh SHED dalam merawat luka diabetik adalah peningkatan tahap antioksidan, pengurangan pengoksidaan lipid dan oksidasi protein, pengumpulan 'hydroxyproline', peningkatan ekspresi penanda Hsp70, menggalakkan proses angiogenesis melalui ekspresi gen pembaikan luka, peningkatan pengumpulan kolagen dan pengurangan sel-sel inflamasi.

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LIST OF SYMBOLS AND ABBREVIATIONS

AOPP	:	Advanced oxidation protein products
ASC	:	Adipose tissue-derived mesenchymal stem cells
bFGF	:	Basic fibroblast growth factor
BM-MSCs	:	Bone marrow mesenchymal stem cells
BMP	:	Bone morphogenetic protein
CCL3	:	C-C motif chemokine ligand 3
CCR2	:	Chemokine (C-C motif) receptor 2
COL1A1	:	Collagen type I alpha 1 chain
CTGF	:	Connective tissue growth factor
CXCL11	:	C-X-C motif chemokine ligand 11
DPSCs	:	Dental pulp stem cells
EGF	:	Epidermal growth factor
ESCs	:	Embryonic stem cells
F3	:	Coagulation factor III, tissue factor
G-CSF	:	Granulocyte colony-stimulating factor
Gli	:	Glibenclamide
GM-SCF	:	Granulocyte-macrophage colony-stimulating factor
GNLY	:	Granulysin
GSH	:	Total Glutathione
GZMB	:	Granzyme B
HGF	:	Hepatocyte growth factor
HLA-DR	:	Major histocompatibility complex, class II, DR alpha
Hsp70	:	Heat shock protein 70
IGF-1	:	Insulin like growth factor 1

IGFBP-7	:	Insulin-like growth factor binding protein 7
IL-1	:	Interleukin-1
IL1A	:	Interleukin 1 alpha
IL-6	:	Interleukin-6
IL-8	:	Interleukin-8
IF	:	Immunofluorescence
IHC	:	Immunohistochemistry
iPSCs	:	Induced pluripotent stem cells
KGF	:	Keratinocyte growth factor
MDA	:	Malondialdehyde
MCP-1	:	Methyl-accepting chemotaxis protein
MMP	:	Matrix metalloproteinase
MSCs	:	Mesenchymal stem cells
PDGF	:	Platelet-derived growth factor
PDLSCs	:	Periodontal ligament stem cells
PLAU	:	Plasminogen activator, urokinase
PMSCs	:	Placental-derived mesenchymal stem cells
PTGS2	:	Prostaglandin-endoperoxide synthase 2
PTPRC	:	Protein tyrosine phosphatase, receptor type C
P2	:	Passage 2
P4	:	Passage 4
RAC1	:	Ras-related C3 botulinum toxin substrate 1
RANTES	:	Interactions of the chemokine CCL5
SELE	:	Selectin E
SELP	:	Selectin P
SD	:	Sprague Dawley

SHED	:	Exfoliated or extracted deciduous teeth
SOD	:	Superoxide dismutase
SPARC	:	Secreted protein acidic and cysteine rich
STAT3	:	Signal transducer and activator of transcription 3
STZ	:	Streptozotocin
TGF-α	:	Transforming growth factor-α
TGF-β1	:	Transforming growth factor beta 1
TLDA	:	TaqMan Low Density Arrays
TNF	:	Tumor necrosis factor
TNFRSF18	:	Tumor necrosis factor receptor superfamily, member 18
UCMSCs	:	Umbilical cord mesenchymal stem cells
VEGF	:	Vascular endothelial growth factor
WJ-MSCs	:	Wharton's Jelly stem cells

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CHAPTER 1: INTRODUCTION

Diabetes mellitus (DM) is a non-communicable disease which affects all age groups. In 2000, approximately 171 million people worldwide were diagnosed with DM and this figure is projected to increase to 366 million by 2030 (Wild et al., 2004). In Malaysia, DM cases stood at 2.5 million in 2013 and are expected to increase to 3.2 million by 2020 (Feisul & Azmi, 2013). Uncontrolled DM could lead to microvascular damage such as retinopathy, nephropathy, and neuropathy and result in increased treatment costs. Among all the complications of DM, diabetic foot ulcer (DFU) is the most common forming around 25% of the total diabetes population. In the United States alone the cost of treating this complication was US$132 billion in 2002 and is expected to rise to US$192 billion by 2020 (Hogan et al., 2003). A retrospective study conducted in a Malaysian government hospital for 2012 to 2013 revealed that the total cost to treat DFU was US$11,000 (Lam et al., 2014).

The underlying problem of DFU is delayed wound healing due to hyperglycemia, poor blood circulation, and nerve damage. Diabetics not only have to deal with the pain, infection, repeated hospital admissions, and possible amputations but also the poor quality of life that they and their families experience. In addition, the cost of treatment for DFU has been escalating. Therefore, an alternative wound-healing treatment regime for this chronic condition is timely (Naves et al., 2016).

The current treatment modalities practiced in hospitals for DFU are wound debridement, care and dressings with topical antibiotics (American Diabetes Association, 2014), topical negative pressure (Xie et al., 2010), hyperbaric oxygen therapy [HBOT] (Londahl et al., 2013), and platelet derived-growth factors (Villela & Santos, 2010). Despite undergoing such treatment, much patience is required by both patients and

doctors as the healing process is slow. This has led researchers to seek more advanced treatments for wound healing including cell-based therapy.

Stem cells have attracted great interest amongst researchers due to their tissue regeneration and wound healing capabilities. Wound healing is enhanced by cell adhesion, migration, and proliferation through various signaling pathways within the stem cells. The candidate stem cell populations for therapeutic use include embryonic stem cells (ESCs), adult mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs).

ESCs could be a good source for the treatment of wounds with Kim et al. (2015) reporting that mouse embryonic stem cells (mESs) can trigger wound healing signaling-pathways. Despite this promising finding, the immunogenicity and tumorigenicity of ESCs make it unsuitable for clinical use (Wu et al., 2007). Further, the ethical and legal restrictions of using ESCs need to be addressed before clinical applications become feasible. This has led to the discovery of iPSCs that allow for the generation of autologous pluripotent stem cell populations derived from differentiated adult tissues. This avoids ethical issues associated with human ESCs and immunogenic complications. In a recent in vitro study, Itoh et al. (2013) reported that 3-D skin equivalents composed of human iPSC-derived keratinocytes and fibroblasts were successfully generated. Even though iPSCs have several advantages over ESCs, there are still risks for tumorigenicity in an undifferentiated state. These risks need to be resolved and improvements made on their safety profile, efficiency, and cost-effectiveness before widespread clinical adoption becomes possible.

MSCs from various sources are also commonly used in wound healing studies. Bone marrow stem cells (BM-MSCs) have been shown to promote wound healing in mice (Wu et al., 2007) while adipose tissue-derived mesenchymal stem cells (ASCs) provide

good skin regeneration for wound healing in rats (Ozpur et al., 2016). In another study, a large scale expansion of Wharton's Jelly stem cells (WJ-MSCs) on gelatin microbeads showed their capacity for wound healing (Zhao et al., 2015). In multiple preclinical trials, placenta-derived mesenchymal stem cells (PMSCs) and human umbilical cord mesenchymal stem cells (hUCMSCs) have been found to significantly enhance cutaneous wound healing. This is achieved by an increase in blood vessel formation and elevated vascular endothelial growth factor (VEGF) expression which promotes healing through paracrine signaling (Wang et al., 2016).

Dental-derived stem cells are another alternative source of MSCs in wound healing treatments. The root apices of the third molar are often still open at the age of eighteen and contain a noticeable pool of undifferentiated cells which reside within the "cell rich zone" of the dental germ pulp (Iohara et al., 2004; d'Aquino et al., 2009). Stem cells from human exfoliated or extracted deciduous teeth (SHED) could also be a unique resource for wound healing treatment. It has been reported that SHED expresses VEGF which is an essential growth factor in stimulating the angiogenesis process in wound healing (Yang et al., 2013). SHED also exhibits good potential in wound healing by providing a better rate of wound closure with increased Type 1 collagen in SHED co-cultured with fibroblast treated group (Nishino et al., 2011). Other advantages of using SHED are that it involves a simple and painless procedure and the ethical concerns differ from the use of ESCs (Miura et al., 2003; Rai et al., 2013).

Several studies in the use of MSCs suggest that the healing was attributed to the secretion of growth factors, cytokines, and chemokines (Chen et al., 2009; Lee et al., 2012; Mehanni et al., 2013). The MSCs employed were of ASCs and BM-MSCs. Thus to fill the gap in the current literature, it is important to study the secretions of biological

molecules in SHED if it is to be used for wound healing treatment. Further, the culture conditions and passages of SHED have to be understood in order to cultivate it as a potential source for such healing.

The overarching goal of this research was to study if dental derived stem cells were a good candidate in the treatment modality for diabetic wound healing. The following research questions were addressed:

i. Are dental-derived stem cells a good cell source for cell-based therapy in wound healing?
ii. Do low serum culture conditions affect the therapeutic potential of dental derived stem cells in relation to wound healing?
iii. Are dental derived stem cells effective in the healing of diabetic wounds?

1.1 Aims of the study

This study had four interrelated aims as outlined below.

Aim I: To obtain MSCs from human extracted deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs) from primary sources suitable for wound healing. The objectives were to:

1. isolate stem cells from SHED, PDLSCs, and DPSCs from their primary sources,

2. characterise and confirm the status of MSCs,

3. evaluate the differences of SHED, PDLSCs, and DPSCs in terms of immune genes in long-term cultured stem cells using the human immune qPCR array, and

4. assess the validation of randomly chosen immune genes from SHED, PDLSCs, and DPSCs using the cytokine protein array.

Aim II: To study the wound healing potential of SHED in low serum culture conditions in different early passages. The objectives were to:

1. assess SHED isolation and compare the morphology, growth kinetics, and senescence level of SHED cultured in passage 2 (P2) and passage 4 (P4) in low serum culture conditions,
2. evaluate human wound healing genes in SHED in P2 and P4 when cultured in low serum culture conditions using qPCR,
3. assess malignant transformation of SHED in P2 and P4 when cultured in low serum culture conditions using transformation assay, and
4. assess the validation of randomly chosen wound healing genes from P2 and P4 using cytokine protein array.

Aim III: To study the ability of SHED in reducing oxidative stress in an *in vitro* diabetic wound model in low serum culture conditions. The objectives were to:

1. assess the digital image of *in vitro* diabetic wound healing through *in vitro* glucose-challenged scratch assay with SHED treated in low serum conditions, and
2. evaluate the oxidative stress enzyme activity level in *in vitro* diabetic wounds treated with SHED in low serum culture conditions using transwell assay.

Aim IV: To evaluate the toxicity effect of SHED and to assess the potential of SHED in treating diabetic wounds in an animal model. The objectives were to:

1. evaluate the blood profile of Sprague Dawley (SD) rats in low and high dosages of SHED transplantation,
2. evaluate the histology of liver and kidney in low and high dosages of SHED in SD rats using Hematoxylin and Eosin (H&E) staining,
3. assess the streptozotocin (STZ) induced-diabetic animal model,

4. access the macroscopic wound contraction of diabetic induced SD rats,
5. evaluate the histology of granulation tissue [(H&E) and pkh-26 tagged cells immunofluorescence (IF) and immunohistochemistry (IHC) analysis for Hsp70 upregulation].
6. evaluate the oxidative stress enzyme activity level of the rat skin tissue homogenate; and
7. assess the level of hydroxyproline accumulations in rat skins using hydroxyproline assay.

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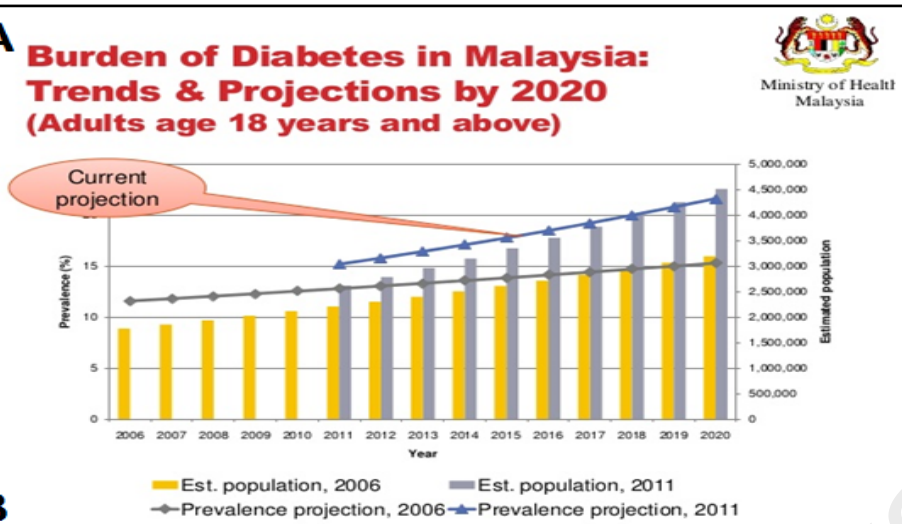
CHAPTER 2: LITERATURE REVIEW

2.1 Diabetes mellitus

Diabetes mellitus (DM) is a major health concern due to its epidemic nature and related health complications. Global trends indicate a sharp increase in type 2 DM especially in developing countries such as Malaysia. Currently, it is estimated that over 200 million people worldwide suffer from DM and the figure is expected to reach 366 million by 2030 (Wild et al., 2004). This prediction was based on population growth, aging, urbanization, and the increasing prevalence of obesity and physical inactivity (Frykberg, 2006). The National Health and Morbidity Survey (NHMS) conducted by Ministry of Health reveals that DM prevalence among Malaysian adults of 30 years and above increased from 6.3% in 1986 to 8.3% in 1996 and further increased to 14.9% in 2006 (National Health Morbidity Survery, 1996; Letchuman et al., 2010). More recent statistics show a further increment of approximately 31% in the 5-year period from 2006 and 2011 (Feisul & Azmi, 2013). These numbers are projected to increase steadily (see Figure 2.1) over the next few years (National Health Morbidity Survey, 2011).

Diabetic foot ulcers (DFU) are a common complication in uncontrolled diabetes and need to be identified early to avoid amputation. Chronic hyperglycemia can contribute to foot ulceration and delay wound healing, and is associated with foot amputations (Frykberg, 1999; American Diabetes Association, 1999; Mayfield et al., 2003). Proper treatment of DFU is crucial to ensure early wound closure to prevent recurrence and reduce the incidence of lower limb amputations (Apelqvist et al., 1993; Caputo et al., 1994; Levin, 1995; Frykberg, 1998; Boulton et al., 1999).

A



B

Complications	2011, n (%)	2012, n (%)
Nephropathy		
Present	5,429 (7.6)	9,707 (7.8)
Absent	51,350 (71.7)	99,016 (79.8)
Unknown	14850 (20.7)	15,256 (12.3)
Retinopathy		
Present	4,627 (6.5)	8,255 (6.7)
Absent	50,455 (70.4)	96,872 (78.1)
Unknown	16,547 (23.1)	18,853 (15.2)
Ischaemic Heart Disease		
Present	3,467 (4.8)	6,508 (5.3)
Absent	53,387 (74.5)	101,630 (81.9)
Unknown	14,775 (20.6)	15,842 (12.8)
Cerebrovascular Disease		
Present	788 (1.1)	1,550 (1.3)
Absent	56,966 (79.5)	106,953 (86.2)
Unknown	13,875 (19.4)	15,476 (12.5)
Diabetic Foot Ulcer		
Present	841 (1.2)	1,527 (1.2)
Absent	58,044 (81.0)	10,8726 (87.7)
Unknown	12,744 (17.8)	13,725 (11.1)
Amputation		
Present	387 (0.5)	721 (0.9)
Absent	58,487 (81.6)	109,652 (88.4)
Unknown	12,755 (17.8)	13,605 (11.0)

Figure 2.1: (A) Diabetes Mellitus cases in Malaysia. (B) Prevalence of diabetes among adults in Malaysia is projected to rise to 21.6% by 2020. (B) The incidence of DFU increased from 841 cases in 2011 to 1527 in 2012 (Feisul & Azmi, 2013).

2.2 Skin morphology

The skin is the largest organ in the body and comprises the epidermis and dermis (Figure 2.2). Its function is to protect the organism from the external environment and damage caused by invading microorganisms and foreign materials. Damage or injury to the skin is known as a wound. Injuries to the skin can be due to several factors such as mechanical, physical, or chemical causes. In both traumatic and intentional type of wounds, bleeding occurs followed by the formation of a blood clot as a result of the

rupture of the blood vessels. Skin injuries can also be caused by pressure ulcers and ischemia, for example in cases of arterial leg ulcers. In such wounds, there will be an occlusion of blood within the blood vessels which leads to necrosis and ulcer formation (Dealey, 2008).

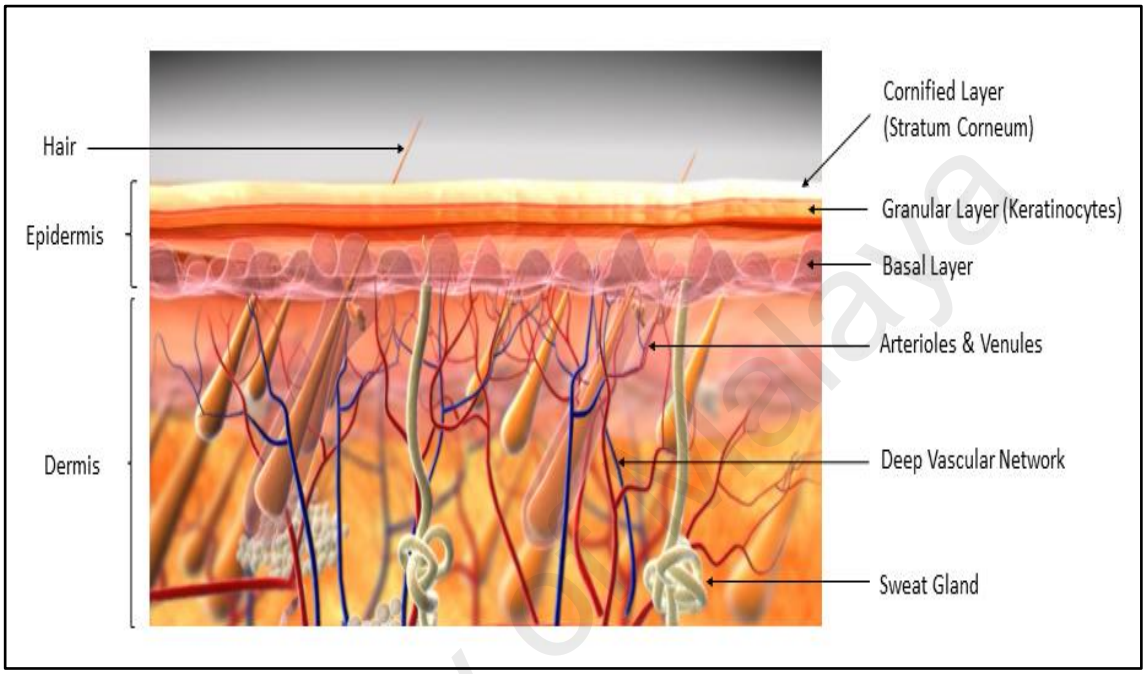


Figure 2.2: The human skin layer has two major parts consisting of the epidermis and dermis.

2.3 Wound healing process

Wounds can be categorised as either acute or chronic. Chronic wounds can be due to tissue deficit caused by wounds that are unable to heal over a prolonged period of time or that are frequent in occurrence. Pressure ulcers, leg ulcers, and DFUs are examples of chronic wounds (Fowler, 2005). Acute wounds on the other hand are known as sudden onset or short duration wounds which heal easily. These wounds include surgical, traumatic, and burn wounds.

Wound healing is the process of tissue repair and proceeds through three phases, namely inflammatory, proliferative and maturation (Figure 2.3). When a tissue is

damaged, neutrophils initiate the inflammatory process by removing any contaminating bacteria (Clark, 1996). Further differentiation of monocytes into macrophages induces the pro-inflammatory pathway leading to the secretion of cytokines and growth factors (Mahdavian et al., 2011). Granulation tissue formation begins with the formation of immature fibrin matrix which will be replaced by collagen and scar tissue (Singer & Clark, 1999).

The inflammatory process occurs when damaged blood vessels begin to bleed resulting in their vasoconstriction. This is followed by platelet aggregation and neutrophil migration to the wound to neutralise bacteria on the injury site (Dealey, 2008). Blood circulation reaches the injured tissues once the blood vessel becomes dilated and more permeable. Platelets then release fibronectin and growth factors, or platelet derived growth factors (PDGF), made up of transforming growth factor alpha and transforming growth factor beta (TGFα & TGFβ), which play an important role in cell migration and growth. The growth factors are a subclass of cytokines that function in cellular communication (Greenhalgh, 1996) and are involved in forming new fibroblasts, stimulating angiogenesis, and promoting the proliferation and migration of epithelial cells (Witte & Barbul, 1997).

Cell migration is crucial for the re-epithelialization of damaged tissue (Tsirogianni et al., 2006). TGFβ attracts monocytes to the wound which differentiate into macrophages while fibronectin binds to the surface receptors on the cells promoting phagocytosis. This process can only occur when there is a sufficient amount of oxygen (Cherry et al., 2000). Both macrophages and lymphocytes are present in wounds from day one, with the former populations peaking between day three and six and the latter between day eight and fourteen (Martin & Muir, 1990). Throughout the inflammatory process,

macrophages not only phagocytize bacteria but also aid wound debridement as well as secrete collagenases, growth factors, and other enzymes. In addition, macrophages also play an important role in recruiting fibroblasts and keratinocytes, which are important for tissue formation (Tsirogianni et al., 2006).

Fibroblasts are involved in the production of the extracellular matrix, secretion of growth factors, promotion of angiogenesis, and protease synthesis (Broughton et al., 2006) while keratinocytes help in resurfacing the wound and maintain the barrier function. This inflammation stage lasts about four to five days and is followed by the formation of granulation tissue. Macrophages promote wound healing by producing platelet-derived growth factors (PDGF) and fibroblast growth factors (FGF) which later divide into producing collagen fibers. Fibronectin also plays a role in wound healing by enhancing fibroblast activity (Orgill & Demling, 1988).

Collagen is a tertiary protein that can be seen from the second day of wound healing with Collagen type III being the most common. Contraction is the process of wound closure by approximately 40-80% that occurs between the fifth and sixth day. Wound healing by first intention will be completed in approximately 24 days depending on the type and size of the wound. The number of macrophages and fibroblasts will gradually reduce throughout this process.

During the maturation process, the wound becomes less vascularised with the old collagen replaced by newly synthesised collagen and this occurs at its highest peak from day 14 to 21 (Cherry et al., 2000) followed by the formation of scar tissue. A hypertrophic scar is the result of impaired wound healing caused by excessive fibrous

tissue deposition during the healing process and leads to extreme accumulation of collagen with a thick wound scar (Munro, 1995).

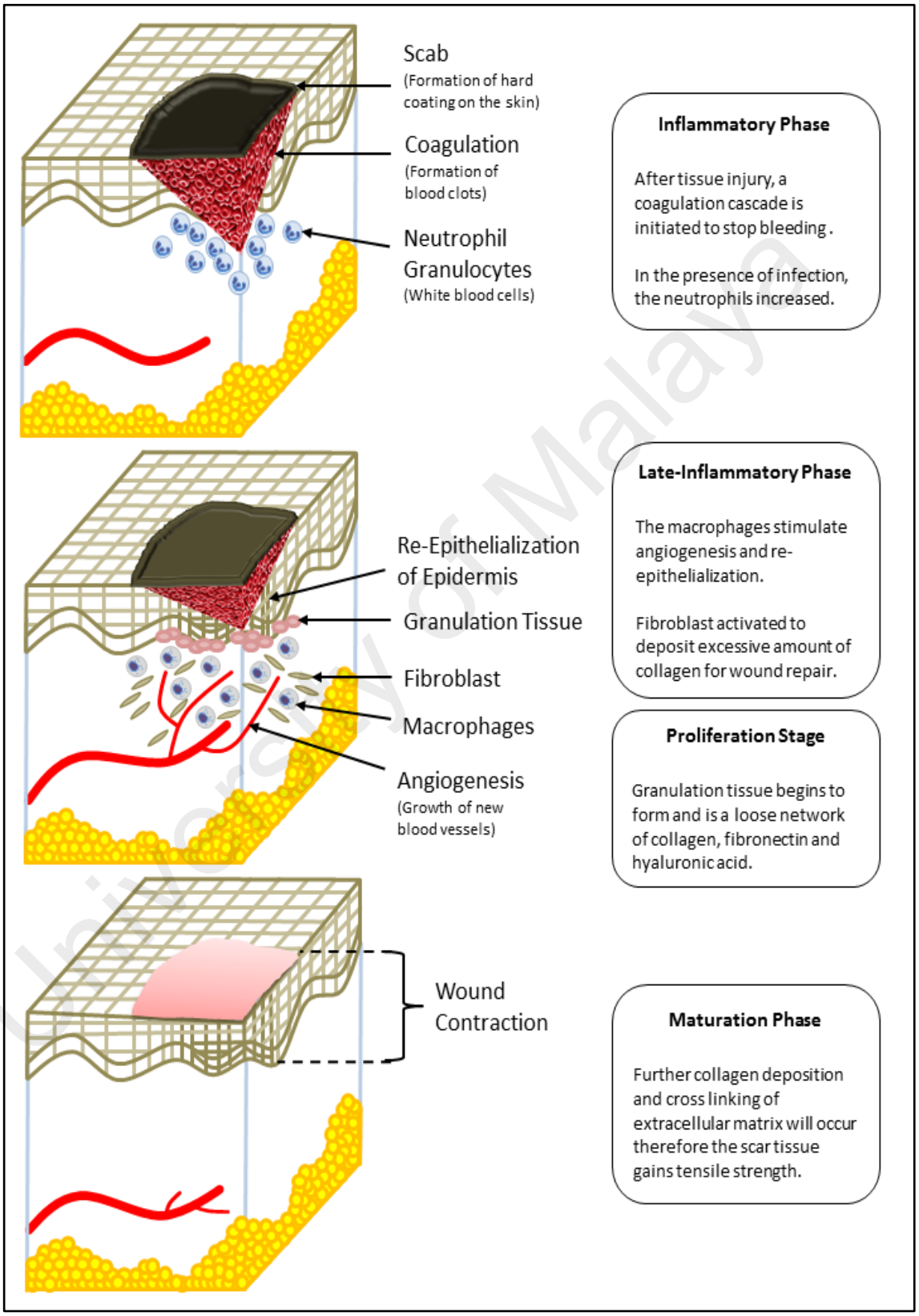


Figure 2.3: The major phases that involve in wound healing. Adapted from Jayaraman et al., 2013.

2.4 The current treatments for chronic wounds

Current treatment methods for chronic wounds include surgical (Hampton, 2015), enzymatic (Konig et al., 2005), and biological debridement (Jones & Wall, 2008; Klaus & Steinwedel, 2015), hyperbaric oxygen therapy (HBOT) (Bakker, 2000), negative pressure wound device (NPWD) (Suess et al., 2006), and plasma mediated bipolar radiofrequency ablation (PBRA) (Nusbaum et al., 2012). These treatments have been developed with the basic aim of promoting angiogenesis. However, major pitfalls due to cost and healing inconsistencies have not improved the process of wound healing and this has led to advanced treatment methods such as stem cell therapy (Figure 2.4).

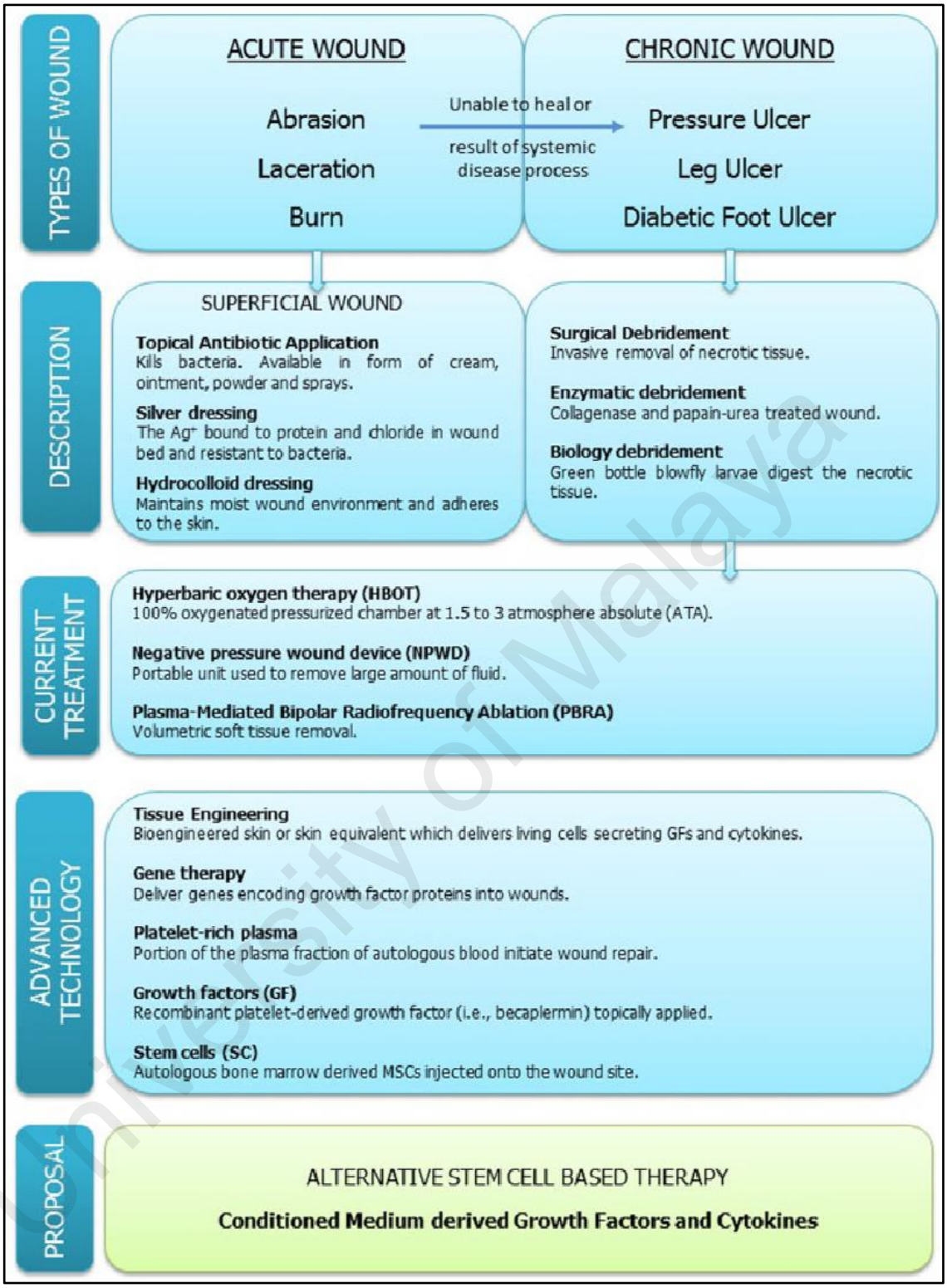


Figure 2.4: The current treatments available for chronic wound healing. Adapted from Jayaraman et al., 2013.

2.4.1 Types of topical applications

The wound healing process can be enhanced by applying topical agents followed by dressing administered externally onto the body. A topical agent is a medicine applied

externally onto the wound while dressing is done to cover the wound to promote healing and to avoid further injury (Dealey, 2008). Topical medicines are available in the form of lotions, creams, ointments, powders (talc), and solutions (liquids) which can also function as a transport medium when specific doses of medication are added to them. Topical lotions are water-based and thin, readily absorbed into the skin, and usually invisible after application. Topical creams are thicker and visible on the skin after application and require more time for the medication to be absorbed into the skin. Ointments or unguents are the thickest form of topical medication applications in which the medicine is suspended in a greasy substance and adheres to the skin until the medicine is absorbed (Zaghi & Maibach, 2007).

Topical wound healing management products include antiseptics which need to be in contact with the bacteria for about 20 minutes to destroy them (Russel et al., 1982). For example, cetrimide is useful for the cleansing of traumatic wounds. Lammers et al. (1990) noted that providone iodine showed increased debridement and a decline in bacterial count. Other than that, silver has also been used as an antiseptic and found to have significant results in controlling wound infections from burns (Lansdown & Williams, 2004). Silver-coated dressings on burn wounds cause less pain on removal and reduced infection and this could also reduce dressing and nursing costs (Tredget et al., 1998). Carneiro and Nyawawa, (2003) suggest that phenytoin significantly reduces pain, exudate levels, and wound sizes in diabetic ulcers and burn wound patients. Biafine, another topical emulsion, is known to increase the number of macrophages migrating to the wound site to treat skin damage caused by ulcers, burns, and dermatitis (Cohen et al., 2007).

2.4.2 Current Technology

Advanced technologies have been developed in medical and healthcare institutions to improve wound care treatments. Recombinant growth factors is one of the early technologies that have been performed and show high rates of healing among foot ulcer patients (Knighton et al., 1990) while skin gene therapy has been seen to generate a number of different growth factors (Guarini, 2003). Galeano (2003) noted the possibility of transferring the virus vector-mediated VEGF gene to the burn wound of an animal model. Hyperbaric Oxygen treatment involves placing patients in a 100%-oxygen chamber, and Kalani et al. (2002) suggest that it is of benefit to most diabetic ulcer patients. Topical negative pressure (TNP) or vacuum assisted pressure (VAC) devices involve applying universal pressure to the wound which improves blood flow and tissue granulation (Luckraz et al., 2003; Loree et al., 2004). The system is cost-effective but appropriate skills are required in handling the devices. Tissue culture has been well-established since the 1980s and has become a preferable method of topical application. Skin from the patient or the donor is cultured in the laboratory to form a large sheet of cells and grafted onto a granulating wound. Kumagai and Uchikoshi, (1997) suggest that autologous cultured epithelial grafting procedure is a promising treatment for patients with hypomelanosis. In addition, tissue engineering is an advanced step of tissue culturing where human dermal fibroblasts are cultured on a biosynthetic scaffold. The fibroblasts then proliferate, secrete protein and growth factors, and eventually generate into three dimensional human grafts that can be applied over wounds. They can also be described as cultured human skin equivalents (HSE) and have significantly high healing rates for diabetic ulcers (Marston et al., 2003).

2.4.3 Stem cells as an alternative to wound repair modality

Stem cells are becoming an alternative source for topical applications in many medical care sectors. This method seeks to heal wounds with minimal scar formation. There is a medical need for methods and compositions to promote wound healing by cellular regeneration therapy and work has been done on exploring the capability of stem cells in cell regeneration and enhancing the wound healing process. Clinicians understand the use of growth factors and their capability in growing cells *in vitro* and this method has resulted in stem cell advances as an alternative source for wound healing (Bell et al., 1981; Boyce, 2001).

Wound healing factors include recombinant growth factors that heal chronic wounds, epidermal growth factor (Falanga et al., 1992), keratinocyte growth factor-2 for venous ulcers (Robson et al., 2001), fibroblast growth factor, and platelet derived growth factor (PDGF) for pressure ulcers (Robson et al., 1992; Pierce et al., 1994). Keratinocyte sheets are an example of a bioengineered product which has been developed and tested on human wounds.

The bioengineered skin is a type of material that delivers living cells which release growth factors and cytokines onto the wound sites. Bioengineered skin may work by delivering living cells which are known as a "smart material" because they are capable of adapting to their environment. There is evidence that some of these living constructs are able to release growth factors and cytokines (Mansbridge et al., 1998; Falanga et al., 2002). However, this cannot be fully implemented as some of these allogeneic constructs are unable to survive more than a few weeks when placed in a chronic wound (Philips et al., 2002).

2.5 What is a stem cell?

Stem cells are known as undifferentiated cells that are able to differentiate into specialized cells and categorized mainly into embryonic and adult stem cells. Despite the pluripotentiality of embryonic cells they are not preferred in the research field due to ethical issues and adult stem cells play a major role in regenerative medicine instead. Nevertheless, both embryogenic and adult stem cells are known to have the potential to differentiate into different cell types such as liver, skin, muscle, bone, and others. Adult or somatic stem cells have the ability to divide and generate a range of cells types. They are found in many parts of human body tissues such as bone marrow, blood, muscle, brain, skin, and the liver. Embryonic stem cells (ESCs) are derived from a fertilized embryo four or five days of post fertilization which is known as blastocyst. Blastocysts consist of an inner (embryoblast) and an outer (trophoblast) cell mass. The outer part of the cell mass becomes part of the placenta while the inner mass will differentiate into a complete organism (Thomson et al., 1998).

Researchers have recently discovered induced pluripotent stem cells (iPSCs) which are known to have the characteristics of embryonic stem cells. These cells are created by inducing the specialized cells to express genes present in the embryonic stem cells which control cellular functions. Adult stem cells derived from human adult skin tissue are reprogrammed to give pluripotent capabilities. The generated pluripotent stem cells are useful in regenerative medicine but the technique used to induce them has to be carefully refined (Itoh et al., 2013).

2.6 Types of stem cells

2.6.1 Embryonic stem cells (ESCs)

The first human ESCs were isolated in 1998 by Dr. Thomson and colleagues from *in vitro* fertilization (IVF) clinic embryos (Thomson et al., 1998). ESCs with their pluripotent capabilities exhibit indefinite self-renewal *in vitro* and can be differentiated into almost every cell type of the body. However, their use has been hampered due to ethical issues, and there are risks of teratoma formation and immune rejection upon transplantation. A study conducted by Lee et al. (2011) maximizes the therapeutic benefit of ESCs for wound healing by using single cell suspension with application of ESC on an excisional wound in the mouse model. The application of ESCs to growth factors helps to stimulate the differentiation of the cells that controls teratoma and improve the wound healing process. In another *in vivo* study, differentiated keratinocyte-like cells derived from mouse embryonic stem cells provided positive effects on a surgically wounded mouse (Vatansever et al., 2013). In addition, Shamis et al. (2011) report that fibroblasts derived from human embryonic stem cells (hESC) have the ability to enable the re-epithelialization of wounds and generated using a 3D tissue model of cutaneous wound healing.

Stem cells derived from human embryos known as hESC are able to accelerate wound healing. The hESC-derived endothelial precursor cells (EPC) have enhanced the tensile strength of wounds after topical treatment and subcutaneous injection in animal models. Further, granulation tissue regeneration and re-epithelialization of wounds were noticed in ischemic tissue. The *in vitro* results of hESC-EPC conditioned medium also suggest the proliferation and migration of dermal fibroblasts and epidermal keratinocytes which increased the synthesis of the extracellular matrix by fibroblasts (Lee et al., 2011).

2.6.2 Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (iPSCs) were first produced in 2006 from mouse cells (Takahashi & Yamanaka, 2006) and in 2007 from human cells (Takahashi et al., 2007). ESCs' immune rejection after transplantation and ethical concerns led researchers to the discovery of iPSCs, which are known to have the characteristics of embryonic stem cells. These cells are created by inducing specialized cells to express genes that control cellular functions. Yamanaka first explained the technique where the introduction of four genes (Oct-3/4, Sox2, c-Myc, and KLF4) into an adult human skin cell could reprogram the cell back to an embryonic state. In another study, Kattman et al. (2011) reported that iPSCs promote vascular regeneration by producing vascular cells which express TGF-β, BMP-2, 4, 6 and 7, Nodal and activins.

2.6.3 Mesenchymal stem cells (MSCs)

The use of mesenchymal stem cells (MSCs) is not as controversial as the use of embryonic stem cells. They are abundant in bone marrow, adipose tissue, Wharton's jelly, umbilical cord blood, amniotic fluids, and foreskin. Caplan (1991) was among the first to propose the use of MSC as a therapeutic concept. The ease of isolation, *in vitro* expansion, and hypoimmunogenecity has brought MSCs into the limelight. Despite significant advances in medical and surgical wound care, the current treatment for cutaneous wounds with bone marrow-derived mesenchymal stem cells (BM-MSC) accelerates wound healing kinetics and increases epithelialization and angiogenesis (McFarlin et al., 2006). Apart from bone-marrow, other sources of MSCs that promote wound healing are adipose tissue (Kim et al., 2007, 2009), cartilage tissue (Bos et al., 2008), dental pulp (Nishino et al., 2011), umbilical cord (Zebardast et al., 2010), cord blood (Luo et al., 2010), synovial fluid (Kim et al., 2015), Wharton's jelly (Zhao et al., 2015), and periodontal ligaments (Chen et al., 2012). Therapeutic studies on skin wound

healing have established several tissue-specific stem or progenitor cell types, such as bone marrow-derived mesenchymal stem cells (BM-MSCs), adipose tissue-derived stem cells (ASCs), dental pulp stem cells (DPSCs), and umbilical cord mesenchymal stem cells (UCMSCs) which have been proven to improve neovascularisation.

BM-MSC contributes to enhanced growth of epidermal cells, angiogenesis, wound contraction, and collagen deposition via engineered collagen-based scaffold implantation in excisional wounds on the murine model (Huang et al., 2012). Green fluorescent protein (EGFP) expressing bone marrow cells were isolated from C57BL/6-Tg (ACTB-EGFP) 1Osb/J donor mice and transplanted into lethally irradiated wild-type C57BL/6 mice to investigate the role of bone-marrow derived cells in a burn injury. The study showed that the large number of fibroblasts, inflammatory cells, epithelial cells, endothelial cell, and hair follicles enhanced the rate of wound healing (Rea et al., 2009). A study by Inokuma et al. (2006) indicated that chemokine/chemokine receptor interactions mediate the migration of the bone marrow keratinocyte precursor cells via a cutaneous T-cell attracting chemokine, CTACK/CCL27. There is a possibility that the bone marrow itself is activated by circulating cytokines or some other signal that initiates cell migration to the wound (Wu et al., 2007).

ASCs represent an alternative source of multipotent cells with similar characteristics to BM-MSC (Kim et al., 2009) and are easier to isolate. ASCs improve the healing rate in diabetic mice by secreting growth factors and cytokines, enhancing granulation tissue and capillary formation, and epithelialization (Mizuno & Nambu, 2011). An *in vitro* study by Lee et al. (2012) revealed that adipose stem-cell conditioned medium (ASC-CM) promotes HaCaT keratinocytes and foreskin-derived dermal fibroblast growth by upregulating type I procollagen alpha 1 chain gene in fibroblasts derived from ASC-

CM. In another study, ASCs enhanced the proliferation of human dermal fibroblast (HDF) through co-culturing and culturing with ASC-CM. The secretion of type I collagen stimulated the migration of HDF (Kim et al., 2007). One of the secreted growth factors identified in ASC-CM is vascular endothelial growth factor (VEGF) which promotes vasculogenesis and angiogenesis. The genetically modified ASC reported longer maintainable stem cells, improved tissue regeneration potential, and increased secretion of VEGF (Lee et al., 2012).

UCMSCs has shown pluripotent characteristics, and harvested UCMSC differentiated into dermal fibroblasts in a conditioned induction media upon being induced at third passage (Han et al., 2011). When UCMSC is incubated in appropriate conditions, the umbilical cord epithelium cells can differentiate and organize into an epidermis-like structure (Mizoguchi et al., 2004), and differentiation of keratinocytes were observed at a ratio of 1 to 10 when co-cultured with keratinocytes (Akino et al., 2005).

2.6.4 Types of dental stem cells

Dental stem cells are isolated from different parts of the teeth, such as from human extracted or exfoliated deciduous teeth (SHED), adult dental pulp stem cells (DPSCs), the apical part of the papilla (SCAP), the dental follicle (DFSC), and periodontal ligament stem cells (PDLSCs). SHED are isolated from the pulp of human deciduous teeth and shown to have higher proliferation rates compared to DPSCs (Miura et al., 2003). DPSCs have been isolated and grown from pulp tissue of permanent human dental pulp. SCAP is another type of dental stem cells isolated from human teeth and found at the apex of the tooth root (Bluteau, 2008) while DFSC is a dental follicle surrounding the developing tooth germ that has long been considered a multipotent tissue based on its ability to generate cementum, bone, and periodontal ligaments (Yao,

2008). PDLSCs are isolated from the root surface of extracted teeth and differentiated into cells or tissues very similar to periodontium (Estrela, 2011).

DPSCs have emerged as an alternative source of MSC compared to BMMSC in pre-clinical trials and have created a significant impact in regenerative medicine. Growth factors, such as TGFBs, BMPs, TGFB1, IGFs, and FGFs show up-regulation at the injury site and play an important role in the healing process (D'Souza et al., 1998). An *in vitro* study by Aranha et al. (2010) showed that DPSCs exposed to hypoxic conditions had VEGF expressions in participation of revascularization. Though differentiation of MSCs into tissue-specific cells has been reported, differentiation-independent mechanisms seem to play a more significant role in tissue repair, and need to be addressed further.

2.7 Anatomy of dental pulp

Teeth are made up of hard tissues which are the enamel, dentin, and cementum, and pulp which is the soft tissue. The enamel forms the outer surface of a crown of a tooth which is the hardest tissue in the human body and has the capability to withstand chewing pressures and temperature changes. Dentin includes the main portion of the tooth which is softer than enamel but harder than human bone. It consists of microtubules that have dental fibres to transmit pain stimuli and nutrition throughout the tissues. Cementum is the tissue that covers the root of the tooth in a very thin layer. It is not as hard as enamel or dentin but is harder than bone. It contains periodontal ligament fibers that help to anchor the tooth within the bone (Metivier & Bland, 2013).

2.8 Animal model and pre-clinical studies

Studies on the role of stem cells on wound healing require an experimental design to evaluate its significance in the healing process, and animal models and human subjects play an important part in this. The laboratory mouse is the common animal model chosen and numerous transgenic strains and models have been developed to help researchers study the molecular pathways involved in wound repair and regeneration (Wong et al., 2011). Animal studies have established preliminary evidence on the safety, feasibility, and efficacy of several important endpoints using BM-MSCs and progenitor cells as a potential therapeutic option to induce angiogenesis (Amann et al., 2010). The excisional wound model of diabetic rats has been reported to accelerate epithelialization, granulation, and angiogenesis upon topical antimicrobial therapy. Stem cell application is found to be promising for the treatment of difficult-to-heal wounds in diabetic rats (Falanga et al., 2007). In another study, a topical treatment of soluble factors from fibroblast stimulated regeneration in an *in vivo* porcine model (Peura et al., 2012). Ayatollahi et al. (2014) isolated and cultured rat bone marrow MSCs and studied the comparison of the differentiation potential of human and rat MSCs in selective culture media.

Despite successful research outcomes in animal studies, pre-clinical studies offer more future prospects in wound care management. Using stem cells has been an alternative and better treatment strategy for various skin wounds. There are many published studies (www.ClinicalTrials.gov) which explore autologous cells as an alternative for wound healing (Table 2.1).

Peripheral vascular disease, diabetic foot ulcer (DFU), burn wounds, and other skin deformities have been treated with autologous stem cells. The findings of clinical trials performed on patients from 2005-12 show only 19 pre-clinical studies conducted on

wound healing of which eight were successfully completed. Recently, a randomized design clinical trial on critical limb ischemia diabetics treated with BMMSC successfully induced revascularization in affected limbs and has proven to be safe and feasible (Kirana et al., 2012). Other completed pre-clinical studies have used topical applications of a combination of alginate dressing and mouse epidermal growth factor (Bi et al., 2012), and autologous skin fibroblasts (Zorin et al., 2014). However, as the inflammatory cells in bone marrow cells may cause adverse effects by increasing the inflammatory response and inhibit the regeneration process (Gonzalez et al., 2003), long term clinical studies are required to determine the precise therapeutic effects.

Table 2.1: Approved clinical trials using stem cells for skin wound healing

Clinical trial identification	Status	Disease indications	Investigational drug/study phase	Patients enrolled	Route of injection	Sponsor
PMID:22340556	Completed (2012)	Patients with refractory wound/randomized control trial	Combination therapy with alginate dressing and mouse epidermal growth factor	18	Topical	Dept. of orthopedics Hangzhou, Zhejiang China
PMID:21649569	Completed (2011)	Critical limb ischemia (CLI)	Autologous bone marrow mononuclear cell injection	20	Intra-muscular	Faculty of medicine, Cairo University
PMID:17518741	Completed (2007)	Acute wound from skin cancer surgery and non-healing lower extremity wound	Autologous bone marrow	13	Topical	Roger Williams Medical Center, USA
PMID:17159798	Completed (2006)	Diabetic ulcer	Autologous skin fibroblast and autologous mesenchymal stem cells	Not given	Topical and injection	Comenius University, Slovakia
PMID:19224912	Completed (2006)	Refractory lateral epicondylitis	Skin dermal fibroblasts	12	Injection	Royal National Orthopaedic Hospital, UK

Table 2.1, continued.

NCT01065337	Completed (2012)	Diabetic Foot Ulcer	Autologous bone marrow/Phase II	30	Intra-muscular	Ruhr University of Bochum, Germany
NCT00535548	Unknown (2007)	Pressure Sore	Hematopoietic stem cell/Phase I	5	Injection	University Hospital, Basel, Switzerland
NCT00796627	Recruiting (2010)	Burn wound	HIF-1 Regulated Endothelial Progenitor Cell	100	Not found	Johns Hopkins University, USA
NCT01353937	Not recruiting (2011)	Diabetic foot ulcer	Autologous bone marrow/Phase I	27	Topical	New York University, USA
NCT00434616	Active, not recruiting (2011)	Critical Limb Ischemia	Autologous bone marrow/Phase II & III	90	Injection	Hospital Berlin Vascular Center Berlin, Germany
NCT01232673	Active, not recruiting (2010)	Critical Limb Ischemia	Autologous bone marrow/Phase II	90	Injection	University Hospital Ostrava, Czech Republic
NCT01216865	Not recruiting (2010)	Diabetic Foot Ulcer	Umbilical Cord mesenchymal stem cells Phase II / III	50	Injection	Qingdao University, China

Table 2.1, Continued.

NCT00616980	Completed (2011)	Critical Limb Ischemia	Autologous plasma/ Phase I &II	28	Injection	Northwestern University, USA
NCT00442143	Unknown (2007)	Critical Limb Ischemia	Autologous Bone marrow Derived Mononuclear Cells/Phase 1	10	Injection	Odense University Hospital, Denmark
NCT00113243	Unknown (2005)	Peripheral Vascular Disease	Autologous Bone marrow / Phase 1	20	Injection	Indiana University School of Medicine, USA
NCT00488020	Unknown (2007)	Critical Limb Ischemia	Autologous Bone marrow / Phase 1	10	Injection	Instituto de Molestias Cardiovasculares, Brazil
NCT00871221	Unknown (2009)	Scleroderma, Systemic Sclerosis	Autologous Bone marrow / Phase II & III	30	Injection	National Institute of Allergy and Infectious Diseases (NIAID), USA
NCT01115634	Completed (2010)	Facial deformities	Autologous fibroblast transplantation/ Phase II / III	40	Injection	Royan Institute, Iran
NCT01305863	Recruiting (2011)	Limb ischemia	Autologous adipose tissue/ Phase I & II	60	Vascular graft	Tissue Genesis, Inc., USA

2.9 Stem cell-conditioned medium (SC-CM)

Recently, stem cell derived-soluble factors have demonstrated biological activity such as enhanced wound healing (Heo et al., 2011) and other functions (Barreto & Salgado, 2010). Examples of soluble factors which secrete growth factors from the conditioned medium of stem cells have been identified (Kilroy et al., 2007; Skalnikova et al., 2011).

The stem cell-conditioned medium has generated interest on cell based therapy. However, the paracrine factors secreted by MSC that are responsible for wound healing processes have not been fully elucidated. MSCs are known for their immunomodulatory and anti-inflammatory properties as well as the secretion of pro-angiogenic elements like vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF) (Imberti et al., 2007; Togel et al., 2007; Zarjou et al., 2011). The production and secretion of growth factors by ASCs into the media is known to provide regenerative effects in the skin. Kim et al. (2009) discovered that ASC-CM stimulated both collagen synthesis and the migration of dermal fibroblasts which promote wound healing and improves wrinkling via paracrine routes in animal models. ASC-CM also inhibited melanogenesis and protected dermal fibroblasts from oxidative damage caused by irradiation (Kim et al., 2009). The effect of the conditioned ASC medium was also reported to increase the proliferation of HaCaT cells and dermal fibroblasts in an *in vitro* study by upregulating the transcription of type I procollagen-alpha-1 chain gene of fibroblasts (Lee et al., 2012). The stimulatory effect of ASC-CM also accelerates the proliferation of endothelial cells (Moon et al., 2012; Lee et al., 2009).

Keratinocytes that are activated during wound healing release growth factors and various cytokines that stimulate fibroblasts and endothelial cells, initiate the influx of

immune cells, and produce systemic effects (Shephard et al., 2004; Werner et al., 2007). The RhoA-ROCK signaling pathway in ASC-CM triggers the migration of keratinocytes which enhances *in vitro* wound healing (Moon et al., 2012). The presence of the hepatocyte growth factor (HGF) in *in vitro* scratch assay has been shown to stimulate the proliferation of HaCaT keratinocyte cells (Delehedde et al., 2002). HGF functions to enhance the migration of neutrophils, monocytes and mast cells into wound areas and promotes the secretion of pro-angiogenesis factors (Bevan et al., 2004). Apart from HGF, other growth factors that promote keratinocyte migration include IL-6 (Sato et al., 1999), CTGF (Igarashi et al., 1993), GM-CSF (Mann et al., 2001), and TGF-β3 **(**Bandyopadhyay et al., 2006). However, the secretion of these growth factors may be different when pooled in ASC prepared from different donors (Moon et al., 2012). In another study by Rehman et al. (2004), ASC-CM showed various growth factor secretions by ASC such as PDGF, IGF, KGF, bFGF, TGF-B, HGF, and VEGF, while a study by Kim et al. (2007) reported the secretion of PDGF, FGF, TGF-β1, KGF, HGF, and VEGF.

BM-MSC-CM injected and topically applied onto diabetic and non-diabetic mice significantly increased the rate of wound closure compared to fibroblast-CM treated mice (Chen et al., 2008). BM-MSC-CM initiated chemo attractive and mitogenic effects on endothelial cells which resulted in the recruitment of endothelial and endothelial progenitor cells into the wound. Several analyses including Real-Time PCR, antibody-based protein array, and ELISA showed expression of cytokines and growth factors in BM-MSC-CM. Compared to fibroblast-CM, BM-MSC-CM secreted cytokines including VEGF-alpha, IGF, EGF, keratinocyte growth factor, angioprotein-1, stromal derived factor-1, macrophage inflammatory protein-1 alpha and beta, and erythropoietin. VEGF-alpha, IGF-1, PDGF-BB, and Ang-1 are known as angiogenic

cytokines found in BM-MSC-CM which lead to the proliferation of endothelial cells and neovascularization in wound healing. Fibroblast-CM on the other hand, was found to secrete IGF-1, KGF, PDGF-BB, EPO, G-CSF, and TPO (Chen et al., 2008).

The growth factors or extracellular matrix proteins secreted by stem cells derived from human extracted or exfoliated deciduous teeth (SHED) and SHED-derived conditioned medium (SHED-CM) have the ability to enhance the wound-healing potential of human dermal fibroblasts (HDF). Wound contraction occurs by increasing collagen synthesis and by activating the proliferation and migration activity of HDFs (Ueda & Nishino, 2010). Inoue et al. (2013) reported that the enhancement of vasculogenesis, migration, and differentiation of endogenous neuronal progenitor cells can be initiated by SHED-CM in ischemic brain injuries in the rat model.

Lee et al. (2011) successfully used the conditioned medium of human embryonic stem cell (hESC)-derived endothelial precursor cells (EPC) in the treatment of excisional wound healing in rats. The secretory factors that were detected using multiplex cytokine array were epidermal growth factor (EGF), fibroblast growth factor (bFGF), fractalkine, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, IL-8, platelet-derived growth factor-AA (PDGF-AA), and vascular endothelial growth factor (VEGF), which stimulate angiogenesis.

2.9.1 Wound healing mechanism initiated by secreted cytokines and paracrine signaling in stem cell therapy

Tissue injury causes blood vessel damage and leakage of blood constituents into the wound site. Hemostasis begins immediately after wounding, with vascular constriction and fibrin clot formation. Hemostasis caused by coagulation provides the basic

architecture for initiating the inflammatory phase and tissue formation (Szpaderska et al., 2003).

The clot and surrounding wound tissue release pro-inflammatory cytokines and growth factors such as PDGF, FGF, EGF, TGF-β, transforming growth factor-α (TGF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) (Szpaderska et al., 2003). The migration of inflammatory cells into the wound, or chemotaxis, starts with the infiltration of neutrophils, macrophages, and lymphocytes. Macrophage infiltration is highly regulated by gradients of different chemotactic factors, including growth factors, pro-inflammatory cytokines, and chemokines [macrophage inflammatory protein 1α (MIP-1α, MCP-1, RANTES)] (Gosain & DiPietro, 2004; Broughton et al., 2006).

Mesenchymal stem cells play an important therapeutic role in wound healing. Endogenous MSCs or exogenously delivered MSCs can migrate to the wound site to heal the damaged tissue. Not only can the MSCs participate in the process but the concentrated conditioned medium from MSCs can also modulate the wound healing process without the presence of MSCs in the wound (Li & Fu, 2012). As such, this study on the MSC-conditioned medium will focus on determining its significance in skin wound healing. Figure 2.5 shows the pathway of a stem cell conditioned medium in wound healing. The mechanisms responsible for enhanced wound healing in skin include the possible trans-differentiation of MSC to form cells of epidermal and dermal lineages (Sasaki et al., 2008). Growth factors secreted by MSCs stimulate paracrine effects and this signaling between the cytokines and pro-inflammatory factors enhances wound closure (Kilroy et al., 2007).

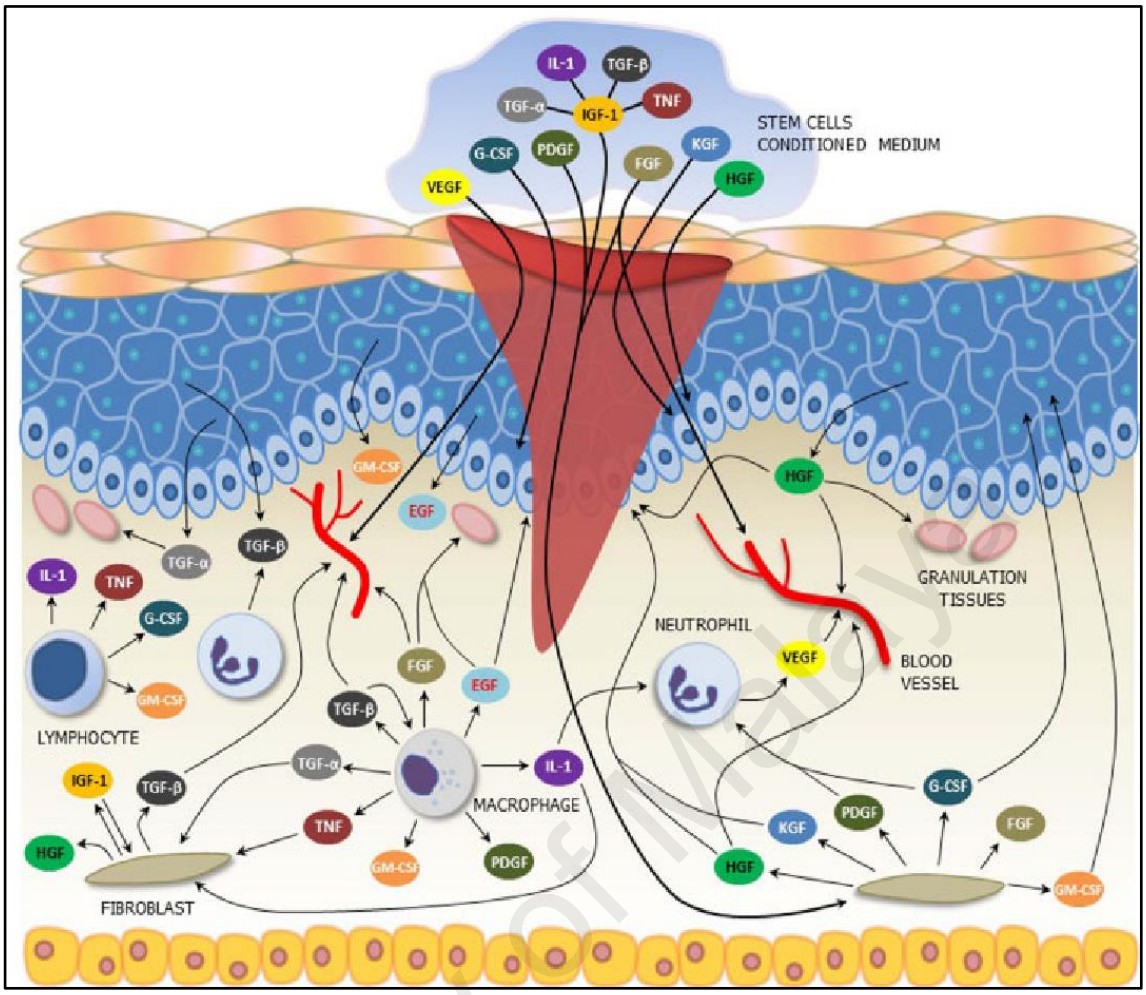


Figure 2.5: Mechanism of wound healing using stem cell therapy. Adaptation from Jayaraman et al., 2013.

A number of wound healing mediators were identified in MSC-CM, including TGF-β1, the chemokines IL-6, IL-8, MCP-1 and RANTES, and collagen type I, fibronectin, SPARC, and IGFBP-7. Table 2.1 shows the types of cytokines secreted during wound healing and their functions. Cytokines play an important role in accelerating the healing process. The enhancement of wound healing is due to accelerated cell migration rather than increased cell proliferation (Walter et al., 2010).

Neovascularization is an important step in wound healing which promotes the growth of new blood vessels to sustain the newly formed granulation tissue and keratinocytes. Wu et al. (2007) discovered that BM-MSCs enhanced angiogenesis in wound healing.

Vascular endothelial growth factor (VEGF) is one of the key factors that promote vasculogenesis and angiogenesis in wound healing (Bueno et al., 2016).

Throughout the process of wound healing, keratinocytes migrate from the basal population around the wound's edge to cover the lesion and later differentiate into a basal phenotype of stratified squamous keratinizing epidermal cells (Metcalfe & Ferguson, 2007). The dermal fibroblasts attracted to the wound site then differentiate into myofibroblasts induced by macrophage-derived cytokines such as TGF-β1 (Serini et al., 1998). These myofibroblasts synthesize ECM that is required for wound contraction (Moulin et al., 1998). Subsequently, the myofibroblasts will be replaced by a second wave of dermal fibroblasts from the surrounding tissue and produce a new collagenous ECM (Sorrell et al., 2008). Therefore, the MSC-CM which contains cytokines and other mediators are essential for stimulating biological activity in order to accelerate the healing process (Walter et al., 2010).

Table 2.2: List of cytokines secreted in stem cell conditioned-medium

Paracrine factors	Function	Phase of Wound healing
TGF-β1	Stimulates migration of macrophages, dermal fibroblasts. Increases angiogenesis and granulation tissue for re-epithelialisation process.	Inflammatory, proliferation
TGF-α	Stimulates epithelial cells and granulation tissue for re-epithelialization process.	Inflammatory, proliferation
bFGF	Increases fibroblast proliferation, angiogenesis and matrix deposition.	Proliferation, maturation
IL-6	Influencing inflammatory cells influx and promotes re-epithelialisation.	Inflammatory, proliferation
IL-8	Promotes skin re-epithelialisation by increasing keratinocyte migration and proliferation.	Proliferation
IL-1	Increases pro-inflammatory cell and fibroblast proliferation.	Inflammatory, proliferation
EGF	Enhance migration of keratinocyte and fibroblast. Increased granulation tissue.	Proliferation
VEGF	Endothelial survival and migration and proliferation. Regulates angiogenesis and granulation tissue formation.	Inflammatory, proliferation
PDGF	Increase macrophage activation, fibroblast proliferation, angiogenesis and collagen metabolism.	Inflammatory, proliferation, maturation
KGF	Stimulation of keratinocytes' proliferation and migration.	Proliferation
G-CSF	Initiate inflammatory cells and increases keratinocytes.	Inflammatory, proliferation
GM-SCF	Proliferation of epidermal cell.	Proliferation
TNF	Increases fibroblast.	Proliferation
IGF-1	Fibroblast and collagen synthesis.	Proliferation, maturation.
HGF	Promotes re-epithelialisation, vasculogenesis and granulation tissue formation.	Proliferation
(MCP-1) and RANTES	Promote dermal wound healing as a chemoattractant to cells of the immunesystem particularly macrophages	Proliferation
Collagen type 1 and fibronectin	Stimulates fibroblast and keratinocycte cell adhesion and migration.	Maturation
SPARC	Cell-matrix interaction.	Maturation
IGFBP-7	Regulate proliferation and migration of keratinocytes.	Inflammatory, proliferation
CTGF	Chemo attractant for fibroblast	Proliferation

CHAPTER 3: ISOLATION AND CHARACTERIZATION OF DENTAL STEM CELLS FROM PRIMARY SOURCES

3.1 Introduction

Stem cell (SC) therapy has entered a new dimension following the conduct of many clinical trials. Among the commonly used cells in clinical trials are mesenchymal stem cells derived from bone marrow (BM-MSCs). Recently, stem cells of dental origins such as permanent teeth (DPSCs), exfoliated deciduous teeth (SHED), periodontal ligaments (PDLSCs), dental follicles (DF), and dental apical papilla (SCAP) have emerged as other attractive sources of cells and have been investigated in clinical trials especially in treating neuro-ectoderm related diseases (Ponnaiyan, 2014; Rodriguez-Lozano et al., 2012; Sedgley & Botero, 2012; Tan et al., 2015). Despite this, a continuous uninterrupted supply of stem cells is needed for successful completion of any clinical trials. Such trials are carried out by culturing the cells in multiple subcultures to attain the required cell number for transplantation purposes. While the process is easier by using BM-MSCs or umbilical cord stem cells, the main caveat surrounding the usage of dental stem cells in a clinical set up is the low cell yield.

This is because, compared to other tissues, the starting material is extremely low and requires additional subcultures to achieve an adequate cell number. For example, a single umbilical cord is able to generate more than 1×10^8 cells within a subculture (Nekanti et al., 2010), while the use of dental tissues to generate a similar cell number requires an additional four to five subcultures, and that too in a pooled sample situation (Govindasamy et al., 2011). Despite this, using stem cells for transplantation has proven to be safe in long term expansion (Huang et al., 2015; Zhuang et al., 2015). It was recently reported that DPSCs cultured at long term expansion are able to maintain cell function, proliferation, and viability thus making them suitable for therapeutic usage

(Martin-Piedra et al., 2014). While these results look promising, most of them focus in the plasticity, stemness, and immunophenotyping. However, another vital aspect worth considering in long term cell culture systems is the property of immunity. In general, stem cells are regarded as immuno-privileged cells and there is convincing evidence that they affect innate and adaptive immune cells in two possible ways; they decrease T and B-cell proliferation by cell-cell contact, and release a wide range of paracrine factors (Meisel et al., 2004). Similar mechanisms were also reported in stem cells of dental origins (Wada et al., 2009; Machado et al., 2013; De Miguel et al., 2012).

There are several methods to evaluate the immune properties of stem cells and one of them is by examining their gene levels. In this context, gene expression profiling is a reliable method and has been commonly used in understanding the biology and ontogeny of the cells (Vasandan et al., 2014; Winkler et al., 2015). Here, we investigated for the first time the immune properties of dental-derived stem cells in long-term expansion. It is believed that understanding the pattern of immune properties of the dental stem cells especially at late culture, will eventually add value to the existing knowledge on the safety of cell transplantation.

To meet the objectives of the study, experimental works were carried out to:

1. Isolate stem cells from SHED, PDL, and DPSC from the primary source;

2. Characterise and confirm the status of MSCs;

3. Evaluate the differences of SHED, PDL, and DPSC in terms of immune genes in long-term cultured stem cells using human immune qPCR array; and

4. Assess the validation of randomly chosen immune genes from SHED, PDLSCs, and DPSCs using cytokine protein array.

3.2 Methodology

3.2.1 Sample collection

This study was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya with Medical Ethics Clearance Number DF CD1411/0087(P), as attached in Appendix 1.1, and with all donors providing written consent. Dental pulp stem cells from extracted deciduous teeth (SHED) [n=3; ages 7-11 years] and permanent teeth (DPSCs) [n=3; ages 24-35] were established as previously described by us (Govindasamy et al., 2010) whereas periodontal ligament stem cells (PDLSCs) [n= 3; ages 7-11 years] were isolated and cultured up to passage 10 (P10) according to previously reported protocols with slight modification (Seo et al., 2004). Cells at P2 and P9 were regarded as early and late passage respectively.

3.2.2 Isolation of dental pulp stem cell from extracted deciduous teeth

The isolation method described by Govindasamy et al. (2010) was used in this study. Root surfaces of the extracted teeth were cleaned with povidone-iodine (Sigma Aldrich, St. Louis, MO, USA) and the pulps extirpated within 2 hours post-extraction and processed. The deciduous teeth were mounted on a specially designed jig and sectioned horizontally, layer by layer using a diamond disc on a straight hand piece until the pulp chamber was exposed. Pulp tissue was removed using barbed broach and rinsed with washing media consisting of Dulbecco's Phosphate Buffered Saline (DPBS) [Invitrogen, Carlsbad, CA, USA] and 0.5% of penicillin/streptomycin (Invitrogen) in a 1:1 ratio. The tissues were transferred into several 1.5 ml tubes (Axygen, Union City, CA, USA) containing Dulbecco's modified Eagle's medium-knock-out (DMEM-KO) (Invitrogen). Mechanical destruction was carried out by mincing the tissues into small fragments using sterile scissors followed by immersion into 3 mg/ml Collagenase Type I solution (Gibco, Grand Island, NY, USA) in a 1.5 ml microcentrifuge tube (Axygen)

which was then incubated for 40 minutes in humidified atmosphere of 95% air and 5% CO2 at 37°C. The mixture was then neutralized by adding 6 ml of culture media (Table 3.1) and the entire mixture then transferred into 15 ml tube (BD Bioscience, Franklin Lakes, NJ, USA) and subsequently centrifuged at 1250 rpm for 6 minutes at 25°C. Supernatant was discarded and the minced pulp was resuspended in 7 ml of culture media, seeded into a new T25 cm2 culture flask (BD Pharmingen, San Diego, CA, USA) and incubated in humidified atmosphere of 95% air and 5% CO2 at 37°C. Non-adherent cells were removed 48 hours after the initial plating. The medium was replaced every 3 days until the cells reached an 80–90% confluence. Cell morphology was captured using an inverted microscope (Olympus model XC-50, Tokyo, Japan). The procedure of the dental pulp tissue extirpation from the extracted tooth is shown in Figure 3.1.

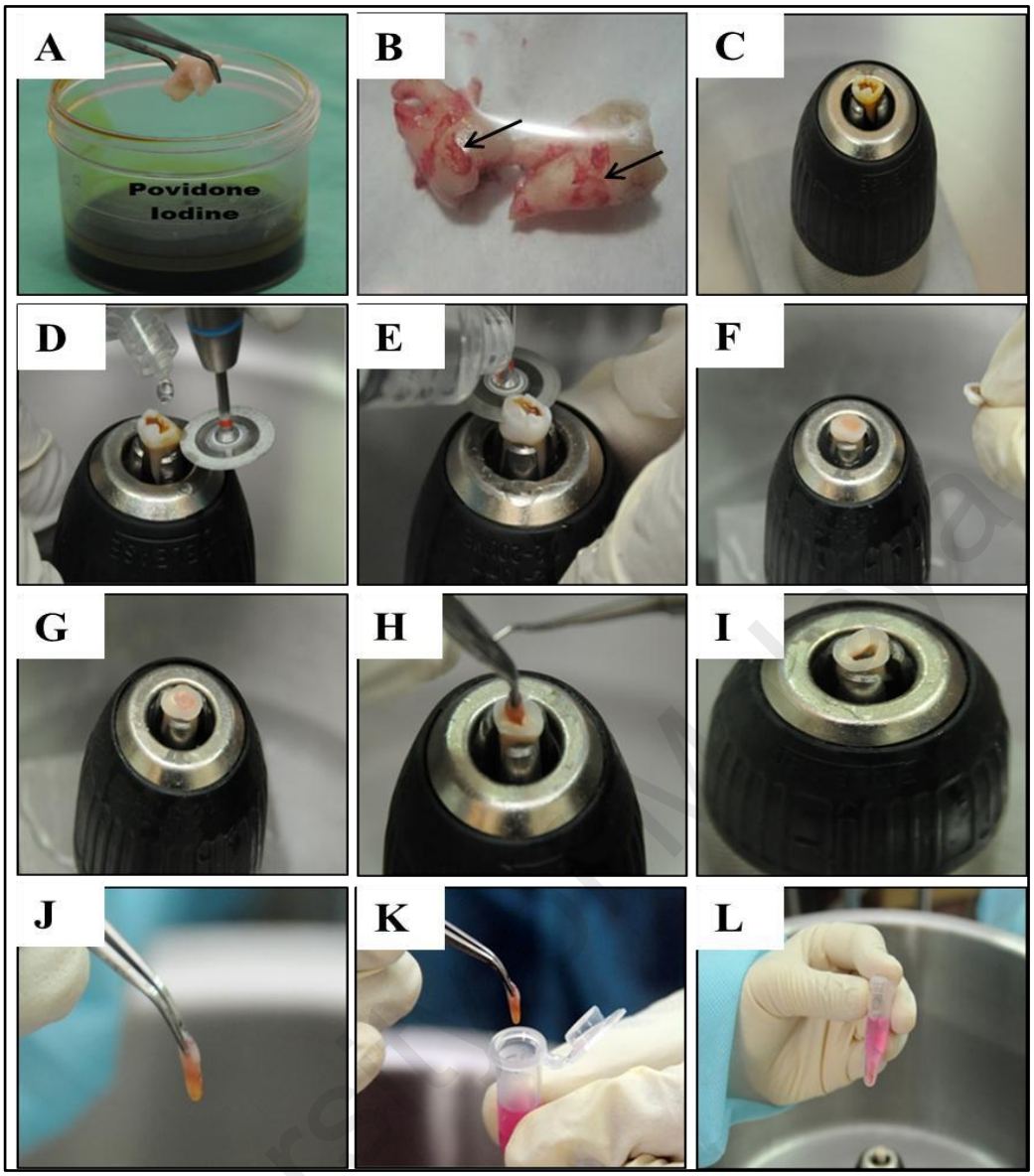


Figure 3.1: Extirpation of pulp and scraping of periodontal tissue (PDL) (arrows). (A) Tooth surface was cleaned with PVP. (B) Periodontal ligament tissue. (C)Tooth was tightly placed onto a jig. (D-G) Crown of tooth was cut at the cementum-enamel junction using sterilized diamond disc to expose the pulp chamber, double distilled water (ddH_2O) was apply during cutting process. (H) The pulp tissue was gently extirpated from the root canals using a sterile tweezer (K-L). The dental pulp and PDL tissues were transported in media (Ronald, 2013).

Table 3.1: Composition of culture media for SHED, DPSCs and PDLSCs

Component	Manufacturer	Volume (ml)
DMEM KO	Invitrogen	442.5
Foetal Bovine Serum (FBS)	Hyclone; Thermo Fisher Scientific Inc, Waltham, MA	50.0
Penicillin/Streptomycin	Invitrogen	2.5
Glutamax	Invitrogen	5.0
Total Volume	-	500.0

3.2.3 Cell culture expansion and cryopreservation

Upon SHED, DPSCs and PDLSCs attaining 80-90% confluence, the cells were rinsed twice with 5 ml of DPBS (-/-) (Invitrogen), detached using 2 ml of 0.05% Trypsin-EDTA (Invitrogen), and incubated at 37°C for 2 minutes before being neutralized with 8 ml of culture media. The solution was centrifuged at 500 g for 6 minutes, and supernatant discarded and cells resuspended in culture media. Aliquot of the solution was used to determine the total cell count. A total of 10µl from the solution was mixed with 90µl of Trypan Blue stain (Sigma Aldrich) and 10µl of the mixture was then transferred to a haemocytometer (Sigma Aldrich) for viewing under a microscope (Olympus). Cells not stained with Trypan Blue stain were counted based on the following formula: [Number of cells x dilution factor x 10^4 x volume of media] used for resuspending the cells. The cryopreservation is a standard method for dental stem cells in which one cyovial contains 1 million cells diluted with freezing media (90% FBS mixed with 10% DMSO).

3.2.4 Cell surface analysis

SHED, DPSCs, and PDLSCs were checked for their cell surface markers expression (CD34, CD44, CD45, CD73, CD90, CD166, and HLA-DR) with a flow cytometer.

Upon reaching 90% confluence, the cells were harvested with 0.05% Trypsin-EDTA (Invitrogen) and resuspended in 10 ml of DPBS (-/-)(Invitrogen).The cell suspension was centrifuged at 500g for 5 minutes at 25°C. The supernatant was discarded and the cell pellet resuspended in DPBS (-/-) (Invitrogen) at a cell density of $1x10^6$ cells/ml. Approximately $1x10^5$ of cells in 200µl DPBS (-/-(Invitrogen) was incubated with the labeled antibodies in the dark for 1 hour at 37°C. The following antibodies were used to mark the cell surface epitopes: CD90-phycoerythrin (PE), CD44-PE, CD73-PE, CD166-PE, and CD34-PE, CD45-fluoroisothiocyanate (FITC), and HLA-DR-FITC. All analyses were standardized against negative control cells incubated with isotype-specific immunoglobulin (Ig) IgG1-PE and IgG1-FITC. At least10,000 events were acquired on a Guava Technologies flow cytometer, and the results analyzed using Cytosoft, Version 5.2, Guava Technologies.

Table 3.2: Antibodies used for cell surface analysis of SHED, DPSCs and PDLSCs

Cell surface antigen	**Volume used**	**Manufacturer**
CD 34-PE	20 µl	BD Pharmigen
CD 44-PE	20 µl	BD Pharmigen
CD 45- FITC	20 µl	BD Pharmigen
CD 73-PE	20 µl	BD Pharmigen
CD 90-PE	20 µl	BD Pharmigen
CD 166-PE	20 µl	BD Pharmigen
HLA-DR- FITC	20 µl	BD Pharmigen

3.2.5 Growth kinetics

The proliferation rate was determined by plating cells of SHED, DPSCs, and PDLSCs per T25 cm2 culture flask (BD Pharmingen) with a cell density of 1,000/cm2. Three replicates were performed for each passage and time pointed for a total of nine passages. The SHED, DPSCs, and PDLSCs that were expanded under the culture

conditions were detached by trypsinisation upon reaching a confluence of 90%. Cells were counted by means of a Trypan Blue stain (Sigma Aldrich) before the next passage, and 25,000 cells were re-plated. Growth kinetics was analyzed by calculating population doubling time (PDT) which was obtained using the formula:

$$PDT = \frac{\log 2\ (time)}{\log\ (\text{initial number of cell} - \text{final number of cell})}$$

3.2.6 Multilineages differentiation

SHED, DPSCs, and PDLSCs (Sections 3.2.2 and 3.2.3) at passage 3 were plated at a density of 1,000 cells/cm^2 in a 6-welled plate (BD Bioscience, Franklin Lakes, NJ, USA) and grown to confluence in a humidified atmosphere of 95% air and 5% CO^2 at 37°C. The cells were differentiated into adipogenic, chondrogenic, and osteogenic lineages.

3.2.6.1 Adipogenic differentiation

Adipogenic differentiation was stimulated by inducing the cells in an adipogenic induction medium consisting of DMEM-KO (Invitrogen), 10% (v/v) FBS (Hyclone), 1% Glutamax (Invitrogen), 200 µM indomethacin, 0.5 µM 3-isobutyl-1-methyxanthine, 10µg/ml insulin, and 1 µM dexamethasone (all from Sigma Aldrich). Lipid droplets in the produced adipocytes were visualized by staining with red oil stain (Sigma Aldrich). The differentiated cells were fixed using 4% paraformaldehyde (PFA) (Sigma Aldrich) for 30 minutes at 25°C and rinsed twice with 1 ml of DPBS (-/-) (Invitrogen). The cells were then washed twice using sterilized distilled water. Oil Red O working solution was gently added and incubated for 50 minutes at 25°C. The stained solution was carefully removed and rinsed thrice with sterilized distilled water. The formation of red lipid droplets was observed under the microscope (Olympus).

3.2.6.2 Osteogenic differentiation

Osteogenic differentiation was stimulated by inducing the cells in an osteogenic induction medium consisting of DMEM-KO (Invitrogen), 10% (v/v) FBS (Hyclone), 1% Glutamax (Invitrogen), 10-7 M dexamethasone (Sigma Aldrich), 10 μM β-glycerol phosphate (Fluka, Buchs, Switzerland), and 100 μM of L-ascorbic acid-2 phosphates (Sigma Aldrich). An assessment of calcium accumulation was visualized by von Kossa staining (Sigma Aldrich). Cells were fixed using 4% PFA (Sigma Aldrich) for 15 minutes at 25°C and rinsed once with 5 ml of sterilized distilled water. The fixed cells were then incubated with 1% silver nitrate (Sigma Aldrich) for 60 minutes under a bright light and rinsed once again with sterilized distilled water. Calcium mineralization was observed under a microscope (Olympus) as a black coloured clump or precipitation.

3.2.6.3 Chondrogenic differentiation

Chondrogenic differentiation was stimulated by inducing the cells in a chondrogenic induction medium consisting of DMEM-KO (Invitrogen), 10% (v/v) FBS (Hyclone), 1% Glutamax (Invitrogen), ITS+1 (Sigma Aldrich), 50 μM of L-ascorbic acid-2 phosphate (Sigma Aldrich), 55 μM of sodium pyruvate (Invitrogen), 25 μM of L-proline (Sigma Aldrich) and 10 ng/ml of transformation growth factor-β type 1 (TGF-β1) (Sigma Aldrich). Cells were washed twice using DPBS (-/-) (Invitrogen) and fixed using 4% PFA (Sigma Aldrich) for 10 minutes at 25°C. A total of 1 ml of Alcian Blue working solution was added into the well and incubated for 30 minutes at 25°C. The cells were rinsed with sterilized distilled water. An assessment of proteoglycan accumulation was stained by Alcian Blue and visualized under a microscope (Sigma Aldrich).

3.2.6.4 Reverse transcription polymerase chain reaction (RT-PCR) for trilineage markers

The RT-PCR was performed using complementary DNA (cDNA) prepared as described in section 3.2.8. A total of 25µl of PCR reaction was prepared by adding the following components: 2.5µl of 10x PCR ($-Mg^{2+}$), 1.25µl of 50 mM $MgCl_2$, 0.5µL of 10 mM dNTP Mix, 1µl of forward primer and 1µl of reverse primer, 0.2µl of Taq DNA polymerase,1µl of template cDNA and 17.55µl of autoclaved ddH_2O. The mixture was centrifuged at 500 rpm for 3 seconds. The cDNA amplification was performed at 94°C for 5 minutes, 94°C for 45 seconds for 30 cycles, 58.5°C for 30 seconds for 30 cycles, 72°C for 45 seconds for 30 cycles, and a final elongation at 72°C for 10 minutes and 4°C using a thermocycler. Polymerase chain reaction (PCR) products were resolved on1.5% agarose gel which was immersed in 1 x Trisborate-ethyl-enediamine tetra borate acid (TBE) buffer which was visualized via ethidium bromide staining (Sigma Aldrich).

3.2.7 RNA extraction and quantification

Total RNA was extracted using either Trizol technique or RNeasy Mini Kit (Qiagen). In the Trizol method, the cells were washed twice with DPBS (-/-) (Invitrogen) and lysed by adding 1 ml Trizol reagent (Invitrogen). The lysate was transferred to a 1.5 ml microcentrifuge tube (Axygen) and incubated for 5 minutes at 25°C. Approximately 200µl chloroform (Sigma Aldrich) was added to the tube and mixed by inverting it 20-25 times followed by incubation for 2-3 minutes at 25°C. The tube was then centrifuged at 12000 rpm for 15 minutes at 4°C following which the aqueous phase was transferred into a fresh microcentrifuge tube (Axygen). To precipitate the RNA, 0.5ml of isopropanol (Sigma Aldrich) was added, mixed, and incubated at 25°C for 10 minutes. Then microcentrifuge tube was centrifuged again at 12000 rpm for 10 minutes at 4°C. Supernatant was discarded and the pellet washed twice with 75% ethanol (Sigma

Aldrich). The pellet was air-dried, dissolved in 30µl of RNase and DNase-free water, and incubated at 37°C in a water bath for 10 minutes. The extracted RNA was treated with DNase (Qiagen) to remove genomic DNA. 1µl RNA was used for quantification and lµl of distilled water was used as a blank. The absorbance was recorded at 260 nm and 280 nm using Nano drop (Thermo Fisher Scientific Inc.) before being stored at -80°C. RNA concentration and purity was calculated using the following equations respectively:

RNA concentration (µg/µl) = OD260 x (40µg RNA/ml), and ratio of reading of OD260 /OD280

3.2.8 Complimentary deoxyribonucleic acid (cDNA)

Synthesis of cDNA was carried out according to the Superscript II Reverse Transcriptase (Invitrogen) or RT2 first strand kit (Qiagen) manufacture protocol. In the Invitrogen protocol, 1µl Oligo dT (50 µM) and 1µl dNTP mix (10 mM) were added to1µg of RNA (Section 3.2.8), and sterile distilled water added to make up the final volume of 13µl in a 0.2ml tube (Axygen). The tube was then incubated at 65°C for 5 minutes and then rapidly cooled on ice followed by a brief spin. A total of 4µl of 5 XFirst strand buffer and 2µl of 0.1 M DTT were added into the same tube and incubated at 42°C for 2 minutes. Then, 1µl of Super Script II (200 units) was added into the tube, mixed by pipetting gently up and down, then finally incubated at 42°C for 50 minutes. The reaction was heat inactivated at 70°C for 15 minutes and the cDNA used for subsequent experiments or stored at -20°C.

3.2.9 TaqMan® Array Human Immune Gene Array

Total cellular RNA from all cell lines at P2 and P9 were isolated with TRIzol® RNA isolation reagent (Invitrogen). The complementary DNAs (cDNAs) were synthesized using 1 µg RNA sample with a reverse transcriptase enzyme kit (Invitrogen). The

immune related genes were profiled by using TaqMan® Array Human Immune Gene Array. Briefly, the cDNAs were loaded on the microfluidic cards for thermal cycling on an ABI PRISM7900HT Sequence Detection System (Fisher Scientific), and expression values for target genes were normalized to the expression of 18srRNA. For estimation of the fold change by TLDA when the initial transcript levels were undetectable, the initial cycle threshold (C_T) value was assigned to be 35, which would lead to a possible underestimation of the actual fold change.

3.2.10 Validation of Human Immune Genes Using Cytokine Protein Array

The secretions of randomly selected immune genes were validated by using a customized human cytokine protein array (Panomics, Redwood City, CA). Briefly, conditioned medium from all cell lines at P2 and P9 were collected and the assay performed according to the manufacturer`s instructions. The analysis was performed using Luminex platform.

3.2.11 Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). Statistical comparisons were made and data analyzed by using analysis of variance (ANOVA) with the significance level set at $P = 0.05$. Tukey post hoc multiple comparison tests were carried out to determine the differences between the groups (Nishino et al., 2011; Nakamura et al., 2013).

3.3 Results

3.3.1 Characterization of MSCs derived from SHED, DPSCs and PDLSCs

To ascertain that the cell lines that were established were bone fide MSCs, we performed some basic MSC characterization studies. All the three types of cells displayed fibroblastic morphology (Figure 3.2 A). We investigated the mesoderm differential potential of SHED, DPSCs, and PDLSCs into adipogenic, chondrogenic, and osteogenic lineages under appropriate media induction and they were able to undergo adiopogenesis, chondrogenesis and osteogenesis respectively (Figure 3.3 B i-ix). To further characterize these cells, immunophenotyping was done by flow cytometry. Both samples were negative for hematopoietic markers CD34 and CD45, whereas more than 85% of the results were positive for MSC markers CD44, CD73, CD90, and CD166 (Figure 3.3 C). The PCR trilineage markers for osteogenesis (Osterix and Osteocalcin), adiopogenesis (Leptin and LPL) and chondrogenesis (Collagen II and Aggrecan) showed the gene expression (Figure 3.3 D).

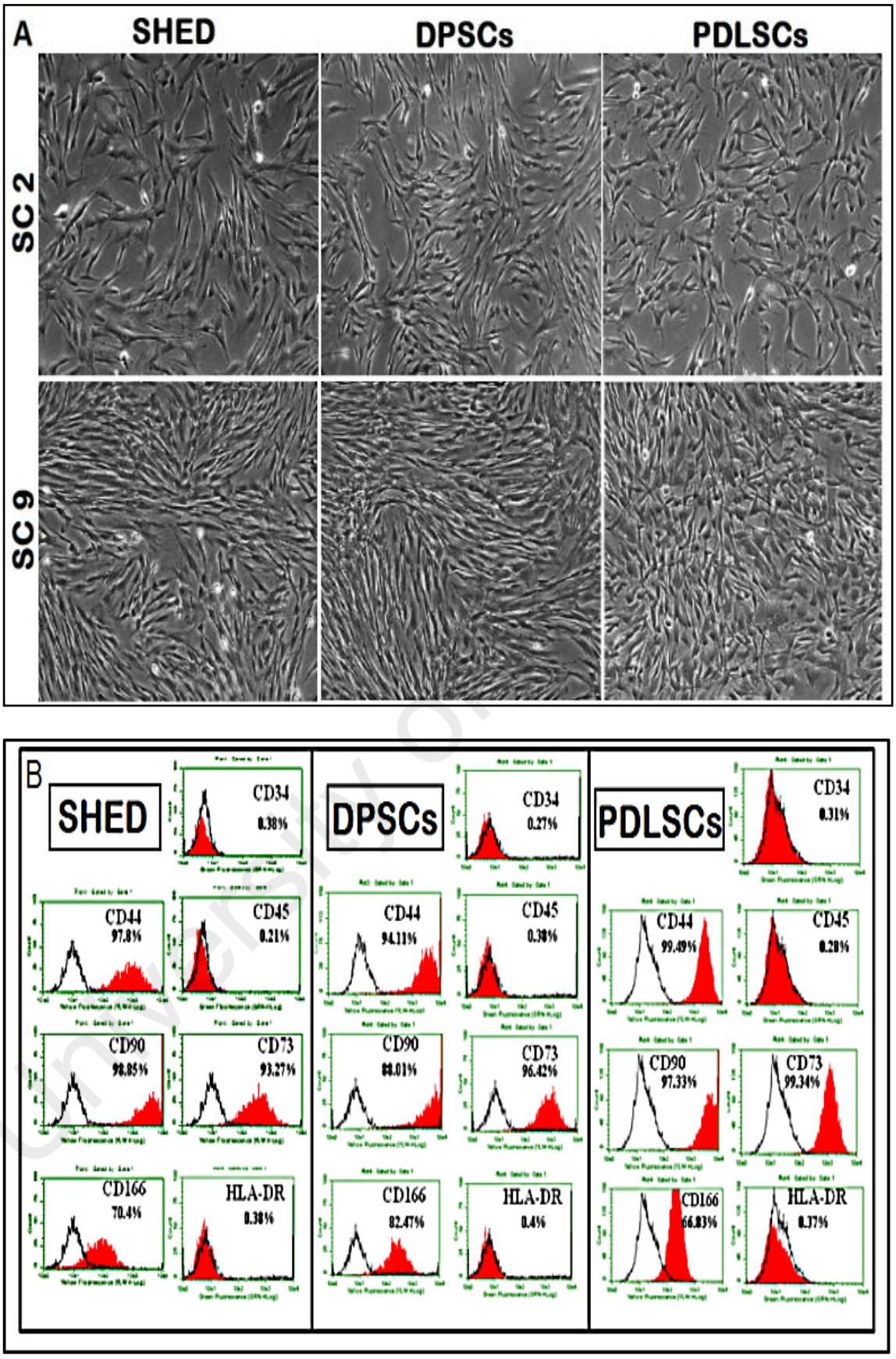


Figure 3.2: A. The morphology of SHED, DPSCs and PDLSCs for passage 2 and passage 9 observed under phase contrast microscope at 10X magnification. B. Immunophenotyping of SHED, DPSCs and PDLSCs which were tested against human antigens CD34, CD44, CD45, CD73, CD90, CD166, and HLA-DR.

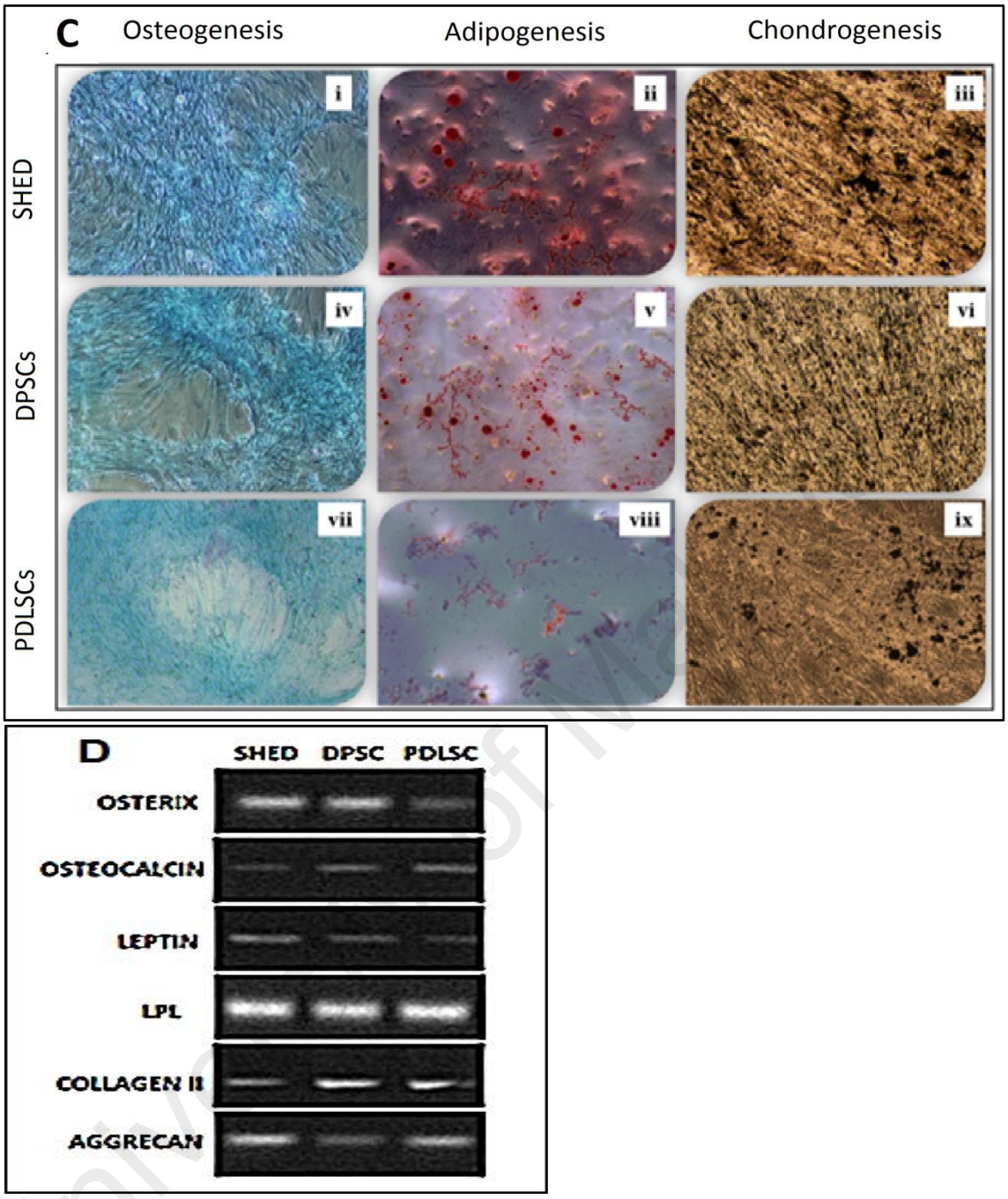


Figure 3.2 C. *In vitro* mesoderm differential potential of SHED (i-iii), DPSCs (iv-vii) and PDLSCs (vii-ix). All the staining was done 21 days after induction. Osteogenesis was confirmed by mineralized matrix deposition stained with von Kossa staining. Adipogenesis was detected by neutral oil droplet formation stained with Oil Red O. Chondrogenesis was detected by the presence of proteoglycans stained with Alcian blue. D. The trilineage RT-PCR markers shown gene expression for all the three dental sources.

3.3.2 Growth Kinetics

The growth curve line graph indicates that the cumulative cell count in per ten thousand, at the end of passage nine, had overall cell yield that was significantly higher in PDLSCs (370,000 cells) followed by SHED (340,000 cells), and DPSCs (300, 000

cells) in T25 cm^2 flask (Figure 3.3). The average population doubling times in hours at P9 for SHED, DPSCs and PDLSCs were (26.8 ±6.41), (26.1 ±9.01), and (25.3 ±6.67), respectively.

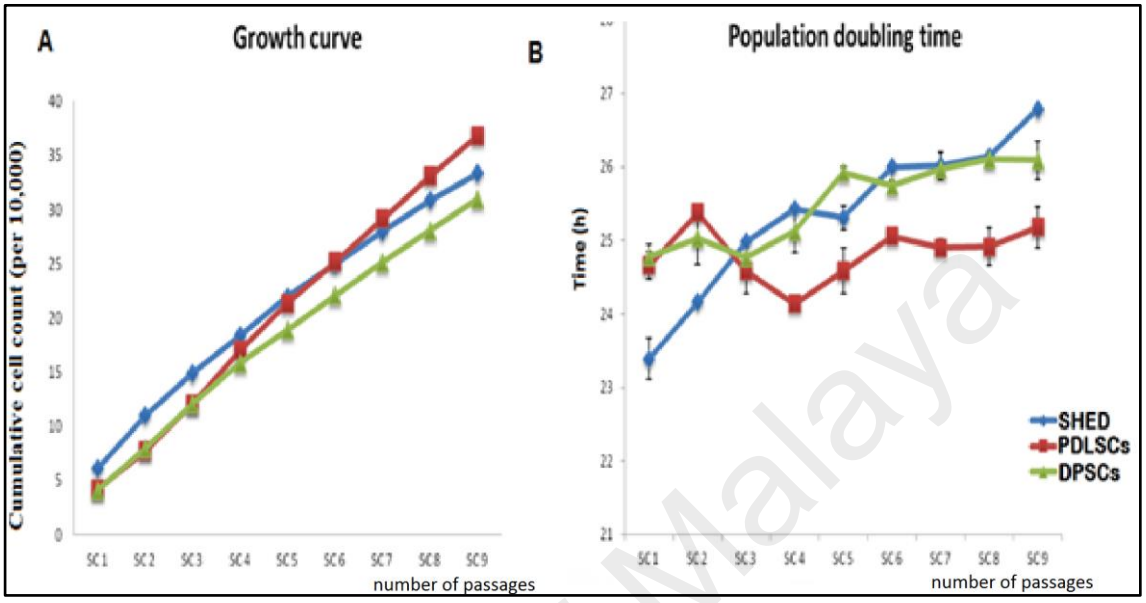


Figure 3.3 A. Long-term growth curves of SHED, DPSCs, and PDLSCs up to 9 passages. B. Comparison of population-doubling time in hours for the dental sources. The results represent the average of six culture replicates (n=6) with standard deviation.

3.3.3 Gene expression variability between SHED, DPSCs, and PDLSCs at early and late passages

To understand the immunological behaviour between these cell lines during their transition period from P2 to P9 (Martin-Piedra et al., 2014), we looked at the expression patterns of immuno-regulated genes. The genes were defined as being 2-fold up or down-regulated in each sample at P2 and P9 when compared to SHED at P2, which acted as a control with an accompanying p value of 0.05. Since huge fold variations were noticed among all the samples, they were tentatively classified into 5 distinct categories based on their fold expression value as follows: up-regulated genes consisting of 3 categories, namely, highly up-regulated (≥ 50 fold), medium-up-regulated (≥ 10 to 49.99 fold), and low up-regulated (≥ 2 to 9.99 fold) followed by unchanged genes (≥ 0.5-1.99 fold) and down regulated (≤ 0.499 fold) categories. In this

regard, in P2, it was noticed that the SHED has indicated mostly unchanged genes compared to DPSCs and PDLSCs. On the other hand, in the medium up-regulated category, no significant differences of gene expression percentage were found among all the cell lines. PDLSCs showed a significant lower percentage ($P<.05$) of genes in the low up-regulated category as compared to SHED and DPSCs. Surprisingly, in P9, a tremendous augmentation of genes percentage were seen in all up-regulated categories within all cell lines ($P<.05$). In terms of the unchanged category, a significant swift ($P<.05$) was observed in SHED, DPSCs, and PDLSCs from P2 to P9 where the genes decreased from 78% to 6%, 57% to 9%, and 60% to 9% respectively. In contrast, a significant increment ($P<.05$) of gene percentages ranging from 8% -17% was observed in all the cell lines between the same passages (Figure 3.4). To better understand the role played by each immune-related gene within the cell lines and passages, we further categorized the genes according to their similar biological functions (Table 3.3-3.5). Meanwhile, SHED expressed many genes at P9 that are related to pro-inflammatory and anti-inflammatory elements such as CCL3, CCL5, IL1A, IL4 and IL8.

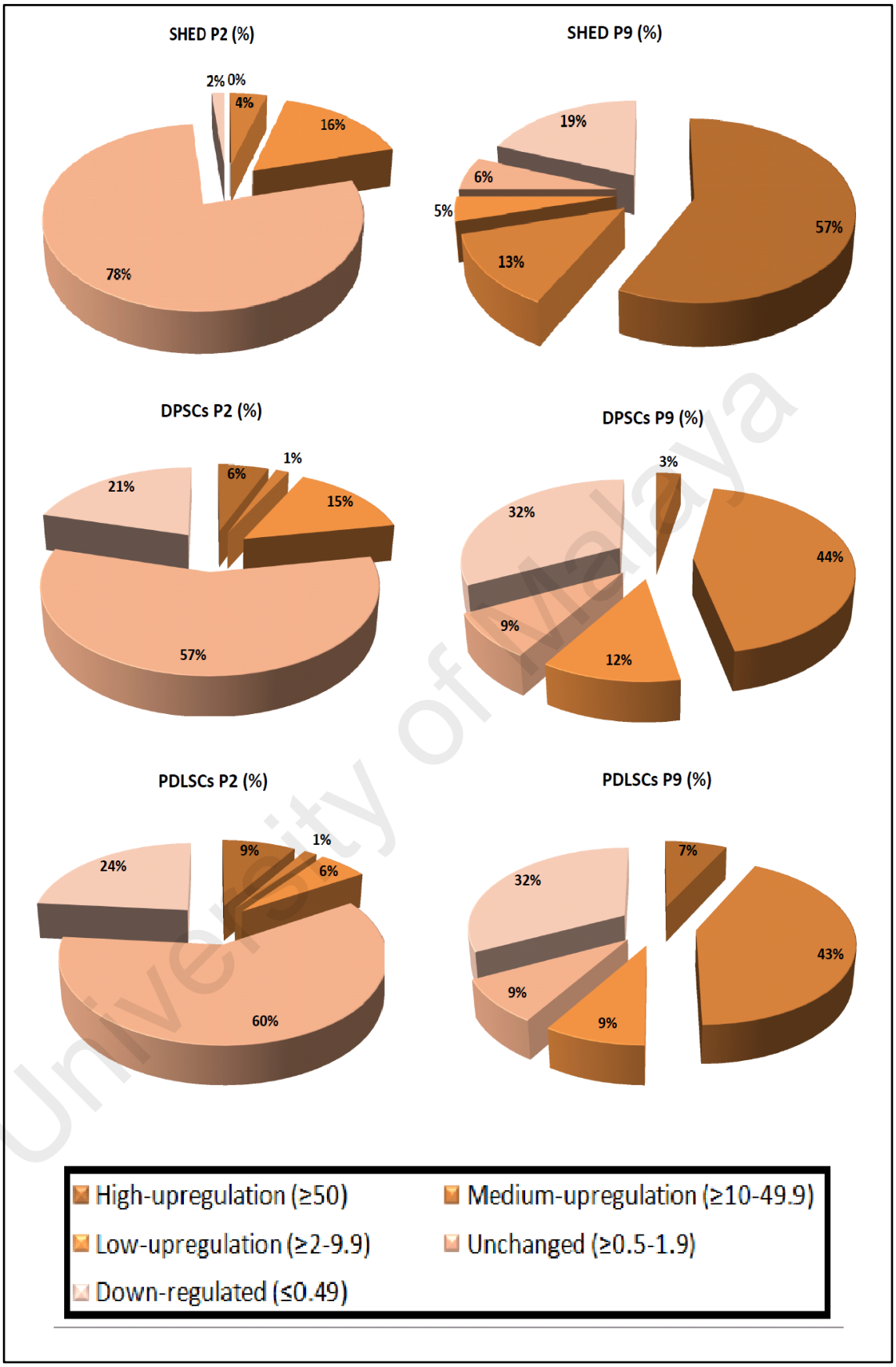


Figure 3.4: Comparison of gene profile between SHED, DPSCs and PDLSCs using a Human Immune gene array with percentages of up-regulated genes in P2 and P9.

3.3.4 SHED cultured at P9 displayed many genes representing pathogen recognition as well as immune signaling and transduction

Among the 15 genes grouped under the category of pathogen recognition as well as signaling and transcription, 7 (HLA-DRB1, CD80, CD34, TNFRSF18, PTPRC, SELE, and SELP; *P*<.001) were highly significant in SHED cultured at P9 compared to those cultured at P2. A similar observation with lesser significant values (*P*<.01; *P*<.05) was observed for DPSCs and PDLSCs cultured in P9 as compared to P2 (Table 3.3).

Table 3.3: Fold changes of genes related to pathogen recognition & signaling and transcription.

PATHOGEN RECOGNITION & SIGNALING AND TRANSCRIPTION						
Gene	SHED		DPSCs		PDLSCs	
	P2	P9	P2	P9	P2	P9
Phagocytes and Antigen Presenting Cells						
HLA-DRA	1.52 ± 0.04	38.77 ± 0.57**	2.50 ± 0.21	2.18 ± 0.12	0.92 ± 0.02	2.40 ± 0.10*
HLA-DRB1	1.48 ± 0.15	171.53 ± 0.50***	0.32 ± 0.03	9.25 ± 0.22**	0.35 ± 0.05	10.53 ± 0.50*
CD40	8.83 ± 0.28	0.78 ± 0.05	2.10 ± 0.10	0.04 ± 0.01	0.69 ± 0.06	0.06 ± 0.01
CD80	1.61 ± 0.04	403.19 ± 0.02***	0.74 ± 0.05	22.31 ± 0.10*	0.71 ± 0.01	25.47 ± 0.50**
CD86	1.68 ± 0.06	0	0	0	0	0
Other						
FAS	1.48 ± 0.05	4.75 ± 0.13*	0.66 ± 0.06	0	0.57 ± 0.06	0
CD34	1.54 ± 0.06	402.73 ± 0.64***	0.73 ± 0.03	22.24 ± 0.21**	0.70 ± 0.01	25.47 ± 1.29***
TNFRSF18	2.90 ± 0.10	42.66 ± 0.57***	0.46 ± 0.01	2.29 ± 0.09*	1.36 ± 0.05	2.70 ± 0.01
Scavenger Receptors						
CD68	16.56 ± 0.48*	0.06 ± 0.01	0.56 ± 0.05	0	0.54 ± 0.01	0
Nuclear transcription factor						
NFKB2	1.45 ± 0.05	0.03 ± 0.01	0.88 ± 0.02	0	0.46 ± 0.05	0
TBX21	2.79 ± 0.09*	0.42 ± 0.02	0	0.02 ± 0.01	0	0
PTPRC	1.45 ± 0.05	1700.49 ±	0.09 ± 0.01	907.17 ± 1.89***	7.49±0.43	561.03 ± 1.05***
Integrin ligands						
ICAM1	11.40 ± 0.36	8.28±0.28	5.57 ± 0.52**	0.46 ± 0.05	1.14 ± 0.15	0.55±0.01
SELE	1.19 ± 0.08	78.23 ± 1.08***	4.35 ± 0.05	42.50 ± 0.50*	69.30 ± 1.13	200.43 ± 0.52
SELP	1.57 ± 0.06	402.06 ± 1.10***	21.90 ± 0.78	238.30 ± 1.54***	25.13 ± 1.03	389.40 ± 0.53***

Highly up-regulated | Medium up-regulated | Low up-regulated | Unchanged | Down-regulated

3.3.5 Pro and anti-inflammatory gene profiling of SHED, DPSCs, and PDLSCs at P2 and P9

The transition of SHED from P2 to P9 resulted in an increment of 7 pro-inflammatory genes namely TNF, ILIB, IFN-ɣ, LTA, IL-12B, IL-1A, and IL-15 (*P*<.001). However, except for IFN-ɣ, not much significance was noticed for the same group of genes in DPSCs and PDLSCs during the transition period. On the other hand, tremendous increments (*P*<.001) of anti-inflammatory genes such as IL-4, IL-5, IL-7, IL-9, IL-10,

and IL-13 were observed in SHED cultured at P9 as compared to P2. Surprisingly, we did not observe a similar expression in DPSCs and PDLSCs, indicating SHED experience immune related genes turbulence when cultured for a prolonged period of time (Table 3.4).

Table 3.4: Fold changes of genes related to cytokines

CYTOKINES						
Gene	SHED		DPSCs		PDLSCs	
	P2	P9	P2	P9	P2	P9
Proinflammatory cytokines						
TNF	1.11±0.10	5850.72±0.87***	0.41±0.03	3.84±0.42*	0.80±0.04	4.37±0.01*
IL6	1.18±0.16	0.94±0.04	7.25±0.25	25.05±0.01	0.44±0.04	0.08±0.02
IL1B	1.95±0.05	191.74±1.56***	0.36±0.05	10.40±0.53**	2.65±0.13	12.43±0.51*
IFNG	1.76±0.05	34.63±0.55***	5799.67±0.58***	25.10±1.01	425.84±0.77	3842.23±0.68***
LTA	2.62±0.13	606.67±1.53***	0.77±0.06	21.59±0.52*	1.87±0.06	24.88±0.82*
IL17	1.18±0.03	0	0	0	0	0
IL12A	22.01±1.00*	1.32±0.09	1.75±0.05	0.08±0.01	0.54±0.04	0.08±0.01
IL12B	1.64±0.06	402.58±0.52***	1.17±0.29	20.92±0.89*	0.74±0.01	27.93±0.90*
IL18	0.85±0.05	14.52±0.50	0.50±0.10	0.80±0.01	0.12±0.01	0.93±0.01
IL1A	1.55±0.05	532.15±1.88***	0.72±0.02	39.37±0.64*	0.74±0.06	27.90±0.85*
IL15	1.63±0.06	429.45±0.51***	0.71±0.02	31.10±0.85*	1.22±0.10	20.80±0.72*
Th-2 type cytokines						
IL10	1.45±0.05	412.78±0.70***	0.75±0.05	27.87±0.61*	0.72±0.03	21.80±1.71*
IL4	1.93±0.06	532.82±0.74***	0.85±0.05	21.27±1.21*	0.76±0.05	25.43±0.51*
IL5	1.41±0.08	402.89±0.84***	1.17±0.15	22.63±0.71*	0.74±0.04	35.53±0.50*
IL13	1.24±0.05	302.74±0.65***	0.84±0.65	21.24±1.16*	5433.67±2.08**	44.80±0.72*
IL7	0.71±0.04	1232.71±1.12***	63.13±1.03*	2.25±0.22	0.28±0.11	588.73±0.64***
IL9	1.63±0.05	450.63±0.55***	1.61±0.08	34.13±0.15**	0.89±0.01	26.60±0.57*
Growth and stimulation factors						
TGFB1	1.42± 0.02	1278.06 ± 0.10***	0.91±0.02	0	1.03±0.03	0
CSF1	1.16 ± 0.14	0.26 ± 0.02	2.69±0.08	0.02±0.01	1.05±0.05	0.02±0.01
CSF2	1.28 ± 0.08	13.70 ± 1.12	1.09±0.10	0.77±0.03	0.23±0.06	0.81±0.02
CSF3	1.65 ± 0.05	411.87 ± 0.81**	0.82±0.02	21.37±0.55*	0.76±0.04	24.48±0.50*
VEGF	4.58 ± 0.08*	0.32 ± 0.02	0.83±0.03	0.03±0.01	1.21±0.36	0.02±0.02
IL2	1.64 ± 0.06	425.07 ± 0.12***	0.73±0.03	22.20±0.18*	0.71±0.03	25.45±0.51*
IL3	1.59 ± 0.09	412.08 ± 1.81***	0.84±0.04	23.42±0.52*	0.88±0.02	27.07±1.68*
IL2RA	2.52± 0.02	452.55 ± 0.51***	0	0	0	0
Chemokines						
IL8	3.24± 0.22*	0.16± 0.01	2.26±0.22	0	0.20±0.10	0
CC chemokines						
CCL2	0.46±0.06	0.36±0.01	10.62±0.54*	0.03±0.01	1357.47±0.50***	0.03±0.01
CCL3	1.36±0.05	23.76±0.67*	1.66±0.66	1.66±0.04	0.05±0.01	1.85±0.05
CCL5	1.65±0.05	402.73±0.64***	4.32±0.07	21.77±0.68*	10.53±0.50	26.13±1.63*
CCL19	1.3±0.10	0	0	0	0	0
CXC chemokines						
CXCL10	1.47±0.21	44.31±0.60*	3.72±0.25	2.44±0.05	1.32±0.07	2.52±0.45
CXCL11	1.78±0.08	423.73±1.10***	2.05±0.05	22.10±1.02*	0.62±0.08	26.13±0.81*
CCR2	1.63±0.03	412.84±0.78***	0.75±0.05	24.90±0.80*	0.81±0.03	25.77±1.08*
CCR4	0.65±0.06	123.46±0.50*	0.06±0.01	125.59±1.51**	1.62±0.07	110.47±1.35**
CCR5	1.55±0.05	440.78±0.70***	0.77±0.03	25.59±0.53*	4.21±0.26	25.13±1.03*
CCR7	1.73±0.10	450.37±0.55***	0.87±0.63	21.00±1.00*	5.84±0.14	26.40±0.53*
CXCR3	0.37±0.03	0	0	0	0	0

Highly up-regulated Medium up-regulated Low up-regulated

Unchanged Down-regulated

3.3.6 SHED cultured at P9 displayed many genes representing several growth and stimulation factors as well as chemokines

It is worth noting that growth and other stimulation factors also play an integral part in immune systems. Accordingly, we noticed that TGF-β1 and other stimulation factors such as CSF3 and IL2RA were highly expressed in SHED cultured at P9 compared to P2 (P<.001) and, again, these genes were stagnant in DPSCs and PDLSCs in similar culture conditions. In terms of chemokine gene expression, only those genes related to the CXC group such as CCL5, CXCL11, CCR2, CCR4, CCR5, and CCR7 were noticeable in all cell lines at P9 (Table 3.5).

Table 3.5: Fold change of genes related to cellular cytotoxicity, surface molecules and apoptosis

CELLULAR CYTOTOXICITY, SURFACE MOLECULES & APOPTOSIS						
Gene	SHED		DPSCs		PDLSCs	
	P2	P9	P2	P9	P2	P9
Cellular cytotoxicity						
GNLY	1.42 ± 0.08	359.40 ± 0.53***	0.68 ± 0.07	22.56 ± 0.51*	1.32 ± 0.16	21.50 ± 0.50***
GZMB	1.9 ± 0.06	50.40 ± 0.69**	1.24 ± 0.06	2.48 ± 0.42	0.14 ± 0.01	3.29 ± 0.26
PRF1	1.89 ± 0.08	305.17 ± 0.29***	1.80 ± 0.10	23.56 ± 1.26*	1.60 ± 0.10	30.63 ± 0.33*
Lymphocytes						
CD3E	1.20 ± 0.10	323.03 ± 0.06***	1.69 ± 0.11	24.00 ± 1.00*	1.62 ± 0.07	24.87 ± 0.81*
CD8A	1.23 ± 0.15	511.73 ± 0.64***	0.24 ± 0.06	21.33 ± 0.31*	0.88 ± 0.16	1993.00 ± 1.73***
CD4	1.72 ± 0.21	11.57 ± 1.37*	0.29 ± 0.09	0.66 ± 0.05	6.43 ± 0.51	0.76 ± 0.05
CD19	1.76 ± 0.18	512.57 ± 0.40***	0.60 ± 0.01	22.44 ± 0.51*	0.85 ± 0.05	31.47± 0.5**
CD28	7.32 ± 0.42	313.00 ± 0.50***	0.63 ± 0.04	16.77 ± 0.69*	0.54 ± 0.03	20.23 ± 0.68**
ICOS	1.75 ± 0.19	0.33 ± 0.11	0	0.04± 0.02	0	0.04± 0.03
CD38	1.49 ± 0.18	239.30 ± 0.21***	0.60 ± 0.05	13.42 ± 0.52*	0.85 ± 0.05	15.36 ± 0.56*
CD40LG	1.77 ± 0.17	256.27 ± 0.25***	0.77 ± 0.03	19.42 ± 0.52*	0.82 ± 0.08	29.17 ± 1.04*
CTLA4	8.30 ± 1.13	844.17 ± 0.29***	0.04 ± 0.01	0.09 ± 0.01	0.05 ± 0.02	0.27 ± 0.15
FASLG	1.62 ± 0.08	0	0	0	0	0
Apoptosis						
BAX	2.59 ± 0.52	0.64 ± 0.04	1.81 ± 0.08	0.10 ± 0.01	1.08 ± 0.07	0.32 ± 0.08
BCL2	8.63 ± 0.55	24.55 ± 0.51*	2.25 ± 0.25	1.42 ± 0.07	1.13 ± 0.03	1.68 ± 0.13
BCL2L1	1.82 ± 0.13	4.52 ± 0.50	0.61 ± 0.08	0.32 ± 0.16	1.07 ± 0.11	0.30 ± 0.20

Highly up-regulated | Medium up-regulated | Low up-regulated | Unchanged | Down-regulated

3.3.7 DPSCs and PDLSCs cultured at P9 expressed less cellular toxicity and apoptosis-related genes compared to SHED cultured at P9

We postulated that the expressions of most of the immune genes in SHED cultured at P9 were most likely contributed by the elevated expression (P<.001) of cellular toxicity

and apoptosis such as GNLY, GZMB, PRF1, CD3E, CD8A, CD19, CD28, CD38 and CD40LG.

3.3.8 Validation of immune genes using cytokine protein array.

We randomly selected a few genes namely IL2, IL4, IL5, IL6, IL7, IL8, IL10, VEGF, and TNF that showed expression patterns in good agreement with the PCR array data (Figure 3.5).

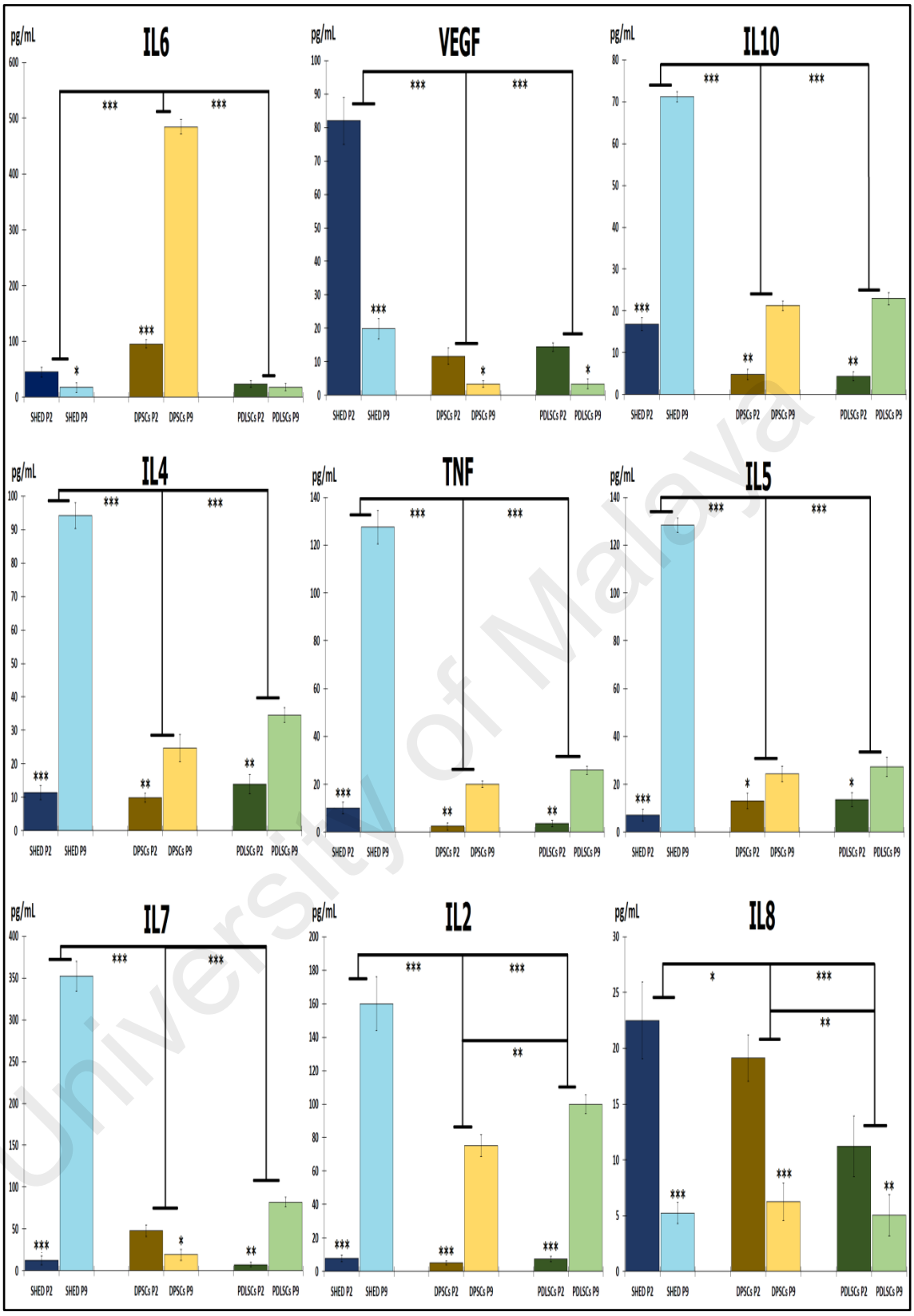


Figure 3.5: Detection of cytokines released by SHED, DPSCs, and PDLSCs at passage 2 (P2) and passage 9 (P9) via Luminex platform. The cytokines selected include interleukin 6 (IL6), vascular endothelial growth factor (VEGF), interleukin 10 (IL10), interleukin 4 (IL4), tumour necrosis factor (TNF), interleukin 5 (IL5), interleukin 7 (IL7), interleukin 2 (IL2) and interleukin 8 (IL8). (*P value <0.05, **P value <0.005 and ***P value<0.001).

3.4 Discussion

The findings of this study prompted a revisit of some of the key aspects of stem cells for clinical usage one being the suitability of using pro-long cultured cells. Generally, cells cultured for long-term are considered to be safe (Bogdanova et al., 2014) although a few studies report that they undergo genomic instabilities (Wang et al., 2013; Roselli et al., 2013). More recent studies have shown that DPSCs cultured up to P14 too have maintained high mitogenic and functionality thus making them a safe source for therapeutic usage (Martin-Piedra et al., 2014). Nevertheless, later studies as well as others in the past (Scheers et al., 2013; Frazier et al., 2013) mainly focused on proliferation and functionability, and may not truly represent the state of the cells. This is true in our situation that despite maintaining proliferation and functionality, a significant shift was observed in the immunomodulatory aspect especially for long-term cultured cells. Interestingly, this shift occurred across all tested cell lines with SHED being the highest.

There was a sharp increase of immune gene expression related to T-lymphocyte activation such as HLA-DR and CD80 across all cell lines at P9. We postulate that the presence of these molecules indicates that the cells are losing their stemness and transforming into terminally differentiated cells (Laurin et al., 2004) and have the potential for creating a graft-versus-host disease scenario if transplanted in allogeneic settings. Further, we noticed a significant increase in the number of pro-inflammatory genes during the transition of the cell lines from P2 to P9. In vivo condition, these genes are expressed at large during the early inflammation phase to eradicate infection, and the over-expression of these genes especially TNF, IL1B, IFN-ɣ, LTA, IL12B, IL1A, and IL2 could potentially harm host tissues (Morales-Garcia et al., 2012; Katz et al., 2014; Xu et al., 2015). Likewise, we also noticed that an increment of several anti-inflammatory genes at P9. In an ideal situation, the expressions of pro-inflammatory

and anti-inflammatory genes complement each other; for example, the pattern of TNF is inversely proportional to IL-10 (Strassmann et al., 1994). In contrast, we found both groups of genes were highly expressed in P9 indicating a disturbance in the genetic makeup though this needs further investigation. It was not surprising to see the elevation of TGF-β expression at P9. The pivotal function of TGF-β in the immune system is to maintain tolerance via the regulation of lymphocyte proliferation, differentiation, and survival (Yoshimura & Muto, 2011) by suppressing pro-inflammatory cytokines. CCL5, CXCL11, CCR2, CCR4, CCR5, and CCR7 were also up-regulated in all cell lines at P9. All these molecules are members of the CXC chemokine family and, apart from playing a role in immunogenicity, they are potent promoters of angiogenesis and mediate their angiogenic properties (Balasubramanian et al., 2012). Perhaps this might contribute to the continuous growth of the cells. It was surprising to notice that despite the cells being cultured under similar conditions, SHED surpassed other cell lines in expressing unwanted immune genes at late passage. This perhaps is attributed to the high expression of cellular toxicity markers such as GNLY and GZMB in SHED as compared to DPSCs and PDLSCs. These genes are known to be involved as intracellular effectors of target cell death, with recent data suggesting that GNLY may have a role in the propagation of immune signals (Cullen et al., 2010).

Collectively, the occurrence of immune genes turbulence from our findings suggest that long-term culture conditions of stem cells may not be feasible for use in transplantation. Having said that, what is the remedy for using cells having a low starting material such as dental pulp or periodontal ligament tissue? To overcome this issue, a large expansion of cells using 3D-bioreactor (Vecchiatini et al., 2015) can be possibly made to dental stem cells at the early passage without triggering any side effects (Elseberg et al., 2015). Apart from that, pooled allogeneic samples are another choice, but further characterization is needed to maintain their functionality. In conclusion, we propose a

comprehensive biological characterization which includes the immune properties before the stem cells cultured at later passage are brought into clinical application.

3.5 Conclusion

1. The growth curve indicates that PDLSCs have the highest accumulated cells at P9, followed by SHED and DPSCs. When observed under the microscope the morphology had no difference either in P2 or P9.

2. The fundamental characteristics of mesenchymal stem cells were observed in all three dental sources.

3. The human immune gene expression in SHED, DPSCs, and PDLSCs in long term cultured cells in P9 has highly expressed genes compared to P2. However, there was increased gene expression of cellular toxicity markers in SHED when compared to DPSCs and PDLSCs. The viability and cell proliferation was also decreased therefore P9 sources are not feasible for clinical usage.

From this study, we can conclude that SHED cultured at P2 has expressed genes namely IL12A and VEGF which plays an important role in angiogenesis process in wound healing which are comparable to other dental stem cells. Therefore SHED at P2 was chosen to further study on its functionality in different culture conditions in our next chapter.

CHAPTER 4: WOUND HEALING POTENTIAL OF SHED WHEN CULTURED IN LOW SERUM CULTURE CONDITIONS

4.1 Introduction

Wound healing is a complex process that undergoes the three major phases of pro-inflammation, proliferation, and maturation. As the wound closes, paracrine signaling occurs within the wound area which activates genes involved in the healing. In addition, upregulation of signaling pathways such as TGFβ1, phosph-ERK, and NF-κBA have shown to enhance the wound healing process by increasing α-smooth muscle actin expression (Wang et al., 2016).

Wound debridement starts with a coagulation cascade that stops the bleeding and an infusion of neutrophils if infection is present. During the late stages of the inflammatory process, macrophages initiate angiogenesis and re-epithelialisation. The proliferation stage follows with the formation of granulation tissues which comprise a collagen and extracellular matrix. Angiogenesis is then initiated leading to the formation of a network of new blood capillaries. The maturation phase is the final phase where wound closure is completed. Here, further collagen deposition and cross linking of extracellular matrix takes place, which allows scar tissue formation (Hutchinson, 1992).

There are a number of genes which contribute to the epithelialisation rate, namely CCL2, IL10, and IL6 at the pro-inflammatory phase; CSF, EGF, and TGF at the proliferation phase; and MMP, PLAU, and COL1A1 which are involved in the maturation phase. The cross-talk between these genes increases the rate of wound healing and reduces fibrosis, which is a type of scar that occurs in delayed wound healing although this does not occur during normal wound healing (Yang et al., 2014).

MSCs in the wound healing process generate good results through the secretion of growth factors, cytokines, and chemokines which lead to angiogenesis (Chen et al., 2015). Stem cells from human extracted deciduous teeth (SHED) can be a unique resource for wound healing treatment. Nishino et al. (2011) have reported that SHED works better together with other compounds or different therapeutic strategies. Their study shows that SHED co-cultured with fibroblast enhances skin wound healing in mice. Hence, more studies need to be conducted on SHED to optimize its function on wound treatment.

Previously, Kanafi et al. (2013) reported SHED's cell migration in 5% of FBS is faster compared to DPSCs. Therefore we have decided to study the efficacy of SHED in treating wound healing in low serum culture conditions (2% of FBS) which reduces animal derived substances in our study and also looking at the expression of wound repair genes.

Experimental works were carried out to meet the following objectives:

1. Assessing SHED isolation and compare the morphology, growth kinetics, and senescence level of SHED cultured in early passages: passage 2 (P2) and passage 4 (P4) in low serum culture conditions;
2. Evaluating human wound healing genes in SHED in P2 and P4 when cultured in low serum culture conditions using qPCR;
3. Assessing malignant transformation of SHED in P2 and P4 when cultured in low serum culture conditions using transformation assay; and
4. Assessing the validation of gene expression by reverse transcriptase and real time PCR

4.2 Methodology

4.2.1 Low serum culture conditions sample isolation and expansion

Dental pulp stem cells from extracted deciduous teeth (SHED) [n=3; ages 7-11 years] were isolated and cultured up to passage 4 (P4) according to reported protocols with slight modification (Jung et al., 2013). In this study, cells at P2 and P4 were both classified as early passages. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS) and glutaMAX 1% (Life Technologies, CA, USA). For 2% FBS, SHED were isolated and seeded into T75 flasks. Once attached, they were expanded into DMEM-KO without FBS and later cultured into 2% FBS, which is a low serum culture media.

4.2.2 Morphology, growth kinetics, and senescence of β-galactosidase cell staining assay

Cells cultured in different culture medium conditions were compared in terms of morphology, growth kinetics, and senescence using β-galactosidase cell staining assay data obtained at P2 and P4. The morphology of cell growth and its population-doubling time were analyzed (Govindasamy et al., 2011). When SHED had reached 60% confluence, growth media was removed from the cells and rinsed with DPBS (Invitrogen) and stained according to the manufacturer's protocol (Cell Signaling Technology, Danvers, Mass., USA). Briefly, cells were maintained in fixative solution for 10–15 min. Then the plates were rinsed 2 times with DPBS (Invitrogen) and subsequently they were stained with β-galactosidase staining solution overnight and visualized under a microscope. The presence of blue-stained cells indicates senescence and the number of senescent cells in 35mm tissue culture dish were counted under a light microscope.

4.2.3 qPCR human wound healing array gene expression analysis

Total RNA was extracted from tissue samples using TRIZOL reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The resulting RNA pellet was then resuspended in 30µl of diethyl pyrocarbonate (DEPC)–treated water. The extracted RNA was quantified by analyzing 1µl of total RNA using the Nanodrop ND1000 Spectrophotometer (Nanodrop Technologies Inc.). Following RNA extraction, complementary DNA (cDNA) was combined with an appropriate amount of RNA through a reverse transcription reaction. Subsequently, messenger RNA (mRNA) expression of 84 wound healing genes found in the human wound-healing PCR array (PAHS-121Z; SABiosciences, Frederick, MD) was investigated for pathway profiling by real-time reverse transcription polymerase chain reaction (RT-PCR). Healthy SHED cultured under 10% FBS were used as controls. Finally, the real-time PCR array data were analyzed using the RT2 profiler PCR Array Data Analysis online software (SABiosciences).

4.2.4 Transformation assay

The quantitation of anchorage-independent growth was performed using Cyto Select 96-well Cell Transformation Assay. Anchorage–independent growth is one of the hallmarks of transformation and which is an indication for *in vitro* detection of malignant transformation of cells. A culture medium of 100 µL containing cell growth activator was added into each well. The cells were incubated for 6-8 days at 37ºC and 5% CO2. Then the cell colony formation was observed under a light microscope and colorimetric detection was performed by measuring the absorbance at 570nm for both the 10% and 2% FBS culture mediums, while immortalized HepG2 cells were used as positive control.

4.2.5 Validation of gene expression by Reverse Transcriptase and Real Time PCR

cDNA amplification was performed in a thermocycler using Taq polymerase supplied with KCl buffer and 1.5 mM/L $MgCl_2$ (Invitrogen) at 94°C/1 min, 58°C/30 sec, 72°C/1 min. Polymerase chain reaction (PCR) products were resolved in 1.5% agarose (Invitrogen) gel in 1 X Tris borate-ethylene-diaminetetraacetic acid buffer. The expression of some primers in the reverse transcriptase-PCR analysis was quantified in duplicate with SYBR green master mix (Applied Biosystems, Foster City, CA, USA). PCR reactions were run on an ABI 7500 Fast Sequence Detection System (Applied Biosystems) and all measurements were normalized by 18s rRNA. For data analysis, the comparative CT method (ΔΔCT) was used. Randomly selected genes were then validated. The validated genes are CXCL2, IL6, TNF, TGFB1, and COL4A1.

4.2.6 Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). Statistical comparisons were made and the data analyzed using analysis of variance (ANOVA) with the significance level set at $P = 0.05$. Tukey post hoc multiple comparison tests were carried out to determine the differences between the groups (Nishino et al., 2011; Nakamura et al., 2013).

4.3 Results

4.3.1 SHED cultured in low serum cultured conditions

In order to understand further on wound repair gene expression by SHED, the areas that we have looked into included its characteristics of morphology, accumulated cells, population doubling time (PDT), cumulative population doubling (PD) and percentage

of senescent cell. The understanding of these areas has indicated that it does not contradict with our findings (Figure 4.1).

As described in the literature, the characteristics of multipotent stem cells (MSCs) were determined by trilineage study on dental pulp stem cells. The results showed adipocyte, chondrocyte, and osteocyte differentiations (Figure 4.2). The safety profile of the SHED cultured in P2 and P4 showed no growth anchorage in different culture conditions against positive control HepG2 (Figure 4.3).

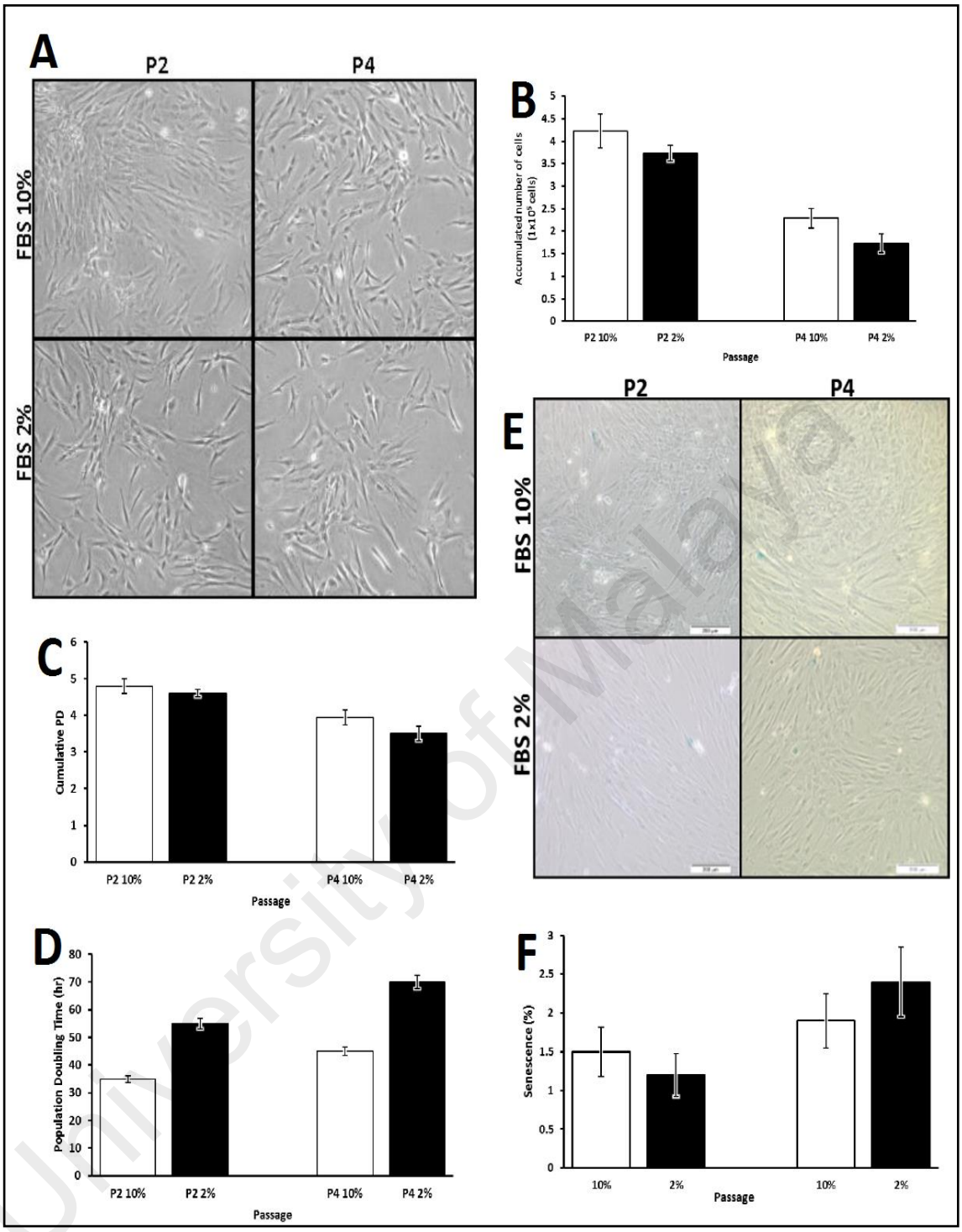


Figure 4.1: A: Morphology of SHED when cultured in different culture conditions and long term cell expansion at 4X magnification. B. The accumulated number of cells (1 X 10^6 cells) has no significance difference in both P2 and P4 SHED culture conditions. C. Cumulative population doubling indicates the number of times cell doubled between passages in different culture conditions. D. Population doubling time (PDT) is time taken in hours by the cells to increase in between passage and different culture conditions. E. The senescence β-galactosidase Cell Staining assay was performed and the presence of blue colour under microscope at 10 x magnification is considered as senescence cell. F. The percentage of senescence cells has no significance difference in between passage and different culture conditions. All experiments were conducted at respective passages with 3 biological replicates for each established cell line.

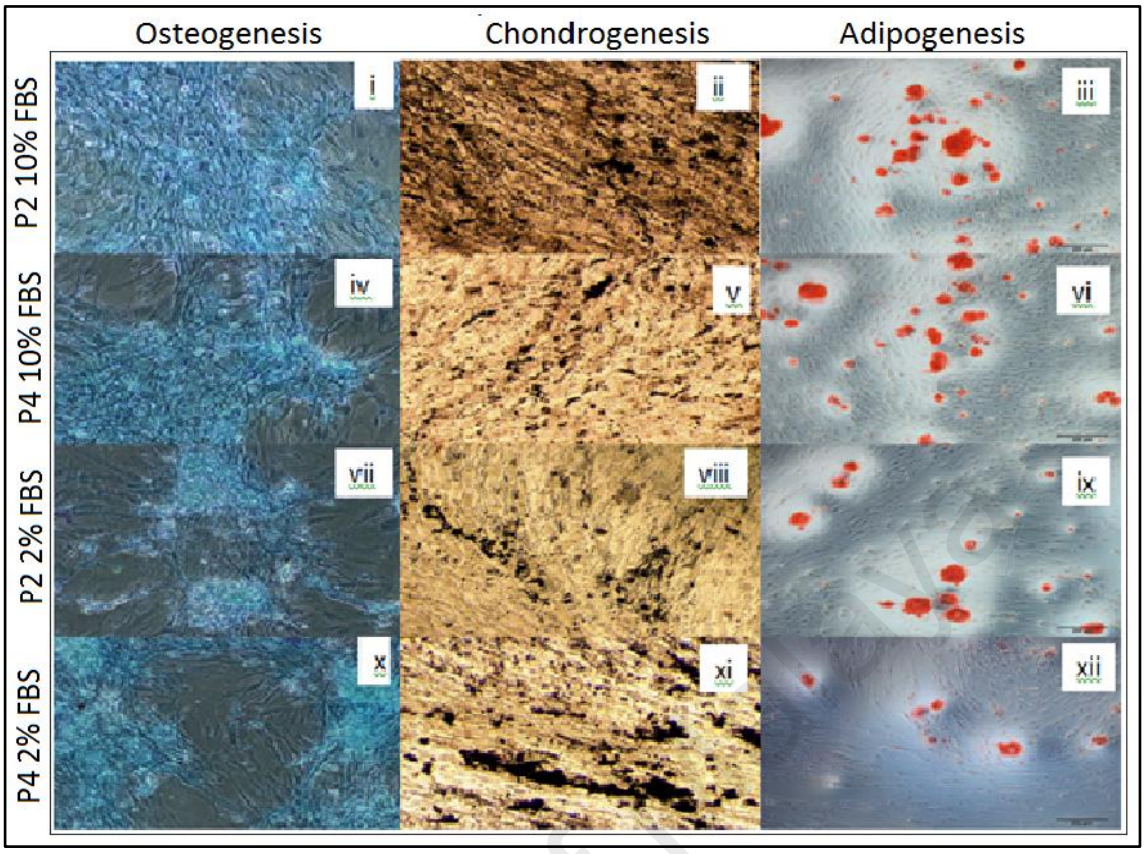


Figure 4.2: Trilineage differentiation of adult multipotent stem cell. Adipogenesis was detected by neutral oil droplet formation stained with oil red O. Chondrogenesis was detected by presence of proteoglycans stained by Alcian Blue. Osteogenesis was confirmed by mineralized matrix deposition stained with von Kossa staining. All the staining were done 21 days after induction for P2 10% FBS (i-iii), P4 10% FBS (iv-vi), P2 2% FBS (vii-ix) and P4 2% FBS (x-xii).

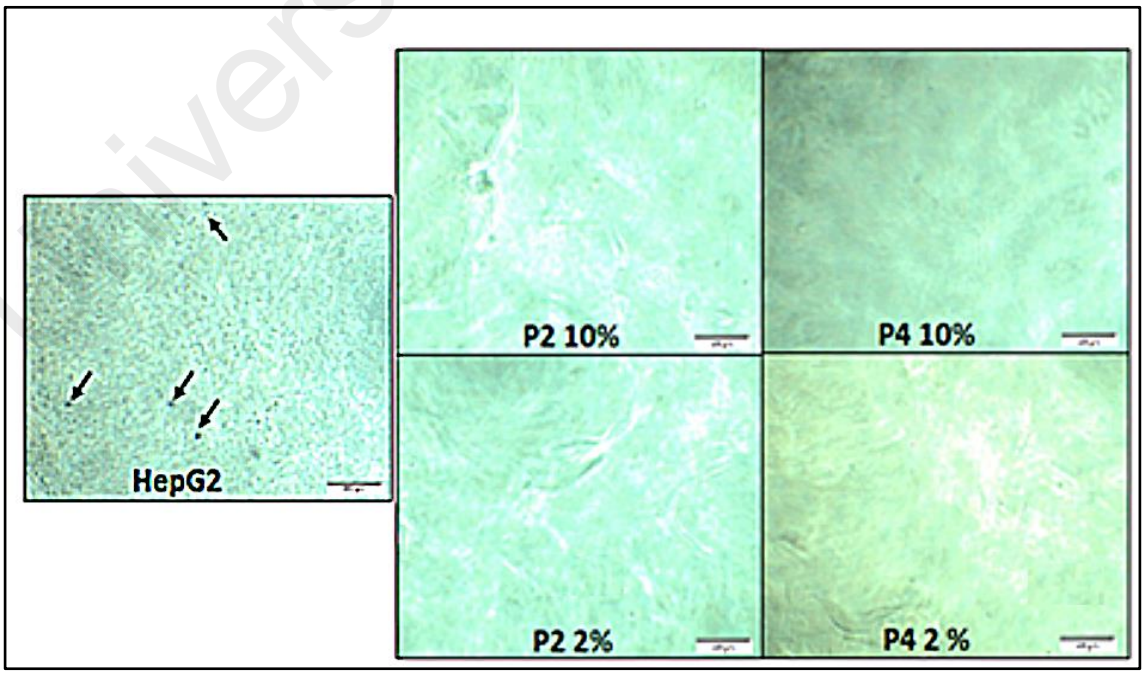


Figure 4.3: The observation of anchorage dependent growth on transformation assay agar matrix layer. HepG2 acts as positive control shown anchorage growth on the layer and none for all other culture conditions. The arrows indicate the anchorage growth.

4.3.2 Wound repair gene expressions pattern in altered cultured conditions of P2 and P4

To better understand gene expression between cell lines, the expression patterns of the functional categories in wound healing genes were observed. The fold changes of expressed markers were determined with ct values. The four categories of ct values were: ct<29 (high expression), ct=30-34 (medium expression), ct=35-39 (low expression) and ct>40 (non-expressed markers). In this regard, wound healing genes were highly expressed in P2 cells cultured in 2% FBS. To better understand the role played by cytokines and growth factors, genes were categorised according to three major phases in wound healing: pro-inflammatory phase (13 genes), proliferation phase (28 genes), and maturation phase (43 genes). The genes were defined as up-regulated or down-regulated based on fold changes compared to SHED cultured in 10% FBS which acted as a control with an accompanying *P* value of 0.05 (Appendix 1.2). Since huge fold variations were noticed among all the samples, only a number of genes (12 genes) were randomly chosen to represent each phase of wound healing.

4.3.3 Pro- and anti-inflammatory gene profiling of SHED at P2 2%and P4 2% FBS shown up regulation

All pro-inflammatory cytokines and chemokines were up-regulated, namely CCL2, CXCL1, CXCL2, IL10, and 1L6. Pro-inflammatory markers such as CCL2 and IL6 and anti-inflammatory cytokines, namely 1L10 were highly expressed with significant difference ($P<0.05$) in comparison to the control group (10% FBS). SHED cultured in P2 shows higher gene expression compared to P4 (Figure 4.5).

4.3.4 Proliferation genes which improves the impaired wound has seen up regulated in SHED P2 2% FBS

Proliferation is a crucial phase that leads to the healing of a wound. Most of the markers observed indicated significant difference ($P< 0.05$). Some highly expressed markers include EGF, TGFA, PTGS2, STAT3, and TNF (Figure 4.6). There were no up regulated genes in P4 except for PTEN and WISP 1 gene. Conversely, there were more down regulated genes in P4 2% FBS compared to P2 2% FBS (Appendix 1.3). Most markers in SHED P2 2% FBS were up-regulated, hence increasing the healing rate.

4.3.5 SHED P2 2% FBS has possibilities to improve the healing condition by increasing the number maturation genes

We postulated that the increase in proliferation markers by SHED P2 2% has contributed to the rise of maturation genes, which are involved in the final phase of wound healing. Most of the maturation genes, noticeably COL4A1, COL4A3, MMP2, MMP7, MMP9, PLAU, and PLG were more highly expressed in SHED P2 2% with significance difference ($P < 0.05$). However, RAC1 and F3 genes were highly expressed in SHED P4 2% (Appendix 1.4). Highly expressed markers in SHED P2 2% such as COL543 was up-regulated more than 10-fold (Figure 4.7)

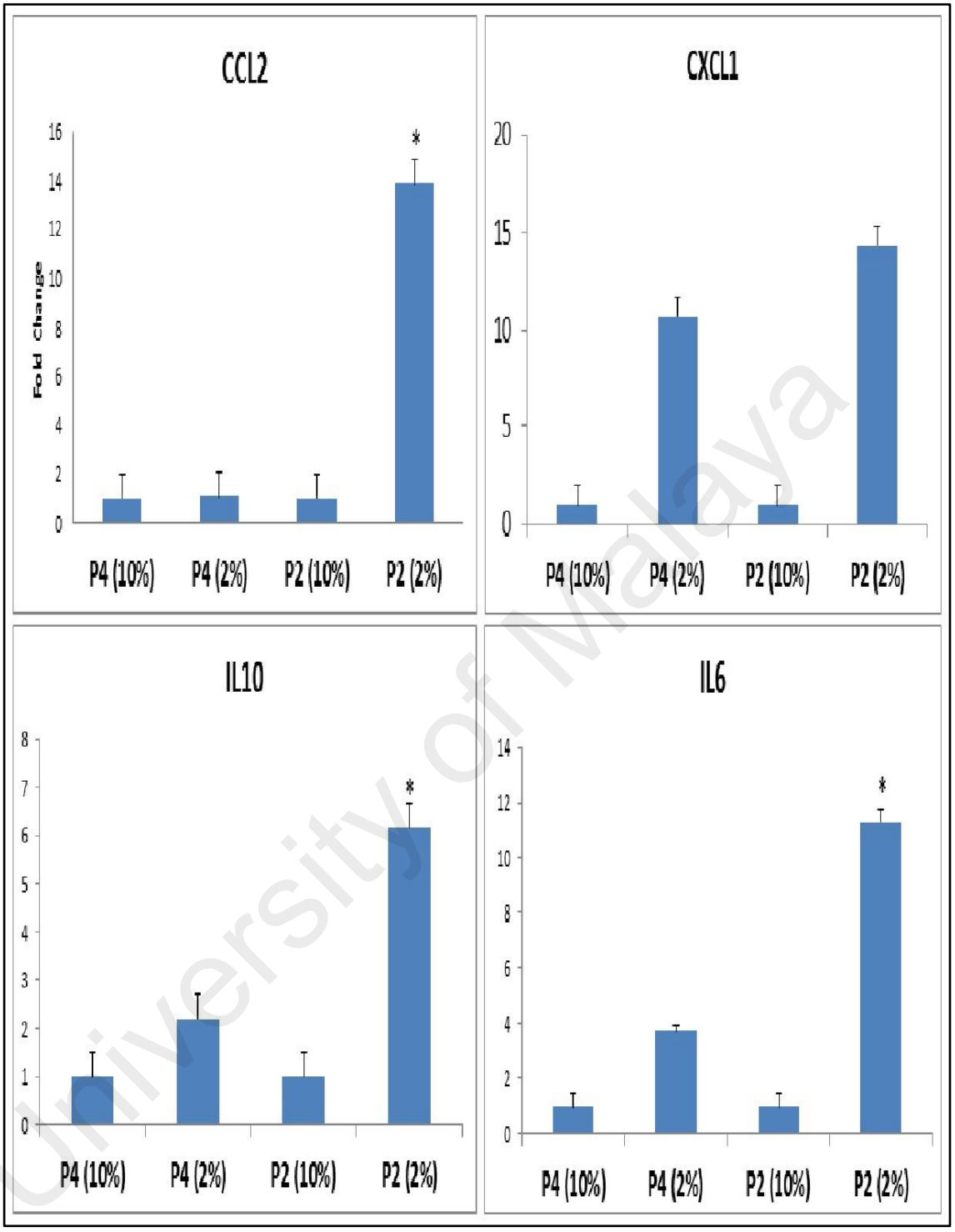


Figure 4.5: Wound healing genes categorized under pro-inflammatory phase. All the experiments were conducted at respective passage with 3 biological replicates for each established cell line. * $P < 0.05$ indicates significance difference.

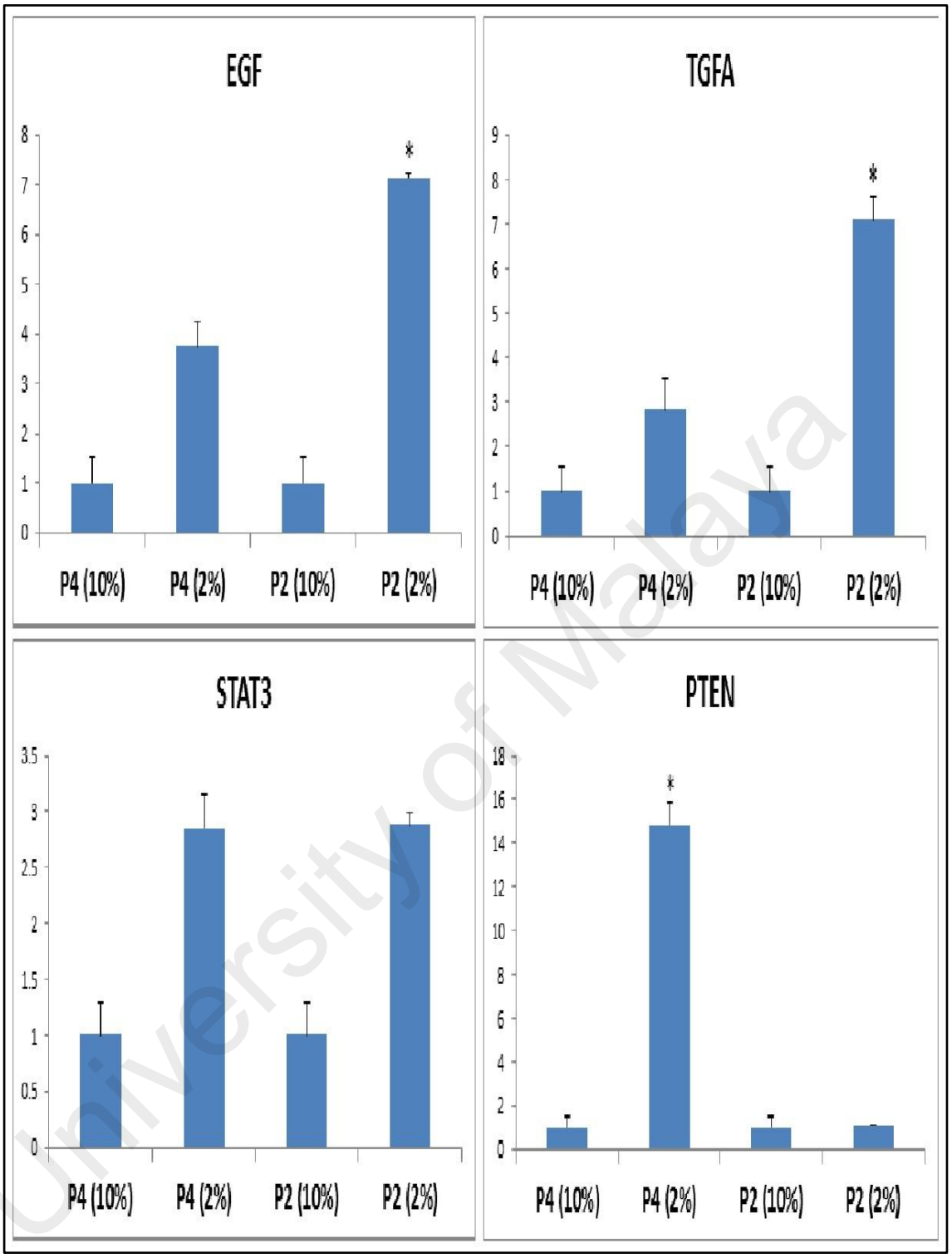


Figure 4.6: Wound healing genes categorized under proliferation phase. All the experiments were conducted at respective passage with 3 biological replicates for each established cell line. * $P < 0.05$ indicates significance difference.

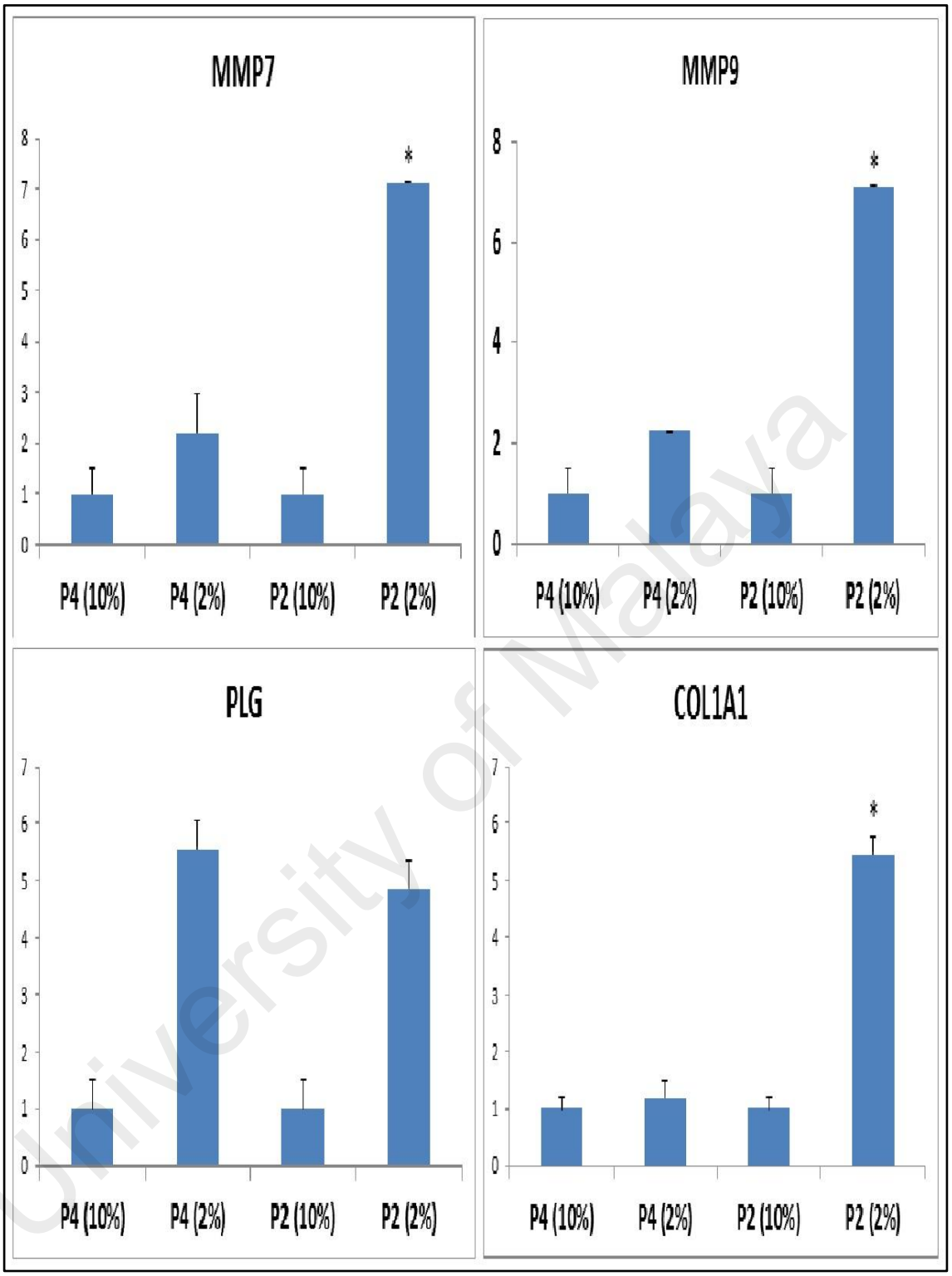


Figure 4.7: Wound healing genes categorized under maturation phase. All the experiments were conducted at respective passage with 3 biological replicates for each established cell line. * $P < 0.05$ indicates significance difference.

4.3.6 Validation of gene expression by Reverse Transcriptase and Real Time PCR

Randomly selected genes showed expression patterns in good agreement with the PCR array data (Figure 4.8).

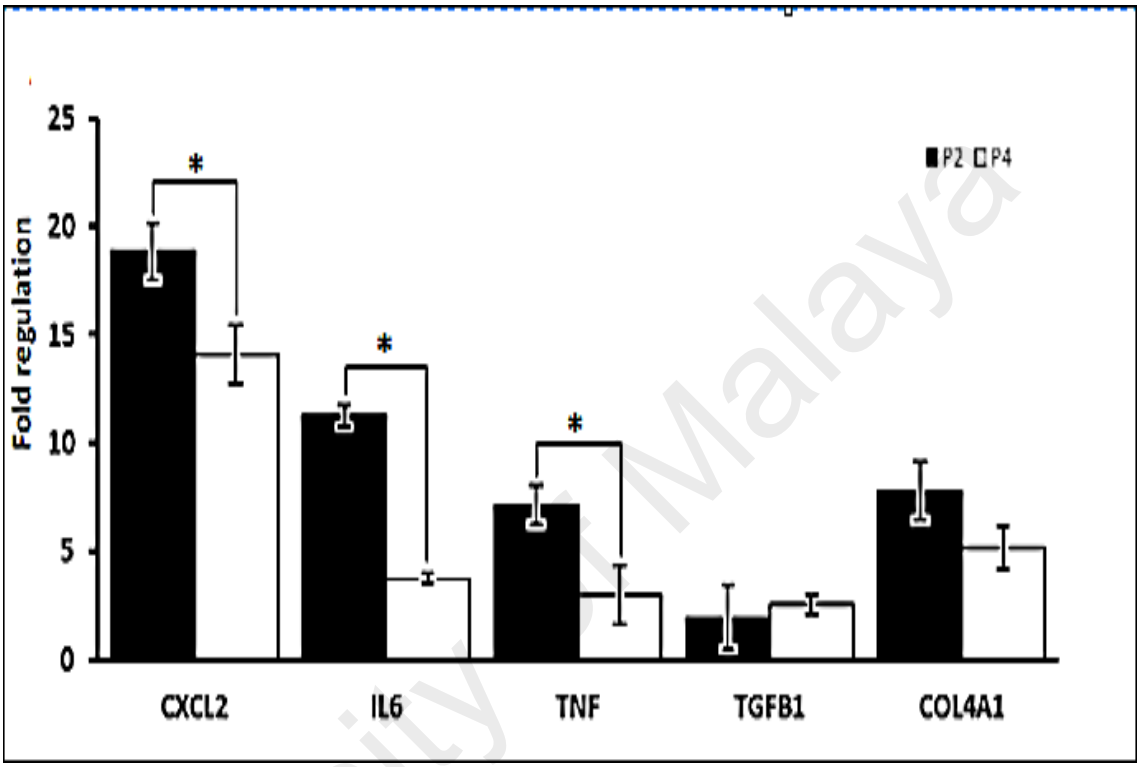


Figure 4.8: Quantitative of gene expression by Reverse Transcriptase and Real Time PCR significance difference for CXCL2, IL6 and TNF with $P<0.05$. All the experiments were conducted at respective passage with 3 biological replicates for each established cell line.

4.4 Discussion

The findings of this study have encouraged further analysis of reduced serum in cultivating dental pulp stem cells for clinical usage. The reason for using FBS in culturing stem cells is to increase the cell growth and supplement for cells. However, there were drawbacks such as safety issues where animal derived proteins can seep into the media. On the other hand, our research also prompted us to know more about wound healing genes expression when cultured in different passages and culture conditions. Dental pulp tissue is mesenchymal cells that need to be cultivated in a large scale

expansion to harvest large numbers of cells for the needs in clinical usage. It was observed that despite maintaining its proliferation and safety profile, a significant difference was seen in expression of wound healing genes in FBS 2% in P2. There was a high increment of pro-inflammatory genes seen in FBS 2% in P2 for all the genes in this category. A sharp increase in these genes can cause a delay in wound healing. However, the presence of anti-inflammatory genes, namely IL10 and IL4 actually complement the process of wound healing. Generally, pro-inflammatory genes such as CCL2, CXCL2, and CXCL5 initiate the healing process by reducing infection by initiating macrophage response. A research conducted by Wood et al. (2014) reported that CCL2 promotes healing by increasing the macrophages response.

For the proliferation category, the highest number of genes were expressed in FBS 2% in P2, namely CSF, EGF, and VEGF. These genes are also crucial in the wound healing process. A full skin thickness wound in mouse model has shown better healing with the expression of EGF and VEGF markers (Kim et al. 2015). The genes found in this category help the vascularization process in wound healing. Ram et al. (2015) has reported that VEGF is one of the key factors in enhancing wound healing in diabetic rats. HGF and VEGF play an important role as angiogenic and pro-survival factors in treating critical limb ischemia (Prochazka et al., 2015).

The maturation phase is the final stage of the complex wound healing process. The noticeable markers at this stage are MMP1, MMP2, MMP7, COL141, COL14A1 and COL14A2. A recent study on wound healing has discovered that COL13A1 and PLAU promote the extracellular matrix formation (ECM) in maturation stage (van den Broek et al., 2015). CSF2 and MMP9 markers were seen expressed in the wound three days after the skin incision was made in mouse (Kameyama et al., 2015).

Most therapeutic research on mesenchymal stem cells (MSC) studied cytokines, growth factors, and paracrine signaling. Nonetheless, these studies were done based in standard *in vitro* culture conditions. This study has established the potential for a large number of wound repair genes to be up-regulated in FBS 2% P2. These results strongly support the notion of SHED cultured in low serum conditions has enhanced the angiogenesis and wound healing process with the expression of namely CCL2, IL10, EGF and MMP7. In low serum conditions, the cells were in a circumstance to produce and release paracrine signals with chemotactic and pro-angiogenic activity (Fierro et al., 2015).

4.5 Conclusions

1. The morphology, growth kinetics, and senescence level showed no significant difference in the lower serum culture conditions in P2 and P4 SHED.
2. There is a marked increase of wound healing genes expressed in SHED P2 2% FBS culture conditions compared to SHED P4 2% FBS.
3. There is no growth anchorage for malignant transformation of cells in P2 and P4 SHED.
4. The wound repair genes upregulated in pro-inflammatory, proliferation and maturation phases were from SHED P2 2% FBS.

From this study, we can draw a conclusion that SHED cultured at P2 low serum condition which is 2% of FBS had shown an increase in wound healing genes namely IL10, IL6, MMP7 and COL1A1 when compared with standard condition of 10% FBS. Hence we proceed with SHED P2 2% FBS in our *in vitro* diabetic wound model in our next chapter.

CHAPTER 5: SHED IN LOW SERUM CULTURE CONDITIONS REDUCES OXIDATIVE STRESS IN AN *IN VITRO* WOUND MODEL

5.1 Introduction

Oxidative stress occurs due to the overproduction of reactive oxygen species (ROS) that are generated in aerobic organisms by the electron transport chain (ETC) of mitochondria. The ROS readily attacks the major organelles within the cells including nucleic acids. Under physiological conditions, the complex I of the ETC is the major location of ROS production (Leverve & Fontaine, 2001; Magder, 2006). Hruda et al. (2010) reported that high glucose increases susceptibility to oxidative-stress-induced oxidative damage in K-562 commercial cell lines. The metabolic state of the cells was affected by the toxic effect of the oxidizing agent. In clinical practice, similar conditions occur in intensive care when a patient with varying blood glucose control and energy substrate availability is exposed to oxidative agents.

The adverse effect of hyperglycemic condition in wound healing is that it alters the angiogenesis process (Lerman et al., 2003). The migrations of cells in diabetic mice are lower at 75% compared to the non-diabetic group; however the underlying mechanism needs to be identified (Wagner & Wehrmann, 2007). Wound healing is a complex process which involves cells namely keratinocytes, fibroblasts, endothelial cells, macrophages, and platelet. Fibroblasts are the most important cells involved in producing and remodeling the extracellular matrix, and fibroblast cell proliferation and migration play key roles in the formation of granulation tissues and further wound repair (Wagner & Wehrmann, 2007; Kanazawa et al., 2010).

Wound debridement is a phase regulated by number of growth factors. For example, basic fibroblast growth factor (bFGF) modulates the growth, differentiation, migration,

and survival of different type of cells (Bikfalvi et al., 1997). The bFGF also regulates the P13K-Rac1-JNK pathway to promote fibroblast cell migration (Kanazawa et al., 2010). In diabetic conditions this process is halted due to oxidative stress. In our previous chapter, Chapter 4, we found that SHED P2 in low serum condition (FBS 2%) showed increased human wound healing genes. Therefore, it will be chosen to be further studied in the *in vitro* wound healing scratch assay.

Experimental works were carried out to meet the objectives of

1. Assessing digital images captured in *in vitro* diabetic wound healing through an *in vitro* glucose-challenged scratch assay with SHED treated in low serum conditions; and
2. Evaluating the oxidative stress enzyme activity level in *in vitro* diabetic wounds treated with SHED in low serum culture conditions using transwell assay.

5.2 Methodology

5.2.1 Preparation for the *in vitro* scratch assay

A SHED sample was cultured in a 6-well plate with seeding density of 1500cell/cm^2. The concentration of glucose in the culture medium was 5mM for normal glucose and 45mM for high glucose concentrations (Foresti et al., 2015). There were three 6-well plates (CORNING) representing each group.

Group A: co-culture of human foreskin fibroblast from normal glucose together with fibroblast from high glucose.

Group B: only SHED P2 2% FBS cultured in high glucose were seeded.

Group C: co-culture of SHED P2 2% FBS together with fibroblast from high glucose.

Upon reaching 90% of confluency, each of the wells was scratched using 10µl pipette tips. Following the wound (scratch assay) the migration of the cells was observed through a phase contrast microscope at 0, 10, 23, 47, and 90 hours (Walter et al., 2010).

5.2.2 Transwell assay

The analysis of oxidative stress level was analyzed by using a transwell assay. The fibroblast with high glucose condition was seeded into a transwell plate (CORNING) at 1500cell/cm^2. Upon confluency, the fibroblast at the bottom layer was scratched with 10 µl pipette tips. The 24 mm polycarbonate membrane transwell insert with a pore size of 0.4 um was placed on top. Then the top layer was seeded with different cell conditions and categorized into 3 groups, namely SHED P2 2% FBS treated in normal glucose, fibroblast in high glucose, and fibroblast in normal glucose. The permeable insert is very useful in studying tissue remodeling because it permits small molecules like growth factors to be transported to the lower compartment. After the wound closure was complete for the entire group, the fibroblast cells at the bottom layer of the transwell were harvested for SOD, GSH, MDA, and AOPP assays. These tests would determine if the SHED treated in the *in vitro* wound model experienced reduced oxidative damage (Rodriguez-Menocal et al., 2012).

5.2.3 Superoxide Dismutase (SOD) activity assay

The adherent cells, upon the *in vitro* closure, were harvested with a cell scraper in 1 ml of cold 1X Lysis Buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA). The cells were then lysed with homogenation by centrifugation at 12000 x g for 10 minutes. The cell lysate supernatant was collected in a 1.5 ml centrifuge tube and quantified using OxiSelect™ Superoxide Dismutase Activity Assay (Cell Biolabs Inc). Here, the samples were prepared including blanking in a 96-well microtiter plate and 10 µl of pre-

diluted 1X Xanthine Oxidase Solutions were added into each well. The solutions were mixed thoroughly and incubated for 1 hour at 37ºC. Based on OD_{490} measured, the SOD content in each sample was determined on a microplate reader. The SOD activity level was calculated using the formula below.

SOD Activity (inhibition %) = (ODblank-ODsample)/(ODblank) x 100

5.2.4 Malondialdehyde (MDA) quantitation activity assay

The cells were detached from the flask using a cell scrapper and resuspended in PBS and then homogenized by centrifugation at 12000 x g for 10 minutes. The whole homogenate was used in the OxiSelect™ TBARS Assay Kit (MDA Quantitation) Activity Assay (Cell Biolabs Inc).

The MDA assay provided by the standard protein solution which was a series of standard solutions ranging from 0µM-125µM was prepared to construct a standard curve. 100 µL of SDS Lysis Solution was added to both 100µL unknown samples and the MDA standards. The well-mixed samples were incubated for 5 minutes at room temperature in microcentrifuge tubes. A 250 µL of TBA Reagent was added to each sample and standard to be tested. The tubes were closed and incubated at 95°C for 45-60 minutes using a heat block, cooled at room temperature for 5 minutes, and centrifuged at 3000 rpm for 15 minutes. The supernatant were used for analysis of MDA content. Based on OD_{532} measured, the MDA content in each sample was determined on a microplate reader.

5.2.5 Total Glutathione (GSH) assay

The cells were detached from the flask via a serum-free trypsinisation process using Trypsin EDTA Express (Invitrogen). The cells were centrifuged at 500 rpm for 5 minutes at 4ºC and washed with cold 1X PBS. Supernatant was removed and cells

washed with cold 1X PBS. Centrifugation was repeated and the solution was removed. Immediately the pellet was resuspended with 200-500 µL ice-cold 5% MPA for a cell concentration of 1-5 x 10^6 cells. The cells were thoroughly mixed and homogenized and the suspension transferred to a microfuge tube and centrifuged at 12,000 rpm for 5 minutes at 4ºC. The supernatant was collected and analysed for total gluthatione using GSH quantitation activity assay kit (Cell Biolabs Inc).

The GSH assay provided by the standard protein solution, which was a series of standard solutions ranging from 0µM-0.5µM, was prepared to construct a standard curve. Into each well of a 96-well plate, 25µL of the 1X Glutathione Reductase solution was added followed by 25µL of the 1X NADPH solution. Next, 100µL of the prepared glutathione standards and samples were added into each well and the solutions mixed thoroughly. 50µL of the 1X Chromogen was added and mixed briefly. The plate reader was prepared for a kinetic assay and set to read at OD_{405}. GSH readings were taken immediately at 1-minute intervals for 10 minutes.

5.2.6 Advanced Oxidation Protein Products (AOPP) assay

The cells were detached from the flask by the serum free trypsinisation process using Trypsin EDTA Express (Invitrogen), centrifuged at 500 rpm for 5 minutes at 4ºC, and washed with cold 1X PBS. The supernatant was collected and analyzed using the OxiSelect™ Advanced Oxidation Protein Products (AOPP) Activity Assay (Cell Biolabs Inc).

The AOPP assay was provided by the standard protein solution, a dilution series of Chloramine Standard in the concentration range of 100 µM – 0 µM and a positive control of AOPP. A 200µL sample was added to microtiter wells followed by 10µL chloramine reaction initiator to the well, mixed, and incubated on a shaker for 5

minutes. Later, 20µL of stop solution was added to the wells and mixed. The absorbance of AOPP was read at OD_{340} using 0µM chloramine as blank.

5.2.7 Statistical analysis

Data were presented as mean ± standard deviation (SD). Statistical comparisons were made and the data analyzed using analysis of variance (ANOVA) with the significance level set at $P = 0.05$. Tukey post hoc multiple comparison tests were carried out to determine the differences between the groups (Nishino et al., 2011; Nakamura et al., 2013).

5.3 Results

5.3.1 The measurement of wound closure in *in vitro* co-cultured scratch assay

The wound closure rate was fastest in the B group (only SHED P2 2% FBS cultured in high glucose), followed by the C (co-culture of SHED P2 2% FBS together with fibroblast from high glucose) and A (co-culture of human foreskin fibroblast from normal glucose together with fibroblast from high glucose) at 10 hours with $P<0.05$. At 23, 47, and 90 hours, the rate of wound closure was higher in groups B and C compared to A which had no significant difference (Figure 5.1). The *in vitro* wound healing rate was interpreted in percentage by deduction of the initial width of the cells scratched and multiplied by 100 (Walter et al., 2010).

Figure 5.1: SHED only and co-culture of SHED with fibroblast has indicated a faster and even closure of the cells at 10, 23 and 47 hours. All the experiments were conducted at respective passage with 6 biological replicates for each established cell line. *$P < 0.05$.

5.3.2 The SOD enzyme level

The quantitative enzyme was observed to have the highest SOD level in fibroblast treated in normal glucose with scratched monolayer of fibroblast in normal glucose. The SHED 2% which was treated in normal glucose with scratch monolayer of fibroblast in high glucose showed a significantly lower SOD level with ($p<0.05$) (Figure 5.2).

5.3.3 GSH level

The quantification of the quantitative enzyme was observed having the highest GSH level in scratched monolayer of fibroblast in normal glucose when treated with fibroblast in normal glucose, followed by the scratched monolayer of fibroblast in high

glucose when treated with SHED, followed by fibroblast in high glucose with no significant difference (Figure 5.3).

5.3.4 The MDA level

The lipid peroxidation in the *in vitro* scratch assay was analyzed and found to be highest in transwell which was scratched and treated with high glucose. However, when compared to transwell treated with SHED 2% normal glucose and fibroblast normal glucose, the lipid was less with no significant difference (Figure 5.4).

5.3.5 Advanced Oxidation Protein Products (AOPP)

The protein oxidation in the *in vitro* scratch assay was analyzed and found to be highest in the transwell scratched and treated with high glucose. However, when compared to the transwell treated with SHED 2% FBS in normal glucose and fibroblast normal glucose, protein oxidation was reduced with no significant difference (Figure 5.5).

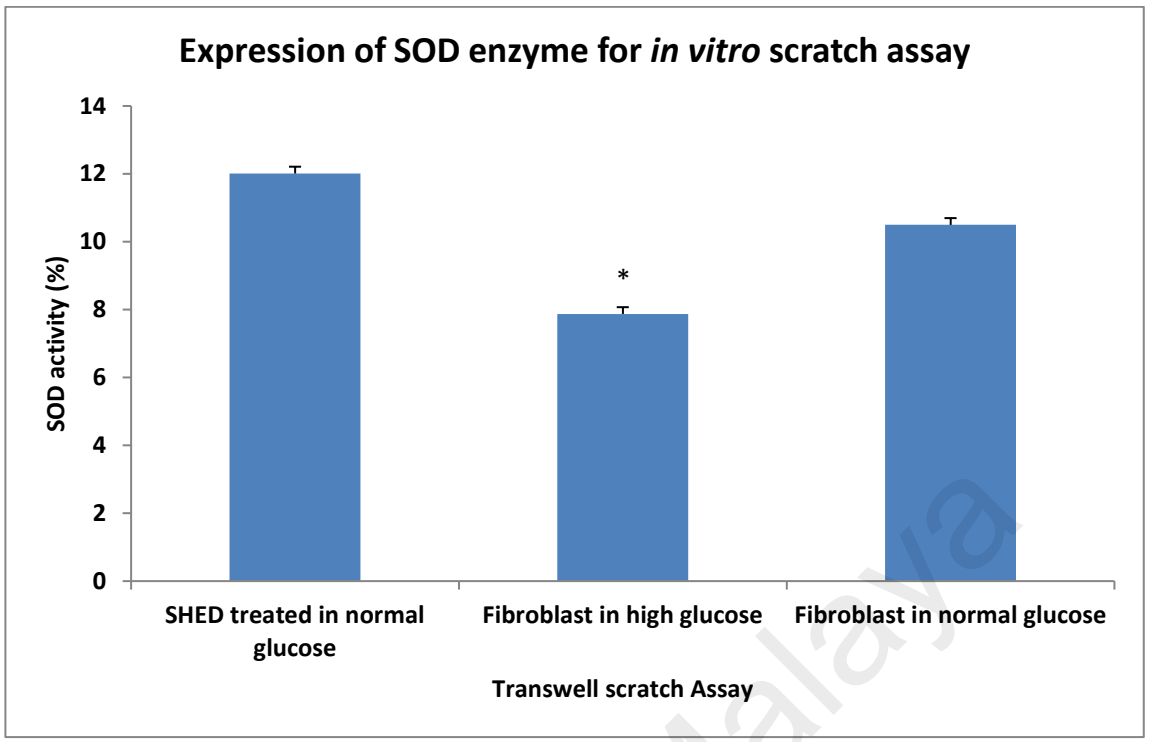


Figure 5.2: Expression of SOD enzyme for the hyperglycemic fibroblast monolayer scratch at the bottom chamber. The treatment were on the upper compartment which are SHED P2 2% FBS, fibroblast in high glucose and fibroblast in normal glucose. *P < 0.05.

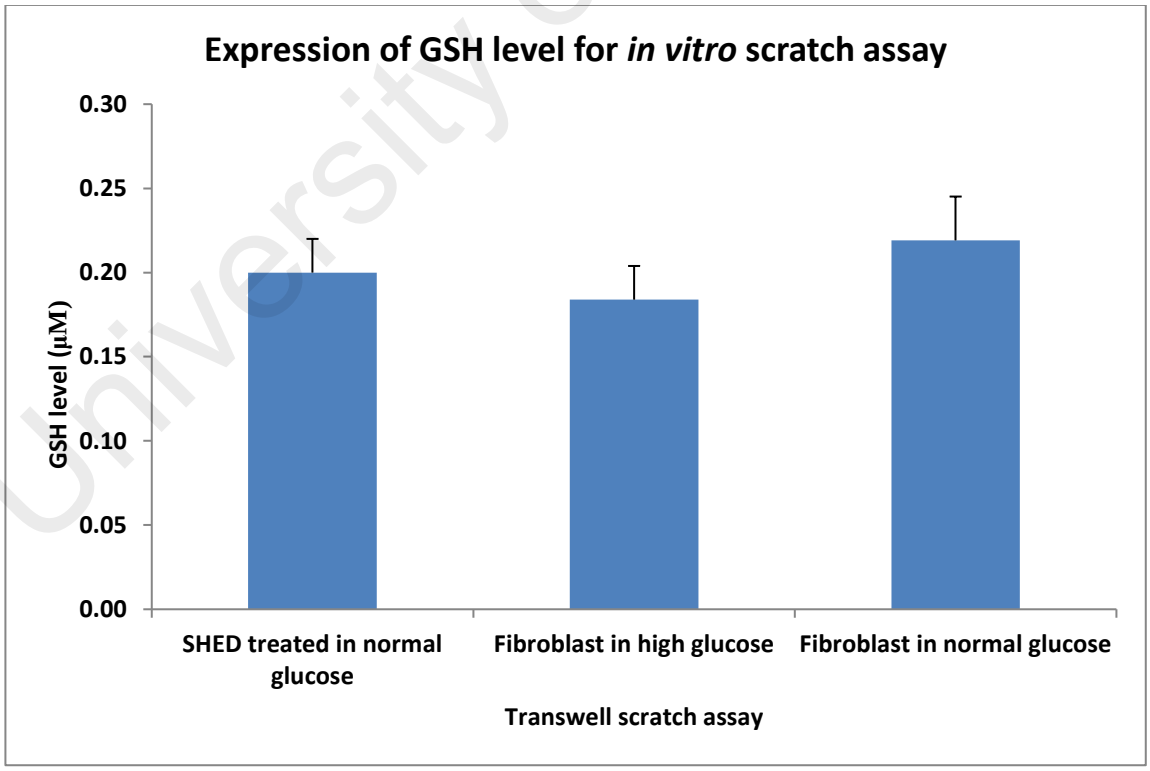


Figure 5.3: Expression of GSH for the hyperglycemic fibroblast monolayer scratch at the bottom chamber. The treatment were on the upper compartment which are SHED P2 2% FBS, fibroblast in high glucose and fibroblast in normal glucose.

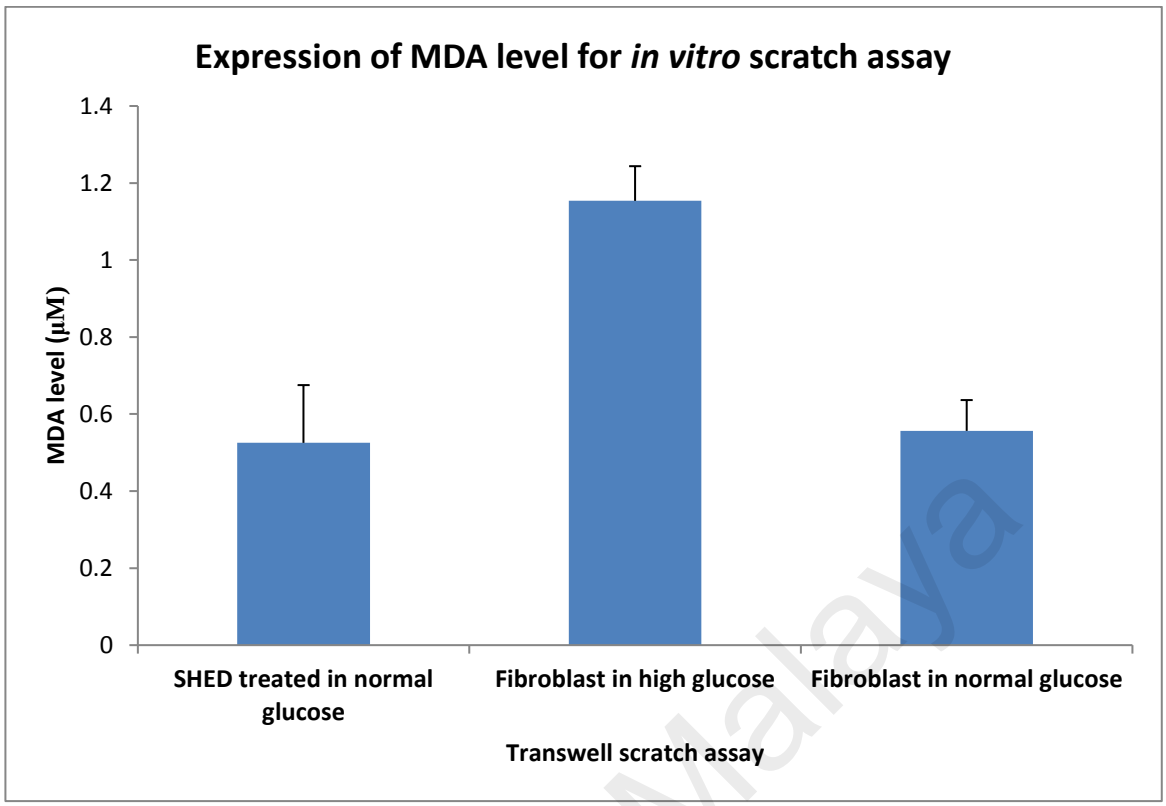


Figure 5.4: Expression of MDA for the hyperglycemic fibroblast monolayer scratch at the bottom chamber. The treatment were on the upper compartment which are SHED P2 2% FBS, fibroblast in high glucose and fibroblast in normal glucose.

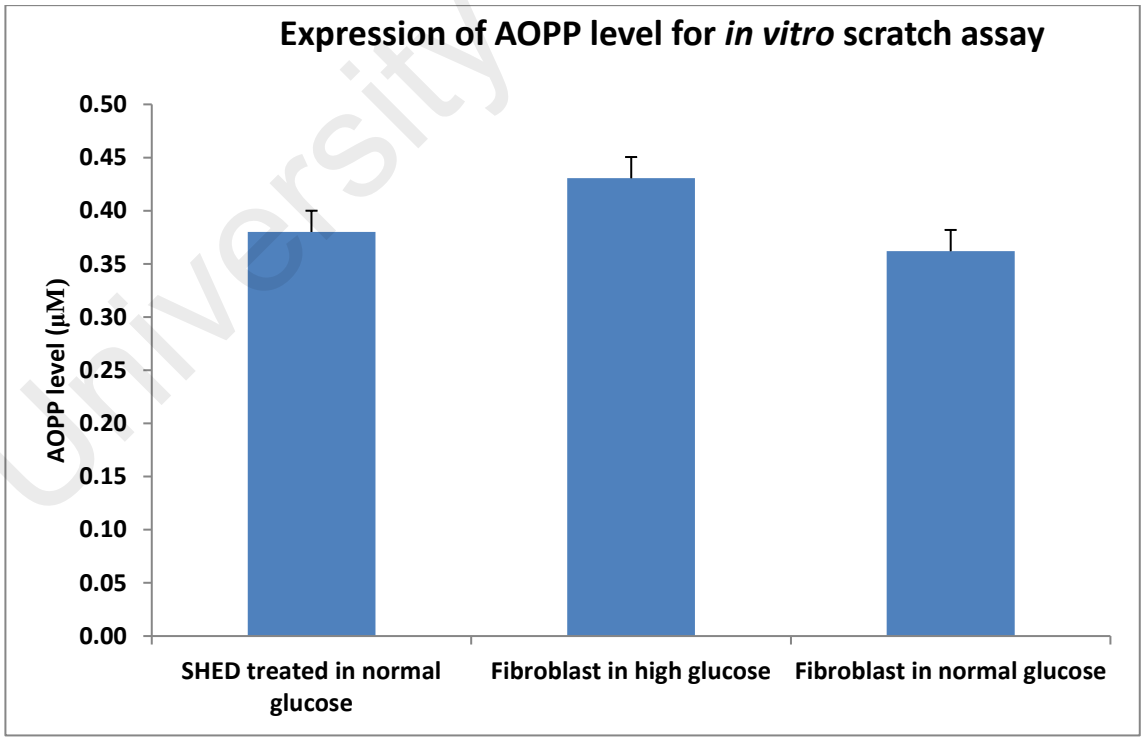


Figure 5.5: Expression of AOPP for the hyperglycemic fibroblast monolayer scratch at the bottom chamber. The treatment were on the upper compartment which are SHED P2 2% FBS, fibroblast in high glucose and fibroblast in normal glucose.

5.4 Discussion

Walter et al. (2010) conducted a similar *in vitro* study on fibroblast and keratinocyte scratch assays. The study indicated that the co-culture of L929 fibroblast and HaCaT keratinocyte in MSC conditioned medium (MSC-CM) enhanced cell migration with the fibroblasts leading the way in closing the scratches. The paracrine factors enhancing wound healing were TGFB1, IL6, IL8, MCP-1, and collagen Type I. In our study, the *in vitro* model suggests that the similar co-culture system had good wound healing potential. The SHED-only scratched assay performed better with significant difference when compared to the fibroblast scratch assay.

The co-culture of SHED and fibroblast also showed increased healing rates compared to fibroblast scratch assay. This indicates that the paracrine factors in SHED enhance the migration of cells and assist in faster wound closure. This *in vitro* scratch assay method mimics the migration of cells *in vivo* and also allows for a study on the interaction between different cell types. The unique advantage of scratch assay is that we can choose and label the cell population with a specific dye (Rodriguez-Menocal et al., 2012). Transwell migration assay performs a different system than co-culture system. In this assay, the interaction that occurs is between soluble factors secreted by the cells at the upper and bottom chambers. The two-chamber dishes prevent direct contact but permit the exchange of soluble diffusible factors (Kim et al., 2007). In our study, fibroblast migration at the bottom chamber was induced by the SHED as a chemo attractant.

This *in vitro* study also evaluates wound closure under high glucose conditions. A high glucose concentration of more than 30mM causes oxidative stress which hinders wound healing (Hruda et al., 2010). This study confirms the previous findings on the high

glucose mediate production of reactive oxygen species (ROS) and oxidative stress (James & Murphy, 2002; Hruda et al. 2010). In the study by Xuan et al. (2014), high glucose concentrations inhibited human fibroblast cell migration in wound healing via the repression of bFGF-regulating JNK phosphorylation. In a recent study, the wound healing capacity of human retinal pigment epithelial cells (ARPE-19) cultured in a medium containing normal (NG, 5mM) or high (HG, 45mM) glucose levels modulates oxidative stress responses regulated by Nrf2. The cell culture condition of HG also reduced ATP production and mitochondrial function (Foresti et al., 2015).

Our study not only sought to measure wound closure but also investigated the ability of SHED in improving oxidative stress conditions among the high glucose-induced fibroblasts monolayer of scratch assay.

The overproduction of ROS diminishes the expression of antioxidant enzymes such as manganese superoxide dismutase, glutathione peroxidase, and catalase (Miranda-Diaz et al., 2016). Therefore, it is important to investigate the antioxidant enzymes in order to know more about damage occurrence in cells. Our study made estimates on the oxidative stress of *in vitro* wound healing by determining the quantitative value of the antioxidant enzymes. The SOD enzyme, for example antioxidative enzyme, that protects cells from further oxidation is found to be highly expressed in the SHED-treated group with $p< 0.05$. This is one the indications in our *in vitro* wound model where fibroblasts at the bottom layer of transwell representing the skin layer scratched in hyperglycemic condition, had increased the wound gap closure following treatment with SHED P2 in low serum at the upper layer of the transwell assay. The upper compartment would only allow small molecules like growth factors and paracrine factors of the SHED to be transported to the lower compartment to induce the closure of the gap. Thus, the evaluation of SOD showed that it was higher in transwell treated with SHED compared to fibroblast in normal and high glucose. This shows that SHED

induces the production of antioxidative enzymes which help to reduce further cell injury. Similar results were noted for the GSH antioxidant enzyme level which shows that both the compartment treated with SHED and fibroblast in normal glucose were high in GSH levels, followed by fibroblast in high glucose. Other than antioxidant enzymes, we also investigated the lipid peroxidation and protein oxidation levels in the *in vitro* wound model.

Lipid peroxidation is a mechanism in cellular damage in animals and lipid peroxides are unstable in forming reactive compounds such as MDA which is a marker for oxidative stress. The MDA expression for the SHED P2 low serum treated group and fibroblast in normal glucose is lower compared to fibroblast in high glucose. This shows that SHED has the ability to further reduce injury and damage to cells. Similar results were obtained from protein oxidation. For example, advanced oxidation protein products (AOPP) are a type of uremic toxin created during oxidative stress. The AOPP is seen elevated in patients with diabetes mellitus, inflammation, and others. Our *in vitro* study shows that the expression of AOPP decreased in fibroblast cells treated with SHED and in normal glucose compared to fibroblasts in high glucose. Therefore, the results for the *in vitro* wound model suggest that protein oxidation is higher in high glucose fibroblasts compared to SHED and fibroblasts in normal glucose.

The studies on *in vitro* wound healing were helpful despite some which showed positive treatment results with the induction of paracrine factors. For example, the conditioned media of adipose derived stem cells (ASC-CM) have reported improvements in *in vitro* wound healing assay (Lee et al., 2012). Another study suggests that the level of SOD and GSH are elevated in human umbilical cord mesenchymal stem cells (UCMSCs) after their treatment *in vitro* and *in vivo* wound models. Wound healing is stimulated by reducing the accumulation of oxidative stress (Zhou et al., 2013).

This study showed the SHED-expressed paracrine factors that would be vital for the healing process. SHED which was cultured in P2 in low serum culture condition (FBS 2%) showed the ability to enhance wound closure in high glucose conditions. In addition, the treatment with SHED also reduced the accumulation of oxidative stress in high glucose conditions. Therefore, SHED improves healing ability in diabetics.

5.5 Conclusion

1. The digital image of *in vitro* diabetic wound healing through in vitro glucose challenged scratch assay with SHED P2 2% FBS showed enhanced wound healing rates at the 10, 23, 47, and 90 hour marks.
2. The antioxidant enzymes SOD and GSH increased while lipid peroxidation and protein oxidation decreased in SHED P2 2% FBS thus improving *in vitro* diabetic wound healing.

From this study we conclude that, SHED cultured at P2 of 2% FBS has reduced the oxidative stress condition and enhanced the in vitro wound healing rate. Therefore we proceed to the pre-clinical study of wound healing using SHED P2 in 2% FBS in our next chapter.

CHAPTER 6: THE TOXICITY EFFECT OF SHED ON THE ANIMAL STUDY AND THE POTENTIAL OF WOUND HEALING ON EXPERIMENTAL STZ-INDUCED DIABETIC RATS TREATED WITH SHED

6.1 Introduction

Diabetes mellitus is a metabolic disease caused by insulin insufficiency (World Health Organization, 2016). Insulin is necessary to break down glucose into glycogen and any impairment of this function leads to high glucose levels in the blood or hyperglycemia (National Institute of Diabetes and Digestives and Kidney Diseases, 2014). One of the common consequences of diabetes is reduced blood flow in the feet due to neuropathy which increases the risk of foot ulcers and infections which could necessitate limb amputation (World Health Organization, 2016).

Current treatments available are wound debridement, care and dressing with topical antibiotics (American Diabetes Association, 2014), topical negative pressure (Xie et al., 2010), hyperbaric oxygen therapy [HBOT] (Londahl, 2013), and introducing platelet derived-growth factors (Villela & Santos, 2010). Since the duration of the wound healing process depends on its severity and is likely to be long, researchers are investigating more advanced treatments such as cell-based therapy.

Stem cells play an important role in regenerative medicine. Several studies have been conducted in using MSCs for the treatment of skin wounds. The studies include BM-MSCs, ASCs, WJ-MSCs, UCMSCs and PMSCs [(Yang et al., 2013; Ozpur et al., 2016; Zhao et al., 2015; Wang et al., 2016; Du et al., 2016)]. Dental derived stem cells are a unique stem cell resource and have shown promise as an alternative wound healing treatment. An *in vivo* wound healing study by Nishino et al. (2011) reported that SHED exhibits good potential in wound healing where it has proven to provide better rates of

wound closure with increased Type 1 collagen co-cultured with a fibroblast treated group. In comparison, it is hypothesized that SHED can be a potential treatment modality for diabetic wound healing by increasing the antioxidant enzymes such as SOD and GSH and reducing lipid peroxidation (MDA) and protein oxidation conditions (AOPP).

Experimental works were carried out to meet the following objectives:

A. General objective

To evaluate the toxicity condition of SHED and its ability to treat diabetic wounds in rats.

B. Specific objectives

To evaluate the blood profile of Sprague Dawley (SD) rats in two different dosages of SHED transplantation. This involved:

1. Evaluating the histology of livers and kidneys in two different dosages of SHED in SD rats using Hematoxylin and Eosin (H&E) staining;
2. Assessing the streptozotocin (STZ) induced-diabetic animal model;
3. Assessing macroscopic wound contractions of diabetic-induced SD rats;
4. Evaluating the histology of granulation tissue [(H&E); pkh-26 tagged cells immunofluorescence (IF) and immunohistochemistry (IHC) analysis for Hsp70 upregulation];
5. Evaluating the oxidative stress enzyme activity level of rats' skin tissue homogenate; and
6. Assessing the level hydroxyproline accumulation in rats' skin using hydroxyproline assay.

6.2 Methodology

6.2.1 Animals for toxicity test

A total of 36 seven-week old male and female SD rats weighing 250-300 grams were obtained from the animal experimental unit (AEU). To determine the safe dosage for the SHED cultured in low serum of 2% FBS, the rats were placed in 3 groups each comprising 6 males and 6 females. A single dose of the SHED which is $0.5x10^6$ cells/ml and $1x10^6$ cells/ml was injected intravenously for each rats. After administration of the doses the animals were observed for 30 minutes, and 24 and 48 hours for toxicological signs. The mortality was also checked for two weeks, and at day 15 the animals were euthanized (Shwter et al., 2016). The experiment was conducted with the approval of and guidelines from FOM Institutional Animal Care and Use Committee, University of Malaya (FOM IACUC, ethics No.2015-180505/DENT/R/SM) (Appendix 1.5).

6.2.2 Preparation of SHED for transplantation for toxicity test

The cell culture protocol is summarized in a previous chapter (3.2.3). The SHED was harvested upon 90% confluency at passage 2 from a T_{125} flask. The conditioned medium was aspirated from the flask and washed twice with DPBS. The cells were detached using Trypsin TrypLE Express (Gibco, USA), collected in a 15 ml Falcon centrifuge tube, and later calculated for total cells. The cell pellet was then centrifuged again and the supernatant discarded. The pellet of SHED which was needed for transplantation was immediately resuspended with 1ml of PBS and ready for transplantation for the rats.

6.2.3 SHED intravenous transplantation for toxicity test

The prepared SHED in 1ml of PBS based on the number of cells was transplanted into the experimental rats in groups via intravenous injection. The SD rats used for this

experiment were categorized into 6 male and female rats for each of the groups as listed:

Group 1: vehicle control (PBS only injected);

Group 2: lower dosage of SHED 500,000 cells/ml in PBS (SHED-L); and

Group 3: higher dosage of SHED 1,000,000 cells/ml in PBS (SHED-H).

Only one rat was injected at a time. The rat was gently grasped around the thorax with thumb and forefingers to secure and control the animal and its head placed in the opening of the restraint plastic. A long thick thread was tied around the base of the tail to prevent the rat from moving backwards. Next, the rat's tail was cleaned with alcohol swab and the injection made at least half a tail length from the body. With the tail under tension, the 21 G needle was inserted into the skin parallel with the vein. The proper placement of needle was at least 3 mm into the lumen of vein and the administration must be steady to avoid rupture of the vessel. Upon completion, the rats were ensured good homeostasis before being returned to the cage (Li et al., 2016).

6.2.4 Blood profile analysis for toxicity test

At day 15 the animals were euthanized at one time with an overdose of ketamine (50 mg/kg via intramuscular) and xylazine (5 mg/kg via intramuscular). Immediately after administration of the doses, a cardiac puncture was made on the rats' abdomens to aspirate 2ml blood in serum separator tubes (SST®) for serum liver and renal profile determination. The blood samples were immediately sent to the laboratory for analysis.

6.2.5 Histological analysis for liver and kidney

After the cardiac puncture was performed on the rats on day 15, the abdomen of the rats was cut open and the livers and kidneys removed and stored in a container containing 10% phosphate buffered formalin solution. The organs were kept in formalin until they

were used for the histological examination (Ding et al., 2004). The histological process includes tissue processing, embedding, sectioning, staining, and mounting (Appendix 1.7). The slides were then ready for observation at 20x resolution microscope (OLYMPUS).

6.2.6 Animals for diabetic wound model

The animal ethics were obtained from FOM Institutional Animal Care and Use Committee, University of Malaya (FOM IACUC, ethic No.2015-180505/DENT/R/SM) for this experimental study. The SD rats were housed under controlled conditions of light (12 h of light and 12 h of darkness) at 50% relative humidity and 24ºC temperatures and maintained on normal chow and water. After an adaptation period of one week, the rats were put into overnight fasting with only access to water and the glucose level was monitored using a glucometer. For this procedure, the rats were restrained with a restrainer, the tail cleaned using 70% alcohol swab, and 10µl of blood was taken from each tail vein using a 25G needle to check glucose levels.

Thirty-six rats weighing 250 grams were included in this experiment with six rats in each group including SHAM group (non-diabetic rats fed with chow and water). For the diabetic rats, ten per group were initially chosen and induced with STZ. These rats were given a single IP injection of STZ (55 mg/kg, Sigma–Aldrich, St. Louis, MO), dissolved in sterile citrate buffer (0.05 mol/L sodium citrate, pH 4.5) (Skovso, 2014). Six days after STZ was induced, the rats were put into overnight fasting, 10 µl of blood was taken from their tail veins, and the glucose levels measured using a glucometer (Accu-Check, Roche Ltd, Swiss). Rats with blood glucose levels exceeding 7 mmol/l after overnight fasting were considered diabetic. The rats which did not turn into diabetics were removed from the experiment. Therefore, of the ten rats in each group, only six

diabetic rats for each group were used in this experiment. All the thirty-six rats in this experiment were separated in six rats per group including STZ-induced diabetic rats from groups 2 to 6.

Group 1 (G1): Negative control (Sham group: normal chow and water consumption);

Group 2 (G2): Vehicle control (PBS only injected);

Group 3 (G3): Treatment 1: low dosage SHED (SHED-L) of 500,000 cells/ml in PBS;

Group 4 (G4): Treatment 2: high dosage SHED (SHED-H) of 1,000,000 cells/ml in PBS;

Group 5 (G5): Topical application of intrasite gel (0.1 ml); and

Group 6 (G6): Topical application of intrasite gel (0.1ml) with Glibenclamide (Gli) given orally.

6.2.7 SHED culture preparation

The SHED used for this study was cultured at passage 2 with a low serum condition. The protocol is summarized in a previous chapter (4.2.1). SHED was harvested upon 90% confluency at passage 2 from T_{125} flask and detached using a serum free trypsin, EDTA Express (Invitrogen). The total number of cells needed for SHED-L (500,000 cells/ml in PBS) and SHED-H (1,000,000 cells/ml in PBS) was calculated and separated in a 15 ml Falcon centrifuge tube.

6.2.8 Wound healing model

Seven days after the rats were confirmed diabetic they underwent general anesthesia with ketamine (30 mg/kg via intramuscular) and xylazine (3 mg/kg via intramuscular). Dorsal hair was removed with electric clippers and the skin disinfected with a Povidone-iodine solution. A skin biopsy was done with a circular blade ranging 6 mm diameter in size on the dorsum of each rats. The blade, which is attached to a pencil-like

handle, is rotated down through the epidermis and dermis and into the subcutaneous fat, producing a cylindrical core of tissue (Zuber, 2002; Nishino et al., 2011).

6.2.9 SHED transplantation to the wound model

The cells which were separated according to SHED-L and SHED-H were centrifuged again for cell pellet and tagged with pkh-26 dye of 80 µM (Appendix 1.6). Using a 21G-sized needle, 1ml of PBS containing stem cells tagged with pkh-26 dye was injected intra-dermally into the adjacent skin of a rat after wounding (Maharlooei et al., 2011). For the vehicle control group, only PBS was injected while for the positive control group, G5, the treatment was a topical application of intrasite gel of 0.1ml around the wound area. For G6, a topical application of intrasite (0.1ml) on the wound area and Gli treatment of 10mg/kg were given orally once a day (Li et al., 2012). The Gli is a type of sulfonyl urea used to treat DM (Kirchheiner et al., 2002) and aids in increasing insulin production in diabetic rats (Mutalik & Udupa, 2004). Therefore, G6 can be considered as a controlled diabetic group (Figure 6.1).

6.2.10 Wound healing analysis

The wound area was measured and digital images taken at days 0, 5, and 10 post-surgery. The wound was measured using permanent markers and transparent papers under light anesthesia with ketamine and xylazine. The transparent paper was then transferred to a graph paper to measure the wound closure area (mm^2) of each rat. The wound closure percentage was assessed for each group by calculating the percentage of wound reduction from the original wound (Moghadamtousi et al., 2015).

6.2.11 Histological analysis

Upon post-surgery at day 10, the skin sample was harvested. The wounded skin area was cut carefully and stored in separately in a container containing DPBS solution for

tissue homogenate preparation and Bouins' fixative solution for skin histology until tissue processing. The slide preparation process is summarized in Appendix 1.7. Histological examination included H&E staining and IF observation for pkh-26 tagged cells.

6.2.12 IHC analysis

The immunostaining was performed using the EGFR pharmDx™ kit (DakoCytomation, Carpinteria, CA, USA). The endogenous peroxidase activity was quenched using a peroxidase block. Tissue sections were then incubated with Hsp70 (1:500, Cat: ab2787, Abcam) (Hajrezaie et al., 2014) biotinylated primary antibody for 15 minutes followed by another 15 minutes of incubation with streptavidin–horseradish peroxidase. The sections were incubated with diaminobenzidine tetrahydrochloride for 5 minutes and then counterstained with hematoxylin and 0.5% ammonia in water. The brown illustrations of samples under a light microscope demonstrated the positive findings.

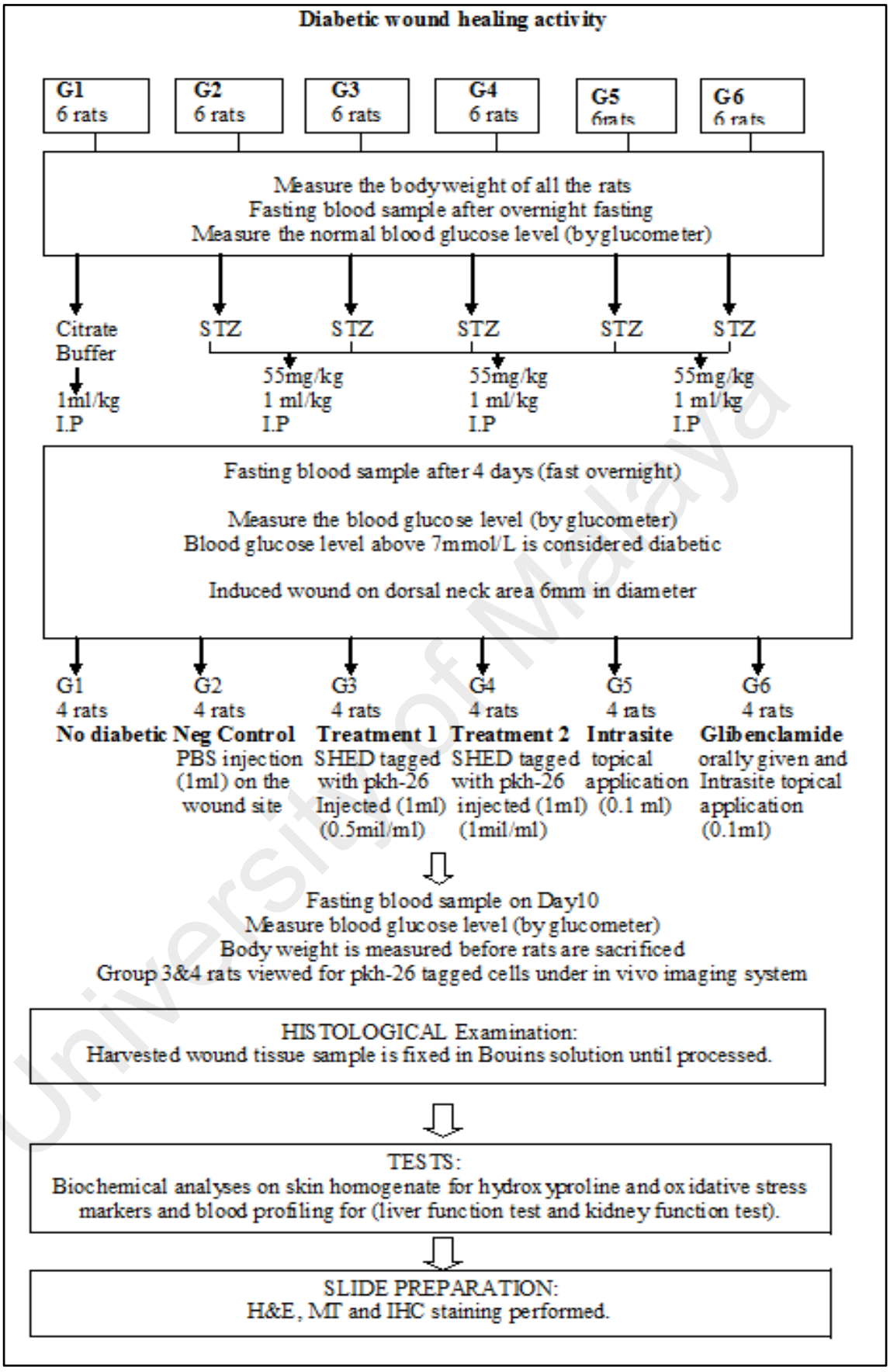


Figure 6.1: Flow chart of SHED ameliorate experimental STZ- induced diabetic wound model in SD rats.

6.2.13 Preparation for rat skin homogenate for biochemical analysis

The skin sample for tissue homogenate (6.2.12) was kept in -80ºC until use. For the homogenization step, 100mg of the tissue was defrosted and grinded by adding 4ml of cold 1X cell lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA) to pre-chilled Dounce homogenizer (SIGMA). The minced tissue was then transferred into a microcentrifuge tube and centrifuged at 4^0C for 10 minutes. The supernatant was collected in a pre-chilled fresh microcentrifuge tube. The tissue lysate supernatant was kept in -80ºC until it was used for biochemical tests (Simpson, 2010).

I. Superoxide Dismutase (SOD) Activity Assay

The tissue lysate (step 6.2.6) were collected into a 1.5 ml centrifuge tube, quantified using OxiSelect™ Superoxide Dismutase Activity Assay (Cell Biolabs Inc). In the assay, the samples were prepared including a blank in a 96-well microtiter plate, and 10 µl of the pre-diluted 1X Xanthine Oxidase Solution were added into each well. The solutions were thoroughly mixed and incubated for 1 hour at 37ºC. Based on OD_{490} measured, the SOD content in each sample was determined on a microplate reader. The SOD activity level was calculated using the following formula:

SOD Activity (inhibition %) = (ODblank-ODsample)/(ODblank) x 100

II. Total Gluthathione (GSH) Assay

The tissue lysate (step 6.2.6) was used for OxiSelect™ Total Gluthatione Assay Kit (GSH Quantitation) Activity Assay (Cell BiolabsInc). The GSH assay provided by the standard protein solution, a series of standard solutions ranging from 0µM-0.5µM was prepared to construct a standard curve. For testing purposes, 25 µl of the 1X Glutathione Reductase solution followed by 25 µl of the 1X NADPH solution were

added into each of the 96-wells of the plate. Next, 100 µl of the prepared glutathione standards and samples were added into each well and the solutions mixed thoroughly. 50 µl of the 1X Chromogen was added and mixed briefly. The plate reader was prepared for a kinetic assay and set to read at OD_{405}. Immediately the GSH readings were taken at 1-minute intervals for 10 minutes

III. Malonaldehyde (MDA) Quantitation Activity Assay

The tissue lysate (step 6.2.6) was used in the OxiSelect™ TBARS Assay Kit (MDA Quantitation) Activity Assay (Cell BiolabsInc). The MDA assay provided by the standard protein solution, a series of standard solutions ranging from 0µM-125µM, was prepared to construct a standard curve. 100 µl of the SDS Lysis Solution was added to both 100 µl unknown samples and the MDA standards. The well-mixed samples were incubated for 5 minutes at room temperature in microcentrifuge tubes. 250 µl of TBA Reagent was added to each sample and the standard to be tested. The tubes were closed and incubated at 95°C for 45-60 minutes using a heat block. Next the tubes were cooled at room temperature for 5 minutes and centrifuged at 3000 rpm for 15 minutes. The supernatant were used for analysis of MDA content. Based on the OD_{532} measured, the MDA content in each sample was determined on a microplate reader.

IV. Advanced Oxidation Protein Products (AOPP) Assay

The tissue lysate (6.2.6) was collected and analyzed using the OxiSelect™ Advanced Oxidation Protein Products (AOPP) Activity Assay (Cell BiolabsInc). The AOPP assay was provided by the standard protein solution, a dilution series of Chloramine Standard in the concentration range of 100 µM – 0 µM, and a positive control of AOPP. 200 µl of samples were added to the microtiter wells, followed by 10 µl chloramine reaction initiator, then mixed and incubated on a shaker for 5 minutes. Later, 20µl of stop

solution was added to the wells and mixed. The absorbance of AOPP was read at OD_{340} using 0μM chloramine as a blank.

6.2.14 Hydroxyproline determination in rat skin tissue

The level of hydroxylproline can be used as an indicator of collagen content. Approximately 10 mg of tissue was added into 100 μl of water and transferred into a microcentrifuge tube. Then, 100 μl of concentrated hydrochloric acid (HCl ~12 M) was added into the microcentrifuge tube containing the tissue sample and capped tightly. It was then hydrolyzed at 120°C for 3 hours using a heating block (CORNING). 50μl of supernatant from each sample group was transferred to a 96-well plate which was placed in an oven at 60°C to dry. 100μl of the Chloramine T/Oxidation buffer mixture was added to each sample and standard well and incubated for 5 minutes at room temperature. Then, 10 μl of Diluted DMAB Reagent was added to each sample and standard well, and incubated for 90 minutes at 60°C. The measurement of absorbance 560 nm (A560) was done using a spectrophotometer (Xue et al., 2015).

6.2.15 Statistical Analysis

Data were presented as mean + standard deviation (SD). Statistical comparisons were made and data analyzed using analysis of variance (ANOVA) with the significance level set at $P = 0.05$. Tukey post hoc multiple comparison tests were carried out to determine the differences between the groups (Nishino et al., 2011; Nakamura et al., 2013).

6.3 Results

6.3.1 Blood profile evaluation for toxicity test

The blood profiling for the toxicity test included the liver function tests (ALT and AST) and the renal function test (creatinine). The results for the experimental group are presented in mean± standard deviation (SD) value (Table 6.1).

Table 6.1: Effect of SHED in liver function test on acute toxicity study in U/L and g/L in mean ± standard deviation of G1: Vehicle, G2: SHED-L and G3: SHED-H

Group		AST (U/L)	ALP (U/L)	TP (g/L)	Albumin (g/L)
1	male	128 ± 0.73	135 ± 0.02	60 ± 2.11	36 ± 0.28
	female	129 ± 0.62	134 ± 0.04	60 ± 1.86	37 ± 0.30
2	male	128 ± 1.34	138 ± 0.03	58 ± 1.98	36 ± 0.11
	female	126 ± 2.23	140 ± 0.02	60 ± 2.44	36 ± 0.27
3	male	130 ± 1.56	137 ± 0.12	58 ± 2.36	36 ± 0.22
	female	131 ± 1.54	134 ± 0.02	58 ± 1.09	37 ± 0.23

The data was presented as mean± SD value. There is no significant difference between the groups when compared to the vehicle control group (AST: Aspartate Aminotransferase; ALP: Alkaline Phosphatase; TP: Total Protein).

Table 6.2 Effect of SHED in kidney function test on acute toxicity study in mmol/L and µmol/L in mean ± standard deviation of G1: Vehicle, G2: SHED-L and G3: SHED-H

Group		Sodium (mmol/L)	Potassium (mmol/L)	BUN (mmol/L)	Creatinine (µmol/L)
1	male	140 ± 0.03	4.4 ± 0.49	5.5 ± 0.01	23 ± 0.28
	female	140 ± 0.19	4.4 ± 0.43	5.5 ± 0.36	24 ± 0.30
2	male	140 ± 0.34	4.3 ± 0.01	5.0 ± 2.27	21 ± 0.11
	female	143 ± 0.23	4.3 ± 0.02	5.0 ± 1.76	20 ± 0.27
3	male	140 ± 0.02	4.3 ± 0.13	5.5 ± 1.03	23 ± 0.22
	female	141 ± 0.04	4.3 ± 0.16	5.5 ± 1.48	23 ± 0.19

The data was presented as mean± SD value. There is no significant difference between the groups when compared to the vehicle control group (BUN: Blood Urea Nitrogen).

6.3.2 Histological observation for toxicity test

At day 10 after SHED transplantation of the liver, the histology indicated a normal liver consisting of a network of hepatocytes. For the kidney histology, the presence of glomeruli indicated no damage to the kidney in the treatment group (Figure 6.2).

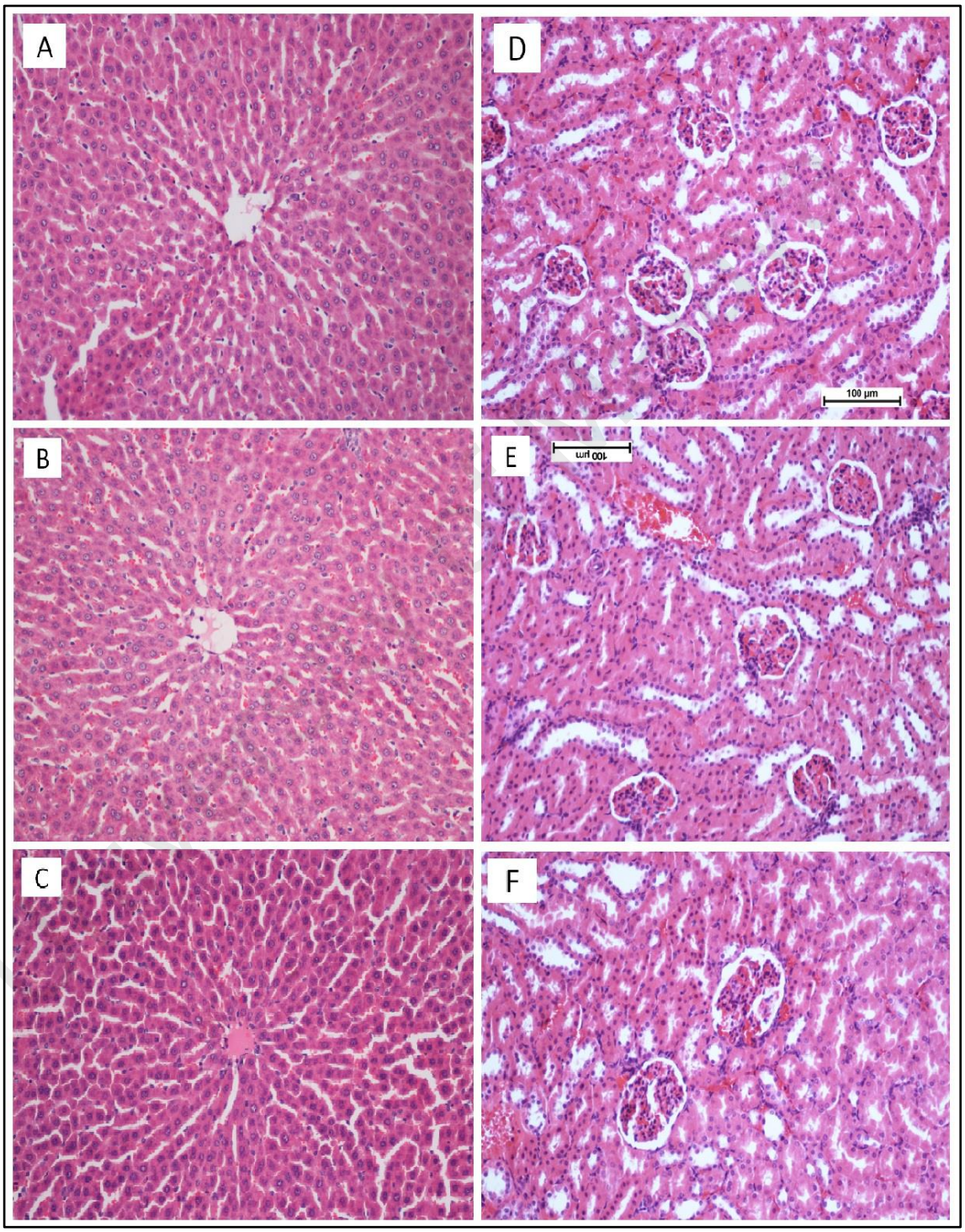


Figure 6.2: SHED's effect on liver and kidney histology for toxicity study. A: liver vehicle control, B: liver (SHED-L), C: liver SHED-H, D: kidney vehicle control, E: kidney SHED-L, F: kidney SHED-H. The H&E stained tissues showed no damage to the liver and kidney. Microscopic resolution: 100µm.

6.3.3 SHED accelerated the wound healing

The rats' percentage of wound closure was higher in the SHED-treated group compared to the vehicle control and intrasite gel treatment groups (Figure 6.3).The digital images captured on days 0, 5, and 10 demonstrate that wound healing was enhanced by SHED (Figure 6.4).

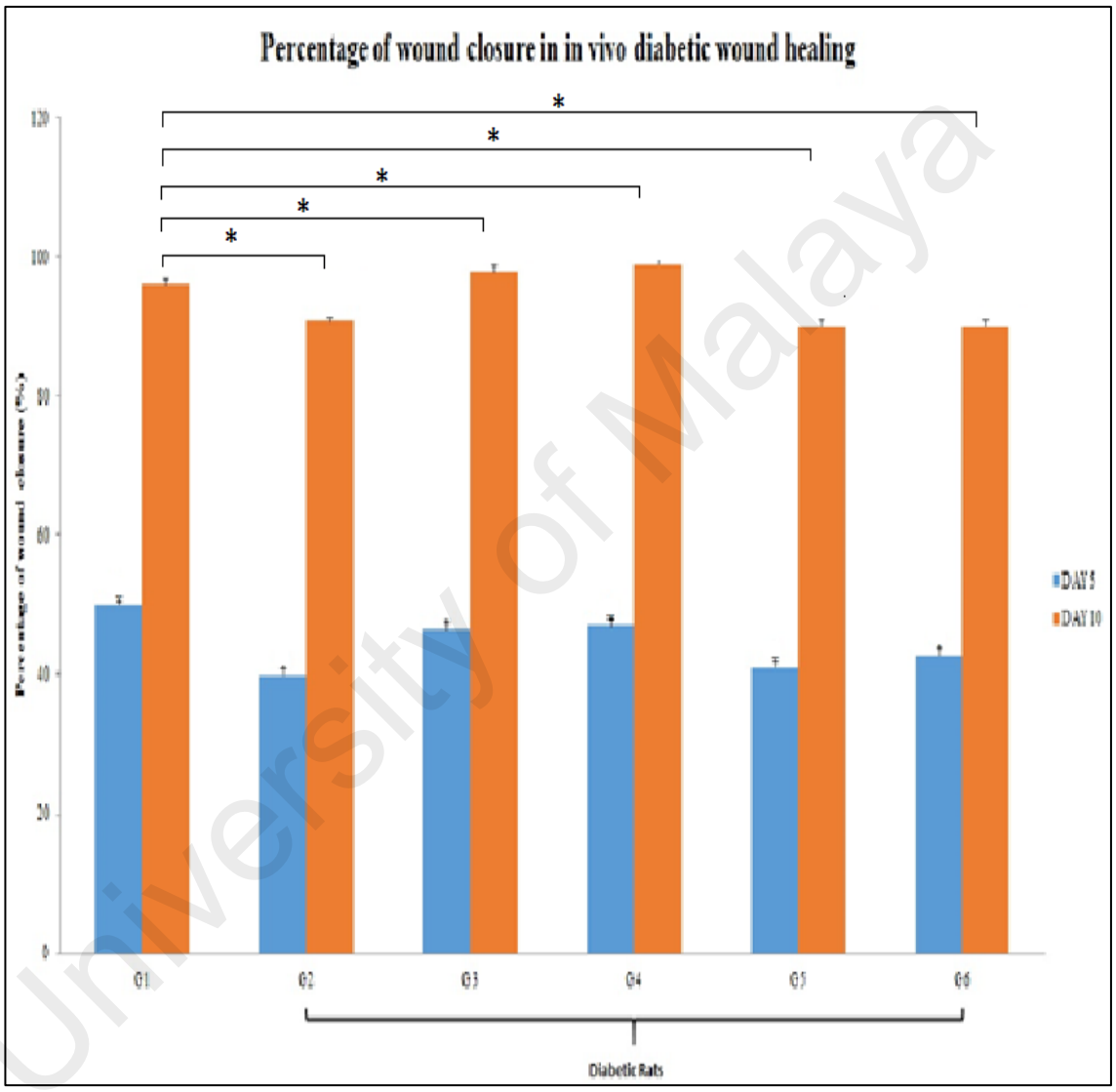


Figure 6.3: Effect of SHED in wound closure rate at day 5 and 10. The G1: normal non-diabetic rats, G2: vehicle group; G3: SHED-L treatment with $0.5x10^6$ cell/ml, G4: SHED-H treatment with $1.0x10^6$ cells/ml, G5: intrasite gel treated group, G6: intrasite gel treated with Gli. Highest rate of wound closure is noticed at day 10 in SHED-H treated group. (* $p < 0.05$ between the groups).

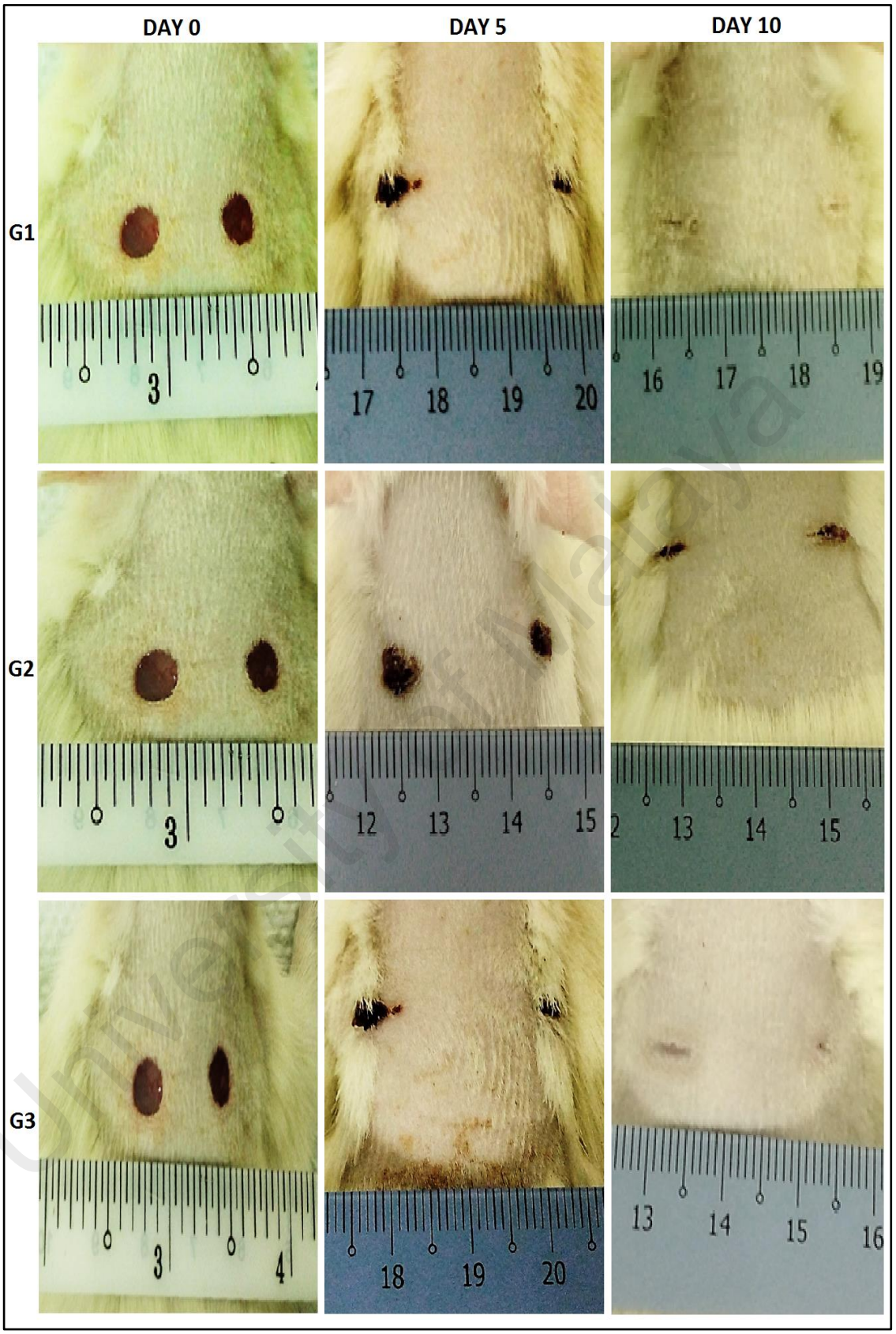


Figure 6.4: Effect of SHED on wound measurement at day 0, 5, and 10 post surgeries. The G1: normal non-diabetic rats, G2: vehicle group; G3: SHED-L treatment with $0.5x10^6$ cell/ml, G4: SHED-H treatment with $1.0x10^6$ cells/ml, G5: intrasite gel treated group, G6: intrasite gel treated with Gli.

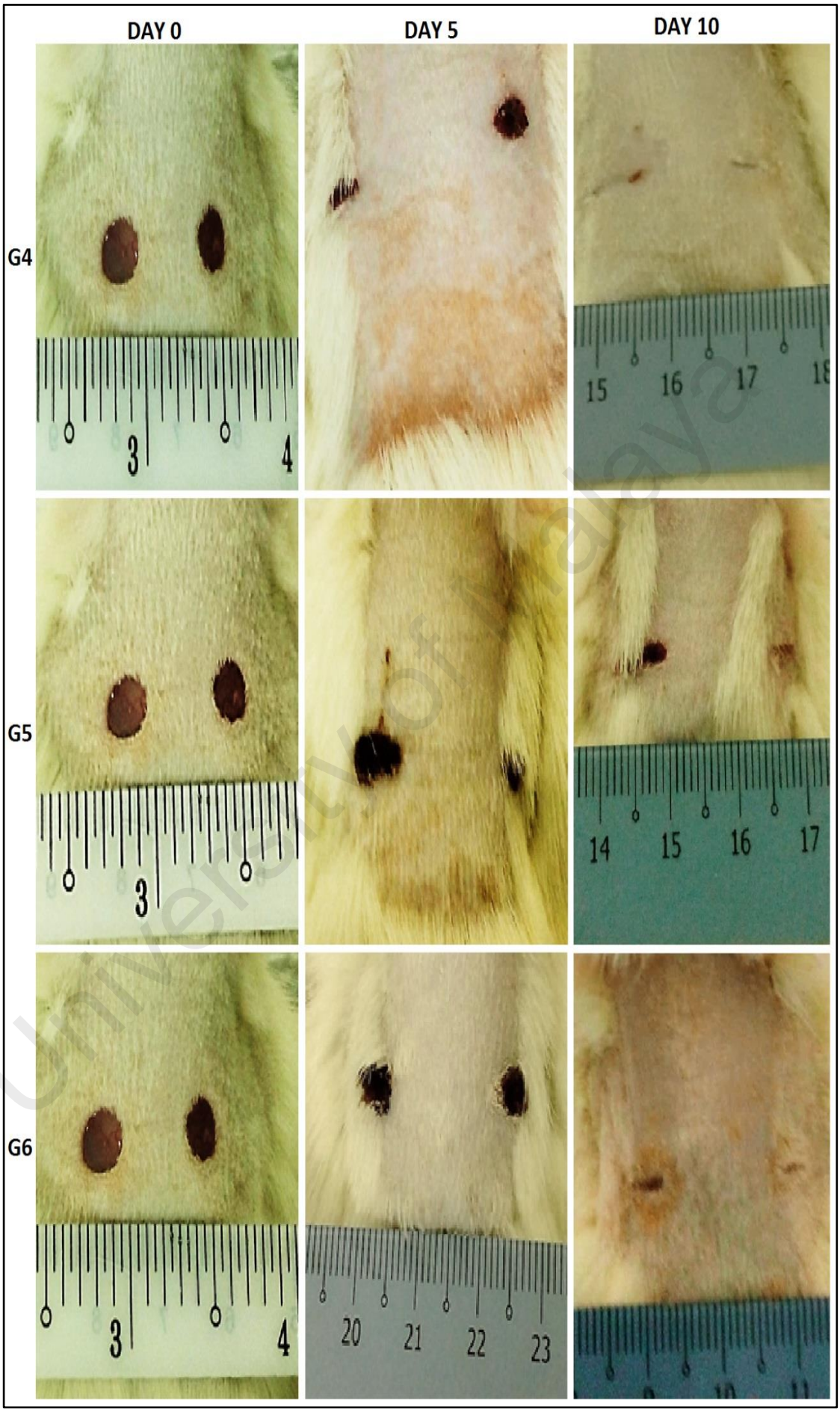


Figure 6.4, continued.

6.3.4 Histological analysis for diabetic wound healing model

Post-surgery at day 10, a thinner keratin layer of rat skin was demonstrated in the SHED-L and SHED-H categories compared to the vehicle control and topical application of intrasite gel groups. The SHED which was tagged with pkh-26 for SHED-L and SHED-H in diabetic rats was observed at this stage (Figure 6.5).

6.3.5 IHC analysis for diabetic wound healing model

Heat-shock proteins (HSPs) are highly conserved family of proteins and among the most copious intracellular proteins (Benjamin & McMillan, 1998). Hsp70 accelerates wound healing by upregulating macrophage-mediated phagocytosis (Kovalchin et al., 2006; Atalay et al., 2009). Higher levels of HSP70 were observed in the SHED treated group compared to the vehicle control group. The brown staining indicated the elevation of Hsp70 protein expression (Figure 6.6).

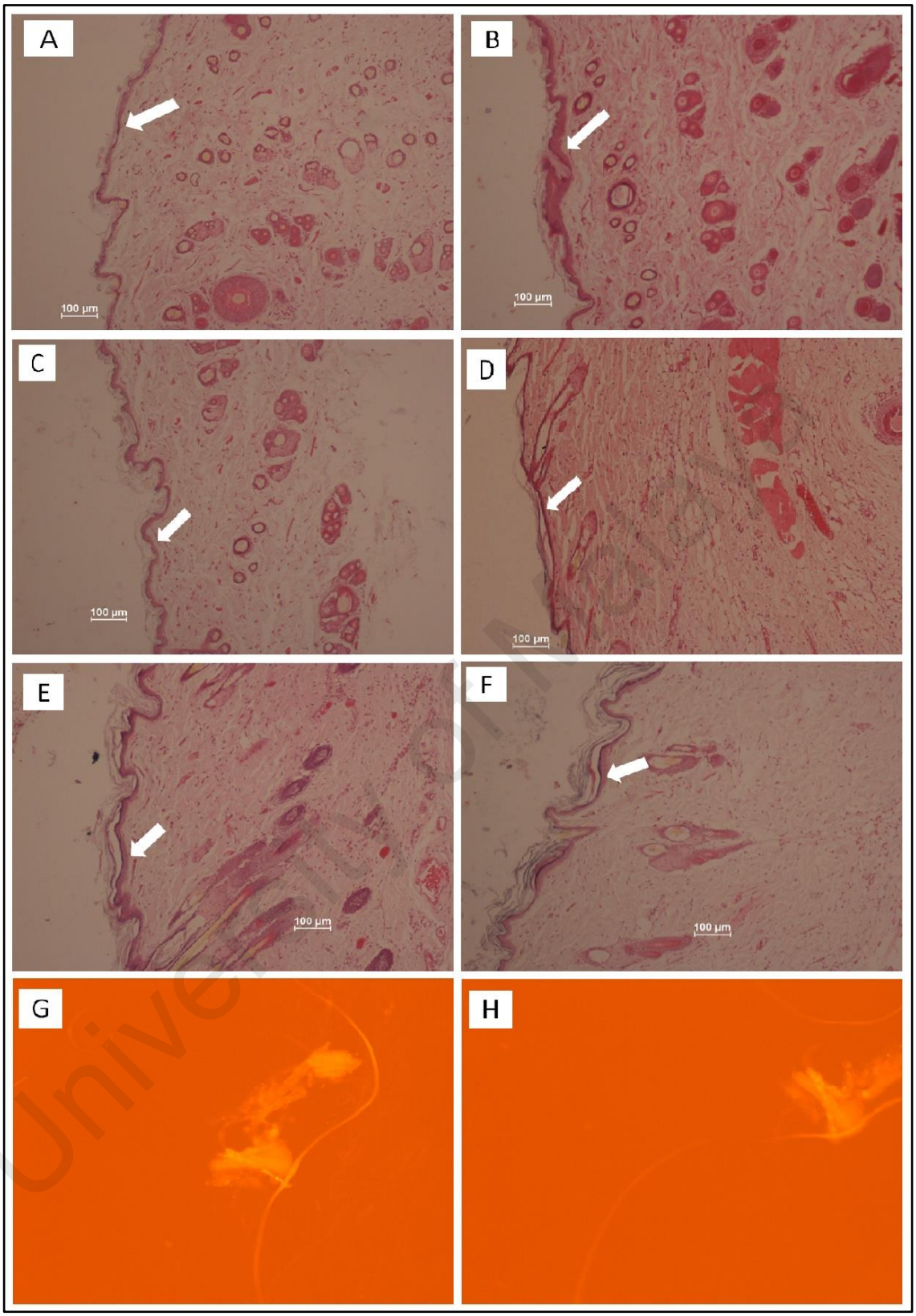


Figure 6.5: Effect of SHED on skin histology of diabetic rats. A: G1: normal non-diabetic rats; B: G2: vehicle group; C: G3: SHED-L treatment with $0.5x10^6$ cell/ml; D: G4: SHED-H treatment with $1.0x10^6$ cells/ml; E: G5: topical application of intrasite gel treated group; and F: G6: topical application of intrasite treated with Gli. The white arrows illustrate the epidermal layer for each group. G: SHED-L pkh-26 tagged and H: SHED-H pkh-26 tagged. Scale bar: 100 µm. The H&E stained skin tissues. Microscopic resolution: 100µm.

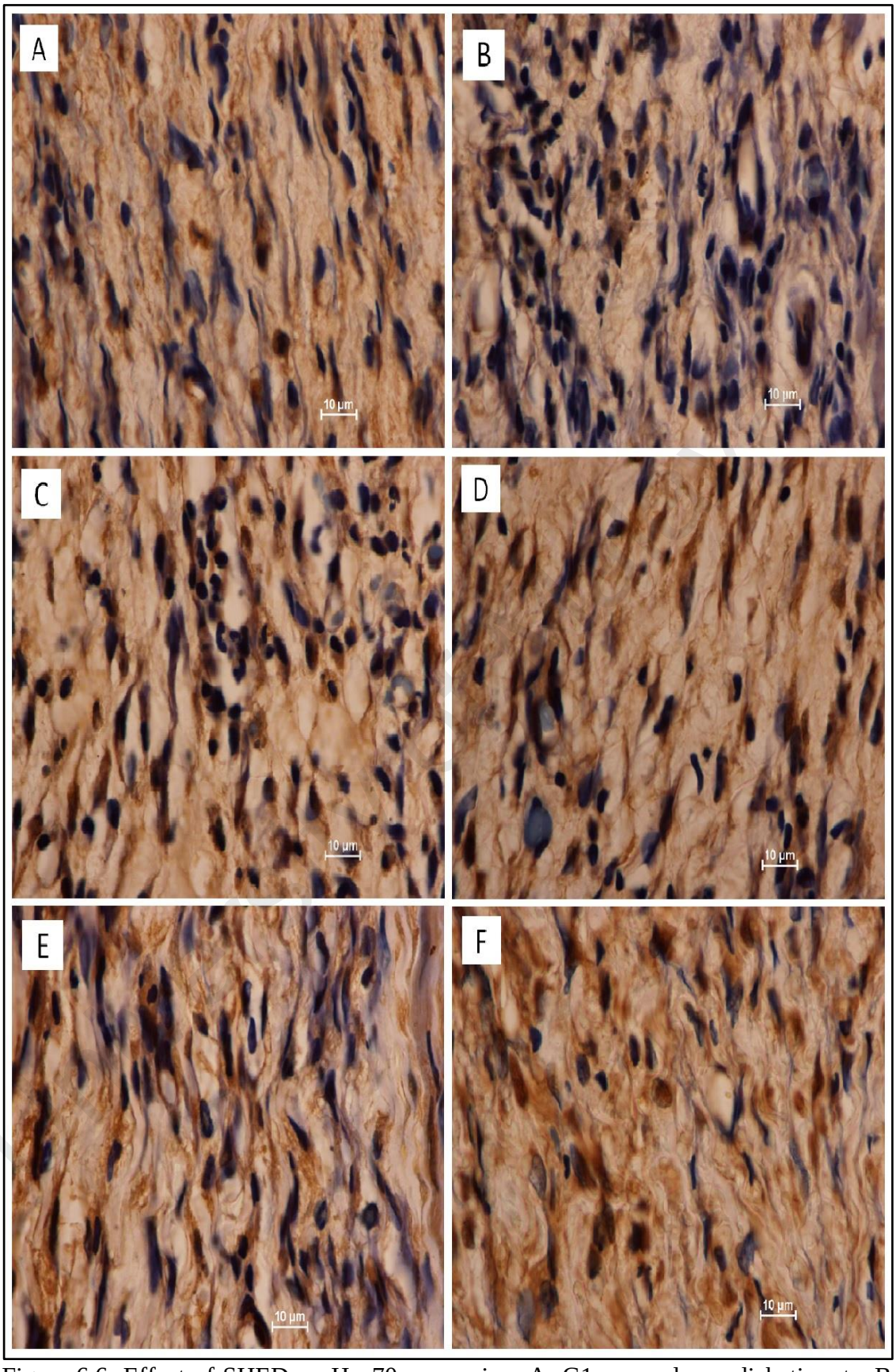


Figure 6.6: Effect of SHED on Hsp70 expression. A: G1: normal non-diabetic rats, B: G2: vehicle group; C: G3: SHED-L treatment with $0.5x10^6$ cell/ml, D: G4: SHED-H treatment with $1.0x10^6$ cells/ml, E: G5: topical application of intrasite gel treated group, F: G6: topical application of intrasite treated with Gli. The immunostaining of skin tissue. Microscopic resolution: 10µm.

6.3.6 Biochemical analysis for oxidative stress markers

The oxidative markers are used for testing oxidative damage and indicate that SHED treatment reduced such damage. The results of the enzyme tested are shown below:

I. SOD level

The changes in SOD activity of the wound tissue homogenate of the rats after the administration of SHED are shown in Figure 6.7. The SOD level shows a significance difference of $P< 0.05$ for G3 (SHED-L) and G4 (SHED-H). The treatment appears to be a protective mechanism to promote the wound healing process. The SHED showed a more protective effect against oxidative damage than the vehicle and the topically applied intrasite gel groups.

II. GSH level

The GSH level in rats was much lower in the SHED treated group compared to the normal rats. The rats in the G5 intrasite treated group had the lowest GSH activity with $P< 0.05$ indicating that the oxidative stress is higher in this group compared to the negative control group (Figure 6.8).

III. MDA level

The lipid peroxidation was measured using MDA level in wound tissue homogenate, and the treatment of SHED showed lower lipid peroxidation compared to the intrasite-treated rats and the vehicle groups with no significance difference (Figure 6.9).

IV. AOPP level

The AOPP level indicates the toxic level of wound tissue and the AOPP content showed that the high-dose SHED treatment group contained lower AOPP amounts compared to the vehicle group with a significance difference of $P < 0.05$. The other groups including the intrasite and SHED low dose group reported lower AOPP content indicating lesser oxidative damage compared to the vehicle group (Figure 6.10).

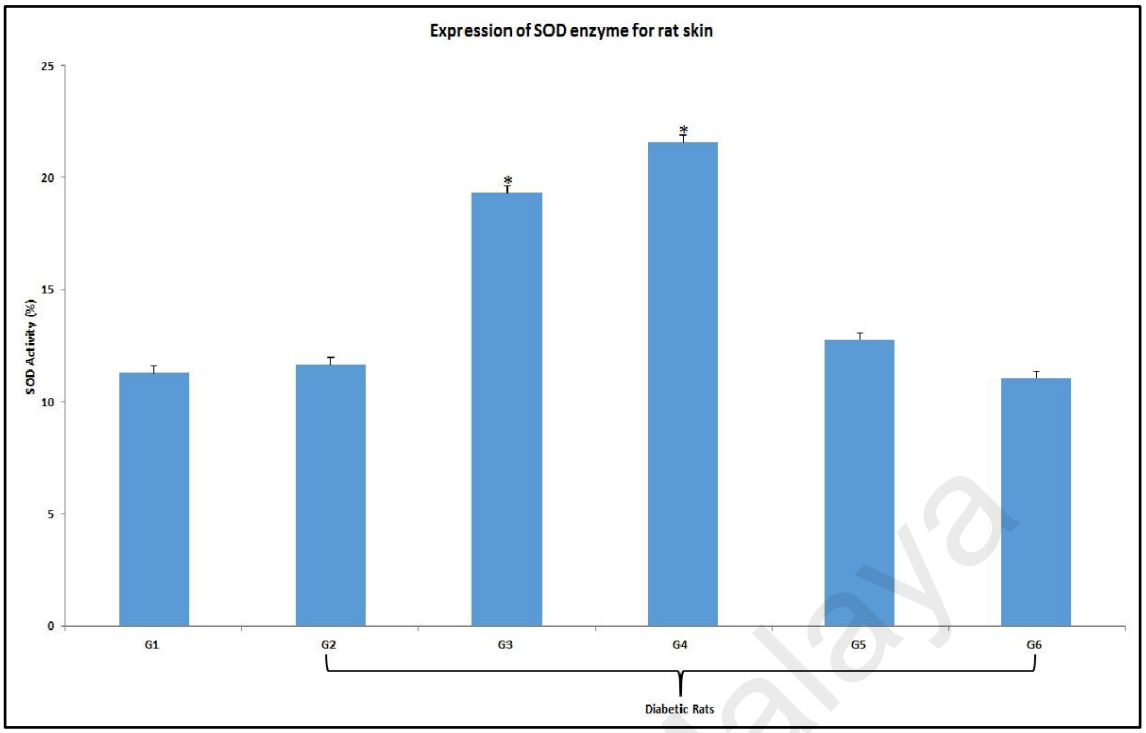


Figure 6.7: Effect of SHED treatment on SOD level in wound tissue. The groups include (G1) negative control, (G2) vehicle control, (G3) SHED-L, (G4) SHED-H, (G5) Intrasite application, and (G6) Intrasite topical application with orally treated Gli. A value of * $P < 0.05$ was considered significant.

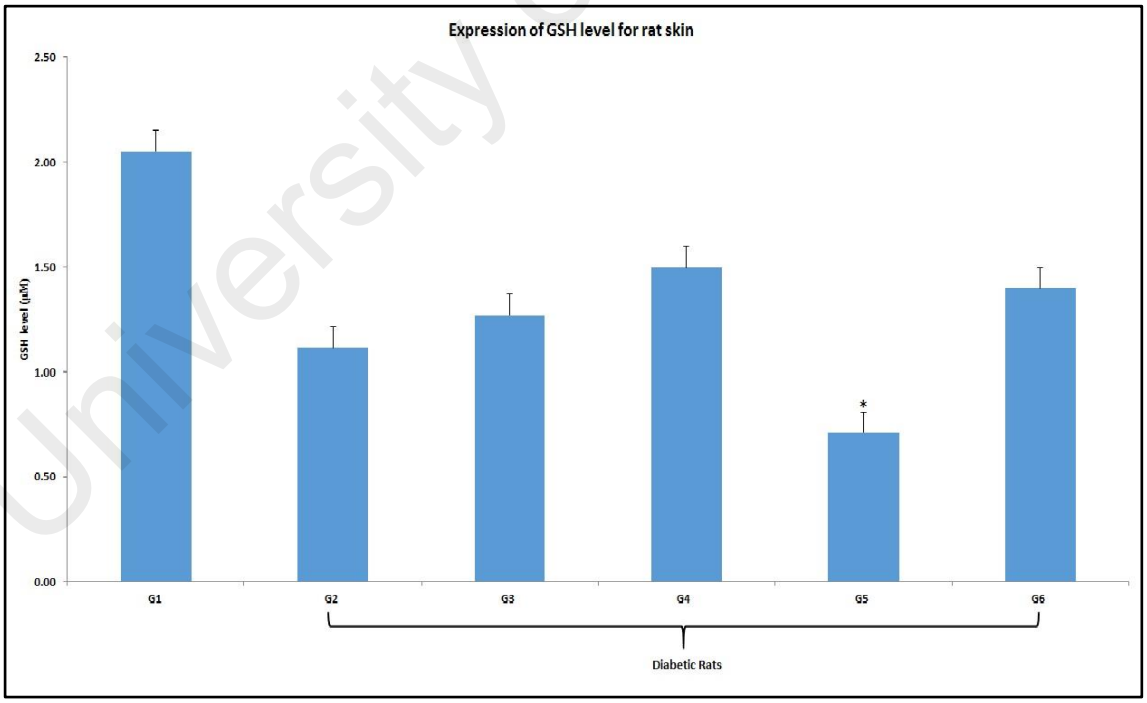


Figure 6.8: Effect of SHED treatment on GSH level in wound tissue. The groups include (G1) negative control, (G2) vehicle control, (G3) SHED-L, (G4) SHED-H, (G5) Intrasite application, and (G6) Intrasite application with orally treated Gli. A value of * $P < 0.05$ was considered significant.

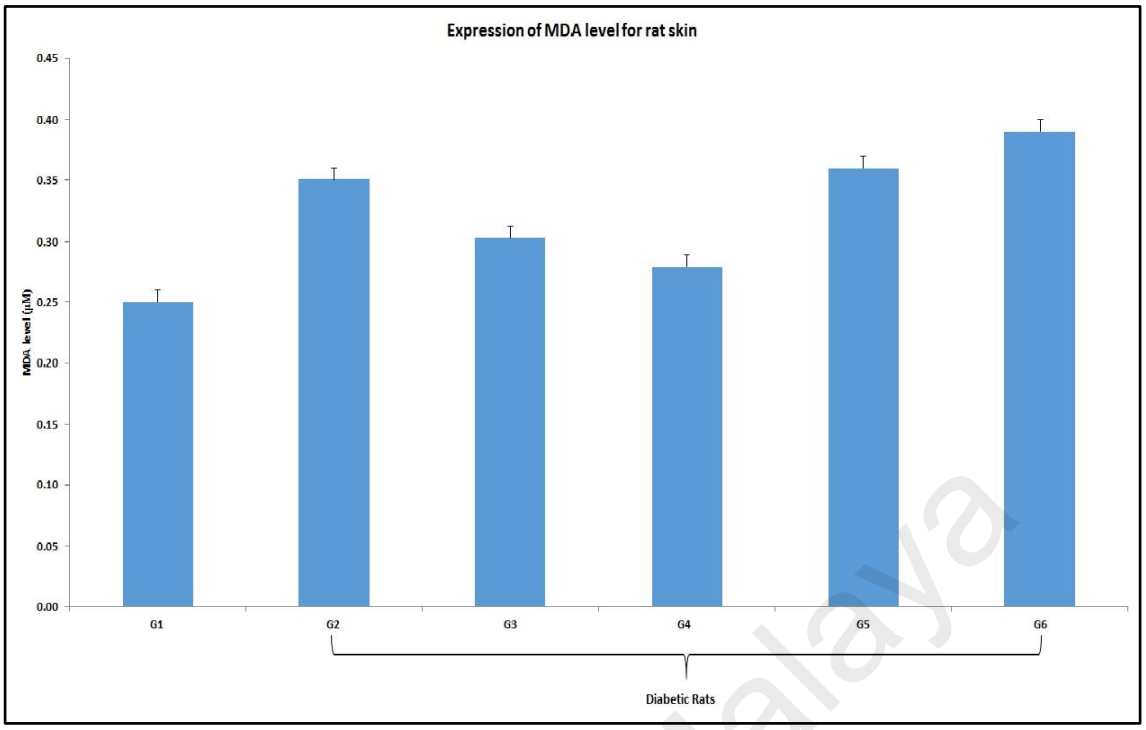


Figure 6.9: Effect of SHED treatment on MDA level in wound tissue. The groups include (G1) negative control, (G2) vehicle control, (G3) SHED-L, (G4) SHED-H, (G5) Intrasite application, and (G6) Intrasite application with orally treated Gli.

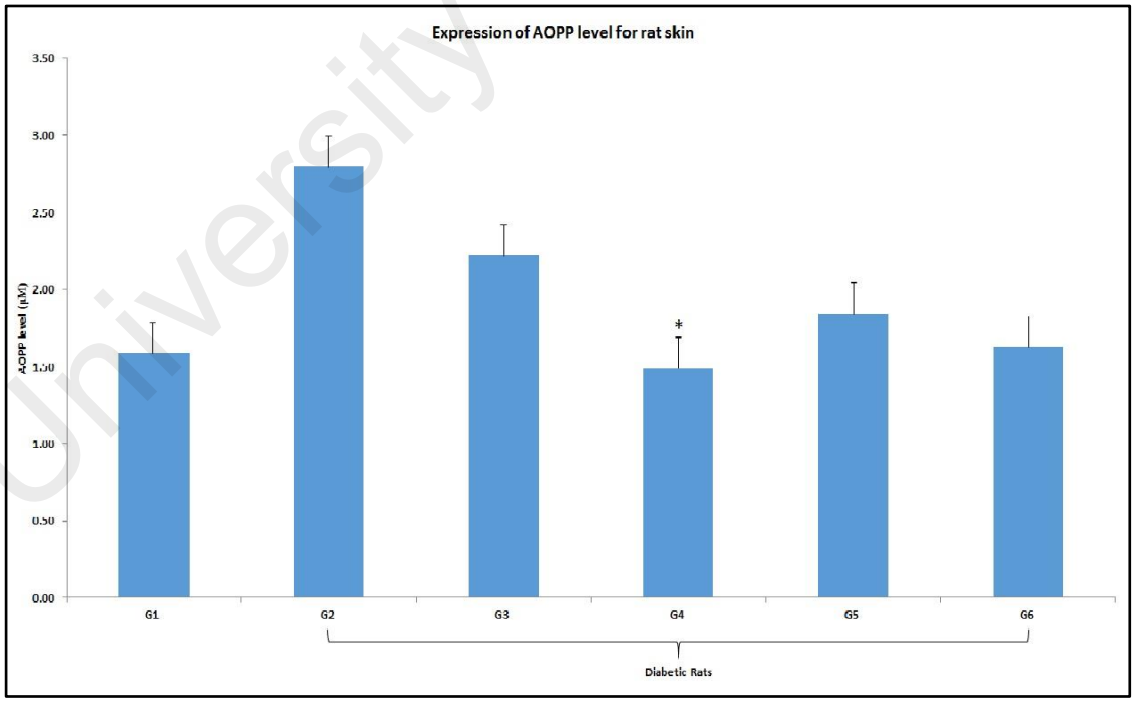


Figure 6.10: Effect of SHED treatment on AOPP level in wound tissue homogenate from six animal groups. The groups include (G1) negative control, (G2) vehicle control, (G3) SHED-L, (G4) SHED-H, (G5) Intrasite application, and (G6) Intrasite application with orally treated Gli. A value of $P< 0.05$ was considered significant. A value of * $P< 0.05$ was considered significant.

6.3.7 Hydroxyproline accumulation in rats' skin

The hydroxyproline accumulation in rats' skin is important for determining the wound recovery of the rats. The amount of hydroxyproline deposition in skin wound homogenate showed the highest deposition in group G3 (SHED-L) and G4 (SHED-H) when compared to vehicle and intrasite group with significance difference $P< 0.05$ (Figure 6.11).

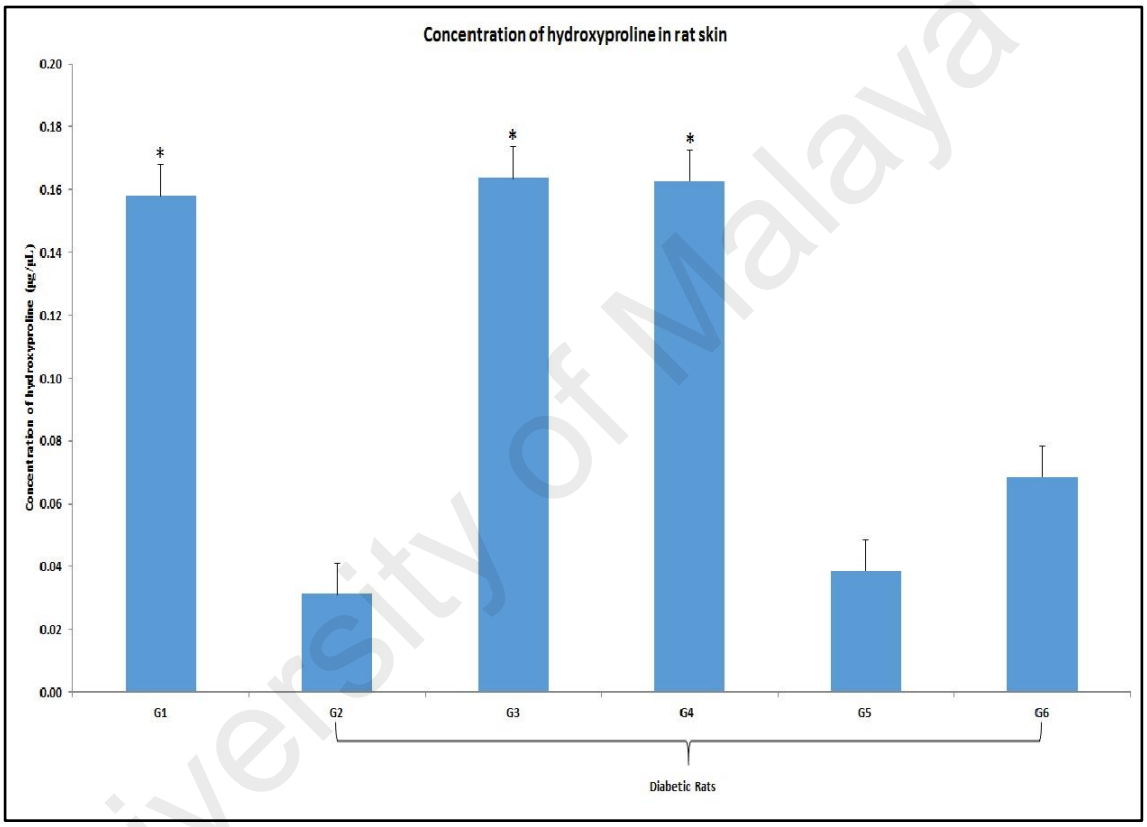


Figure 6.11: Effect of SHED treatment on hydroxyproline level in wound tissue homogenate from six animal groups. The groups include (G1) negative control, (G2) vehicle control, (G3) low dose SHED, (G4) high dose of SHED, (G5) Intrasite application, and (G6) Intrasite application with orally treated Gli. A value of * $P< 0.05$ was considered significant.

6.4 Discussion

Dental pulp stem cells have been attracting attention as a novel stem cell source in recent years (Nishino et al., 2011). The toxicity test for SHED injected intravenously in rats suggests that it is safe to use for transplantation. Nishino et al. (2011) used the 5 x 10^6 million of cells transplanted as a topical application around the wound. Nonetheless, our previous study noted that such cells are well known as having the ability to express a variety of wound healing markers namely IL6, IL10, VEGF, and TGFβ (Jayaraman et al., 2016). Previous studies show that SHED has promising wound healing capabilities. A study revealed that cell transplantation of SHED combined with human fibroblast significantly promoted wound healing in nude mice after 14 days (Nishino et al., 2011). In another study, dental pulp stem cells and their conditioned medium demonstrated capable wound healing effects on seven-week-old nude mice through the angiogenesis mechanism (Yang et al., 2013). When compared with our results, the SHED in low serum culture conditions enhanced wound healing in STZ-induced diabetic rats.

Wound healing studies generally evaluate skin defects created through excisional wound models (Rouhollahi et al., 2015; Moghadamtousi et al., 2015). Seeing the promising wound healing activity of dental pulp stem cells, we created a similar wound healing model. However, a punch biopsy of 6mm was used to achieve a standardized wound defect and closure in this study.

A macroscopical examination indicated that wound closure was enhanced in the SHED-L and SHED-H groups and show a marked wound healing closure rate at day 10 compared to the vehicle control group as well as the two positive control groups of topical application intrasite gelonly and the intrasitegel with Gli. A study by Nishino et al. (2011) also reported enhanced wound closure after treatment by SHED ($5x10^6$ cell/ml) co-cultured with 100ug/ml of bFGF in mice.

Histological analysis showed that the SHED-L (0.5 x 10^6 cells per ml) and SHED-H (1 x 10^6 cells per ml) treated groups had markedly accelerated original tissue regeneration in establishing a thinner epidermal layer thus providing protection against further injury. The granulation tissue contained more collagen fiber accumulation in SHED-L and SHED-H groups compared to the non-treated groups. This supports a study of excisional wounds reported that tissue sections of mice had enhanced wound closure and accumulated Type1 collagen in the SHED treated group (Nishino et al. 2011). Intradermal dental pulp transplantation promoted wound healing in the proliferative phase by stimulating the proliferation and angiogenesis process (Yang et al., 2013).

The HSP 70 has a pivotal role in the wound-healing process through attenuation of the inflammatory responses. And also provides a tissues defense mechanism against injuries by preserving synthesis and conformation of proteins, repairing damaged proteins and enhancing the healing process (Lamore et al., 2010). It was previously established that Hsp70 is the most abundant inducible HSP in the wound bed, therefore its protein expression was determined in this study using immunohistochemistry analysis (Wagstaff et al., 2007). Mosser et al. (1997) suggested that Hsp70 is able to prevent the effector steps of apoptotic cell death. It is known to have resistance to stress-induced apoptosis in which the expression of hsp70 was constitutively elevated.

Wound debridement in diabetics is known to undergo an ischemic condition which promotes generation of reactive oxygen species triggered by inflammatory cells around the injured tissue. The increase in oxygen-derived free radicals in the wound area causes more oxidative damage to the cells, which will lead to delayed wound healing (Bickers & Athar, 2006). In a normal person, the free radicals will be balanced by the body's homeostasis system through the person's endogenous antioxidant capacity. But in

uncontrolled diabetics, the high glucose mediates the production of ROS and oxidative stress that counterparts changes in cell function. The increased ROS disrupts the regulation of oxidative stressors that lead to ROS-induced injuries in diabetic wounds (James & Murphy, 2002).

Cells have evolved an elaborate system of enzymatic and non-enzymatic antioxidants which help to scavenge these indigenously generated ROS. Several enzymes involved in ROS-scavenging have been investigated to understand the role of antioxidant systems. These enzymatic and non-enzymatic antioxidants are useful in promoting environmental stress tolerance (Ahmad et al., 2010). We also examined enzymatic antioxidants namely superoxide dismutase (SOD), total glutathione (GSH), and non-enzymatic antioxidants such as malondialdehyde (MDA) which is a major lipid peroxidation products and advanced oxidation protein products (AOPP).

The superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are types of ROS which are continuously produced during the metabolic process in all living organisms. Nevertheless these disproportionate ROS will lead to cellular injury and, therefore, need to be removed from the cells immediately by antioxidant defense mechanisms. SOD is one of the most important antioxidative enzymes which catalyze the dismutation of O_2^- into $H_2O_2$and O_2 (Lepock et al., 1990; Zelko et al., 2002; Valentine & Hart, 2003). In a previous chapter, chapter 5, the *in vitro* transwell scratch assay reported that SHED demonstrated antioxidant activity in the *in vitro* wound model. When compared to normal glucose fibroblast, the SHED treated group demonstrated the highest SOD antioxidant activity with significance difference of $P< 0.05$. This supports our *in vivo* findings that verified that SOD activity is significantly high in SHED-treated group with $P< 0.05$. Recently, stem cell treatment in wound healing has also revealed that the SOD

implicates significant antioxidant activity condition by protecting the cell from further oxidative damage (Geesala et al., 2016)

Reduced glutathione (GSH) is mostly discovered in living cells. It is an essential antioxidant that helps protect the cells from ROS attacks, maintain exogenous antioxidants such as vitamins C and E, helps peroxide breakdown, and regulates nitric acid cycle. The enzyme glutathione reductase assists in the conversion of GSH from its oxidized form during oxidative stress (Julius et al., 1994; Anderson, 1996; Halliwell, 1999; Mytilineou et al., 2002). In the previous chapter, the GSH level in normal fibroblast was seen to be higher than in the SHED treated group; however the results for GSH in this *in vivo* study indicates that the GSH is higher in the SHED high-dose group compared to the vehicle control and intrasite gel groups with significant difference $P < 0.05$. A different study using bone marrow stem cells for wound healing treatment reported significant GSH antioxidant activity indicating a protective effect in a murine model (Geesala et al. 2016).

Lipid peroxidation is a mechanism that is involved in cellular impairment in living organisms. It produces lipid peroxides which are unstable during oxidative stress in cells and elevates biproducts, for example malondialdehyde (MDA). Therefore an increase in MDA levels is considered high oxidative damage occurrence (Ugochukwu & Cobourne, 2003). When compared to the results presented in the previous chapter, it was suggested that lipid peroxidation increased in high glucose fibroblast than the SHED treated group. In addition our animal study also implicates the same results by revealing high levels of MDA in the vehicle control and the intrasite gel treated groups. This shows that SHED plays an important part in protecting cell injury from lipid peroxidation. Zhang et al. (2014) reported that MDA content decreased in the skin

homogenate after treatment with adipose tissue stem cells in a study on skin damage due to glycation in mice.

The oxidative stress that occurs through the reaction of chlorinated oxidants, such as chloramines and hypochlorous acid, with plasma proteins created uremic toxins is known as advanced oxidation protein products (AOPP). Excessive production of AOPP induces oxdative damage in cells caused by ROS (Witko-Sarsat et al., 1996; Deschamps-Latscha et al., 2004; Servettaz et al., 2007). In our previous chapter, the *in vitro* transwell scratch assay results indicated that high AOPP was produced in high glucose fibroblast compared to the normal glucose fibroblast and SHED. Nevertheless in our *in vivo* study, the AOPP was proven to decrease in the SHED high-dose treated group compared to the vehicle control and intrasite gel groups with significance difference of $P< 0.05$. In another study, the AOPP level was measured to study the ability of novel dairy products in improving tissue repair. The AOPP level was found to decrease in reducing oxidative stress (Kocic et al., 2014).

Hydroxyproline is a major component of collagen which is used to quantitate its content in tissue homogenates. The mechanism is determined by post-translational hydroxylation of proline that produces hydroxyproline. In the *in vivo* hydroxyproline test, the results indicate that the collagen deposition was significantly higher in the SHED group compared to the vehicle control and intrasite gel groups with significance difference of $P< 0.05$. This result suggests that both low and high doses of SHED played an important role in collagen accumulated for the wound repair model. In a study by Xue et al. (2015), type I collagen accumulation with osteocalcin were demonstrated in transfected MSCs as indicated by the high production of hydroxyproline. Another study also showed that increased hydroxyproline levels in

human bone marrow stem cells helps in development of a mature collagen network (Murdoch et al., 2016).

In conclusion, these findings show that SHED is proven to be not hepatotoxic or nephrotoxic upon transplantation on rats. In the *in vivo* diabetic wound model, SHED accelerates wound healing in the maturation phase of healing as indicated by wound contraction, collagen formation (histology), and collagen synthesis (biochemical analyses). Further, it also reduced oxidative damage in the wound area. In addition, lipid peroxidation and AOPP decreased while enzymatic antioxidants, SOD and GSH increased.

6.5 Conclusion

1. The liver and kidney blood profiles indicated no liver and kidney damage in the vehicle control, SHED-L, and SHED-H groups.
2. The histological analysis indicated no damage to the liver and kidney tissues.
4. The macroscopic measurement of wound contraction and the rate of wound closure improved in the SHED-treated group.
5. The skin histology of the rats indicated a thin epidermal layer in the SHED group with collagen fiber accumulation in the maturation stage and no inflammatory cells, and that the wound completely healed at this stage.
6. The immunohistochemical analysis indicated more Hsp70 protein expression in skin wounds treated with SHED compared to the vehicle control group.
7. The oxidative stress activity was assayed and showed increased SOD and GSH activity and lowered MDA and AOPP in the rat skin homogenate.
8. The level of hydroxyproline content in the rats' skin homogenate indicated higher collagen synthesis in the SHED-treated group.

CHAPTER 7: CONCLUSION

Within the limitations of this study the following conclusions can be drawn:

1. The dental derived stem cells, SHED, DPSCs, and PDLSCs showed indistinguishable MSC characteristics. Further, no significant difference was observed in the morphology of all three groups at P2 and P9. Even though a larger amount of immune genes was expressed in P9 as compared to P2, more cellular toxicity markers were detected in P9. This led to the selection of SHED P2 as stem cell source of choice for our wound healing study and SHED also expressed IL12A and VEGF markers which plays important role for wound healing when compared to gene expression by DPSCs and PDLSCs.

2. SHED's morphology, growth kinetics, and senescence level showed no significant difference in the low serum culture conditions in P2 and P4. There was a marked increase of wound healing genes expressed in SHED P2 when cultured in 2% FBS culture conditions compared to SHED P4. Most of the wound repair markers representing the 3 stages of healing were from the SHED P2 2% FBS group.

3. The *in vitro* glucose-challenged scratch assay showed an improved wound healing rate in the SHED-treated group. SHED could also induce the healing rate by increasing the level of antioxidant enzymes (SOD and GSH) and reducing lipid peroxidation (MDA) and protein oxidation (AOPP).

4. The acute toxicity test on an animal model showed no damage to the liver and kidney upon intravenous transplantation. The pre-clinical usage of SHED in the diabetic wound model reported an enhanced wound healing rate with thinner

epidermal layer. The results signify that SHED is a good candidate for diabetic wound healing by enhancing the over-expression of Hsp70 markers in the SHED-treated group. Moreover, SHED reduced the oxidative stress condition by improving SOD and GSH activity as they are scavengers of ROS and decreases the MDA and AOPP level for enhanced healing. The level of hydroxyproline also increased in the SHED-treated group. SHED also induced collagen deposition and less inflammatory cells in the granulation tissue at the wound site.

Study limitations

The limitations of the study are as follows:

1. Developing a precise *in vitro* wound healing model was a challenge as the wound gaps had to be equalised for all test groups. However, we managed to secure the average for more accurate results.

2. Creating the animal model was challenging in terms of detecting the wound proliferation stage because of the tiny wound size. We only managed to study the wound at the maturation stage.

Future studies

Suggestions for future studies are as follows:

1. A better *in vitro* wound healing study using a digital video capturing format would be useful for observation of the co-culture cells on wound closure.

2. A larger wound area would provide a better understanding of the pro-inflammatory, proliferation, and maturation stages of healing on the animal wound healing model.

3. Other immunohistochemistry (IHC) markers such as PCNA and TGFβ markers can be used to study the skin wound healing.

4. Further studies on the paracrine signaling pathways involved in wound healing mechanism is important.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Jayaraman, P., Nathan, P., Vasanthan, P., Musa, S., & Govindasamy, V. (2013). Stem cells conditioned medium: a new approach to skin wound healing management. *Cell Biology International, 37*(10), 1122-1128.
2. Jayaraman, P., Govindasamy, V., Gnanasegaran, N., Kunasekaran, W., Vasanthan, P., Musa, S., & Kasim, N. H. (2016). Expression patterns of immune genes in long-term cultured dental stem cells. *Clinical Oral Investigations,* 20(1), 109-116.
3. Jayaraman, P., Govindasamy, V., Musa, S., Kasim, N. H. (2015). The role of nutrient supply on dental pulp stem cells growth. *International Society of Stem Cell Research (ISSCR) in Stockholm, Sweden.* (Conference Presentation).
4. Jayaraman, P., Abdulla M. A., Musa, S., Kasim, N. H. (2016). The potential of wound healing on experimental STZ-induced diabetic rats treated with extracted human dental pulp stem cells (SHED). *Stem Cell Society Singapore (SCSS) in Biopolis, Singapore.* (Conference Presentation).

Articles in progress for submission:

i. Title: Low serum culture conditions enhances wound repair genes in human exfoliated dental pulp stem cells

ii. Title: Exfoliated dental pulp stem cells (SHED) ameliorate experimental STZ- induced diabetic wound model in Sprague Dawley rats