Wound Healing Potential of Human Neonatal Mesenchymal Cells in an Animal Model of Hyperglycemia

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
Faculty of Dentistry
University of Toronto

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Abstract

Potential targeting of impaired wounds resulting from hyperglycemia using mesenchymal stem cells is a promising approach. We hypothesized that when administered to dermal wounds in hyperglycemic subjects, neonatal mesenchymal stem cells (MSCs) would be more effective than adult mesenchymal cells in accelerating healing. We examined the in vitro impact of various glucose conditions on proliferation and senescence of human umbilical cord perivascular cells (HUCPVCs) and adult bone marrow MSCs (hBM-MSCs). We also investigated the healing potential of both cells in dermal wounds of streptozotocin-induced NOD-seid-gamma (NSG) mice utilizing planimetry and histomorphometry. HUCPVCs showed higher proliferation under normal and hyperglycemic conditions and lower senescence under all conditions compared to hBM-MSCs. Wound closure was better in treated wounds compared to untreated ones. Disease tolerance varied among mice which affected healing. HUCPVCs still holds a potential over adult MSCs for impaired wounds; yet more studies are needed to recognize their bona fide capacity.
“And whoever fears Allah, He will make a way out for him (from any difficulty). And He will provide him sustenance from where he does not expect.”

Excerpt from Al-Qur’an: Surat Al-Talaq; 2-3.

“It is not the critic who counts; . . . the credit belongs to the man who is actually in the arena, . . . who strives valiantly; who errs, who comes short again and again, because there is no effort without error and shortcoming; but who does actually strive to do the deeds; . . . who spends himself in a worthy cause; who at the best knows in the end the triumph of high achievement, and who at the worst, if he fails, at least fails while daring greatly; so that his place shall never be with those . . . who neither know victory nor defeat.”

Theodore Roosevelt
Acknowledgments

First and foremost, I must thank the Almighty Allah for granting me strength, patience, and perseverance to overcome some health- and work-related challenges encountered and to make the completion of this humble work and writing up this thesis a possible reality. I must also give sincere thanks to my family for their continuous prayers, blessings, and encouragement despite the long distance.

Undeniably, living such an experience would not have been possible without the privilege that Professor Davies had given me. JED, I am indebted to your help, guidance, and deep understanding. I appreciate the time you dedicated to observe my surgeries in spite of your busy schedule. It was indeed an honor for me to be my first academic mentor in Canada. Your clear vision and passion for your work is something I greatly admire. Also, I am thankful to the insightful comments, suggestions, and thought-provoking questions provided by my advisory committee members, Drs. B. Hinz and D. Kilkenny.

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Indeed, it has been not only a degree, but also a life- and self-discovery journey which I am deeply thankful for!
Dedication

This work, presented herein, is humbly dedicated to my family

and the soul of my grandmother.

May
### Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>QUOTES</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>vi</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xvi</td>
</tr>
</tbody>
</table>

## CHAPTER 1: INTRODUCTION ........................................................................ 1

### 1.1 Cutaneous wound healing ................................................................. 1

1.1.1 Overview ......................................................................................... 1

1.1.2 Diabetic foot ulcers: Clinical significance ........................................ 2

1.1.3 The biology of dermal wound healing ............................................... 3

1.1.3.A Normal (acute) wound healing ..................................................... 3

1.1.3.A1 Hemostasis ................................................................................. 3

1.1.3.A2 Inflammation ............................................................................. 4

1.1.3.A3 Granulation tissue (GT) formation ............................................ 6

1.1.3.A4 Remodelling ............................................................................. 8

1.1.3.B Impaired wound healing in diabetes ........................................... 9

1.1.3.B1 Cellular impairments ............................................................... 9

1.1.3.B2 Defective extracellular matrix (ECM) ........................................ 11

1.1.3.B3 Signalling factors abnormalities .............................................. 12

1.1.3.B4 Defective neovascularization ................................................... 13

1.1.3.B5 Advanced glycation end-products (AGEs) .................................. 14

### 1.2 Treatment options for diabetic wounds .............................................. 15

1.2.1 Conventional treatment modalities .................................................. 15

1.2.2 Advanced treatment modalities ....................................................... 15

1.2.2.A Growth factors ........................................................................... 15

1.2.2.B Skin substitutes .......................................................................... 16

1.2.2.C Other treatment options ............................................................ 18

1.2.2.D Limitations of current treatment ................................................ 18
1.3 Mesenchymal stem cells (MSCs) for impaired wounds

1.3.1 The perivascular niche

1.3.2 Characteristics of MSCs

1.3.3 Suggested mechanisms of action of MSCs in wound healing

1.3.3A Paracrine effect and immunomodulation

1.3.3B Differentiation/Trans-differentiation

1.3.4 BM-MSCs for impaired wounds

1.3.4.A Preclinical/clinical data

1.3.4.B Limitations of adult MSCs in therapy

1.3.5 Neonatal versus adult mesenchymal stem cells

1.4 Human umbilical cord perivascular cells (HUCPVCs)

1.5 Cell delivery approaches

1.5.1 Hydrogels for in vivo cell delivery

1.5.2 Methylcellulose (MC)

1.6 Animal models of impaired wound healing

1.6.1 Overview

1.6.2 Hyperglycemic animal models

1.6.3 Dermal wounds models

1.6.4 Immunodeficient mice in hyperglycemic wound healing studies

1.6.4.A NSG mice: Strain properties

1.6.4.B Hyperglycemia induction in immunodeficient mouse strains

1.6.4.C WH is impaired in hyperglycemic immunodeficient mouse strains

1.7 Thesis rationale

1.8 Thesis hypothesis and objectives
CHAPTER 2: MATERIALS AND METHODS

2.1 Cells

2.1.1 Source

2.1.2 Culture

2.2 In vitro studies

2.2.1 MSCs proliferation under various glucose culture conditions

2.2.1.A Ki-67 proliferation marker

2.2.1.B Proliferation assay: Experimental design

2.2.1.C Ki-67 Immunocytochemistry/immunofluorescence protocol

2.2.1.D Imaging and data analysis

2.2.2 Mesenchymal cells senescence under various glucose culture conditions

2.2.2.A Senescence Associated-β-galactosidase assay (SA-β-Gal)

2.2.2.B Experimental design

2.2.2.C SA-β-Gal labeling procedure

2.2.2.D Imaging and data analysis

2.3 In vivo study

2.3.1 Ethical approval

2.3.2 Animals

2.3.3 Pilot studies

2.3.3.A Scope

2.3.3.B Developing a hyperglycemic animal model

2.3.3.C Developing a wound healing surgical model

2.3.3.D Evaluating wound healing in normal and STZ-induced NSG mice

2.3.3.D1 Sacrifice and tissue harvest

2.3.3.D2 Skin samples processing for histology

2.3.3.D3 Wound Image analysis

2.3.3.D4 Establish a wound treatment protocol

2.3.4 Main studies

2.3.4.A Experimental outline

2.3.4.B Mice health monitoring

2.3.4.C Sacrifice and tissue harvest

2.3.4.D Skin samples processing for histology

2.3.4.E Wound analysis
2.3.4.E1  Wound image analysis ............................................................. 51
2.3.4.E2  Histomorphometry ................................................................. 51

2.4  Statistical analysis ........................................................................... 53
2.4.1  In vitro studies ............................................................................... 53
2.4.2  In vivo studies ............................................................................... 53

CHAPTER 3: RESULTS ............................................................................. 58
3.1  In vitro studies ................................................................................... 58
  3.1.1  Ki-67 Proliferation index .............................................................. 58
  3.1.2  Senescence: SA-β-Gal expression .................................................. 60

3.2  In vivo studies ..................................................................................... 62
  3.2.1  Behavioral observations in NSG mice ........................................... 62
  3.2.2  Clinical observations in STZ-induced NSG mice ............................ 62
  3.2.3  Random blood glucose (RBG) and body weight profiles ................. 64
         3.2.3.A  Initial observations ............................................................... 64
         3.2.3.B  Main study observations ...................................................... 66
         3.2.3.C  Overall observations .......................................................... 66
  3.2.4  Wound healing (WH) study: Early observations ............................ 69
         3.2.4.A  Healing is delayed in STZ-induced NSG mice ......................... 69
         3.2.4.B  Wound coverage ................................................................. 72
         3.2.4.C  Treatment protocol ............................................................. 73
         3.2.4.D  Wound tissue harvest ......................................................... 74
  3.2.5  Wound healing (WH) results: Main studies .................................... 75
         3.2.5.A  Image analysis results .......................................................... 75
         3.2.5.B  Histologic and histomorphometric observations ...................... 79
                 3.2.5.B1  General observations ................................................. 79
                 3.2.5.B2  Wound histological width ........................................... 80
                 3.2.5.B3  Re-epithelialization .................................................... 81
                 3.2.5.B4  Granulation tissue (GT) formation ............................... 85
CHAPTER 4: DISCUSSION .................................................................................. 89

4.1 In vitro results ........................................................................................................ 89

4.2 In vivo results .......................................................................................................... 92
  4.2.1 Hyperglycemia can be induced in NSG mice ...................................................... 92
  4.2.2 Behavioral observations in NSG mice ................................................................. 94
  4.2.3 Clinical observations in STZ-induced NSG mice ................................................ 95
  4.2.4 Wound healing is compromised after STZ induction .......................................... 95
  4.2.5 No observable effect of cells on wound healing ................................................ 96
    4.2.5.A Review of treatment results in relation to the literature ................................. 96
    4.2.5.B Stress and malnutrition ............................................................................... 97
    4.2.5.C Other possible factors ................................................................................. 99

CONCLUSIONS ...................................................................................................... 102

FUTURE DIRECTIONS ......................................................................................... 103

REFERENCES ........................................................................................................ 105

APPENDICES ......................................................................................................... 122
List of Tables

CHAPTER 1

Table 1.1 Publications summary referencing the pre-clinical use of BM-MSCs in diabetic wound healing animal models. ................................................................. 35

Table 1.2 Publications summary referencing the in vitro/in vivo use of methylcellulose............37

APPENDIX

Table A.1 Adjusted STZ dosage calculation chart.................................................................122

Table B.1 Blood glucose and weight profiles for STZ-induced and non-induced control NSG mice................................................................................................................124

Table C.1 Day 7 wound digital images of healthier and weaker hyperglycemic NSG mice........125
List of Figures

CHAPTER 1

Figure 1.1 The chemical structure of methylcellulose (MC) ................................................................. 29
Figure 1.2 The chemical structure of streptozotocin and D-glucose ......................................................... 31

CHAPTER 2

Figure 2.1 In vivo study timeline ................................................................................................................ 45
Figure 2.2 Wound planimetric analysis ........................................................................................................ 53
Figure 2.3 Schematic illustration of the in vivo study design ......................................................................... 54
Figure 2.4 Summary of the surgical procedure ............................................................................................. 55
Figure 2.5 Wound harvest procedure ........................................................................................................... 56
Figure 2.6 Histomorphometric measurements .............................................................................................. 57

CHAPTER 3

Figure 3.1 Proliferation of HUCPVCs and hBM-MSCs under various glucose conditions ....................... 59
Figure 3.2 Senescence of HUCPVCs and hBM-MSCs under various glucose conditions ....................... 61
Figure 3.3 Differences in the physiological response to hyperglycemia in NSG mice ............................ 63
Figure 3.4 A comparison of blood glucose levels between STZ-induced and non-STZ induced control mice at 2 time-points ........................................................................................................ 65
Figure 3.5 STZ induction success in NSG mice ............................................................................................. 65
Figure 3.6 Blood glucose and body weight profiles for all the STZ-induced and non-STZ induced control mice over a course of 12 days post-induction ............................................................................. 68
Figure 3.7 Wound healing is impaired in hyperglycemic NSG mice ........................................................... 70
Figure 3.8  Differences in skin thickness between hyperglycemic and normal control NSG mice.

Figure 3.9  Histological appearance of dermal wounds of STZ-induced and non-STZ induced control NSG mice at day 7.

Figure 3.10  Dressing appearances in STZ-induced and non-STZ induced control mice.

Figure 3.11  Effect of tissue harvest and processing technique on histological outcome.

Figure 3.12  Distribution trends of wound areas for all treatment groups in hyperglycemic NSG mice.

Figure 3.13  Distribution trends of the percentage decrease in wound size for all treatment groups in hyperglycemic NSG mice.

Figure 3.14  Dressed versus exposed wounds.

Figure 3.15  Wound width of all treatment groups in hyperglycemic NSG mice.

Figure 3.16  Full epithelialization is seen in exposed wounds of different types (Pilot studies).

Figure 3.17  Morphological comparison of the neo-epithelial layer in all groups in healthier and weaker hyperglycemic mice.

Figure 3.18  Neo-epithelium thickness, length, and re-epithelialization % for all treatment groups in hyperglycemic NSG mice.

Figure 3.19  Granulation tissue formation.

Figure 3.20  Wound bed thickness of all treatment groups in hyperglycemic NSG mice.

Figure 3.21  Representative micrographs of the center of the wound bed of all treatment groups in healthier and weaker hyperglycemic NSG mice.

Figure 3.22  Fat connective tissue in the wound bed of some samples.
List of Appendices

Appendix A: Streptozotocin (STZ) dosing chart.................................................................122

Appendix B: Glucose and weight measurements.............................................................124

Appendix C: Wound digital images profile.................................................................125

Appendix D: Protocols used.........................................................................................126

D.1 High-dose STZ induction protocol for mice.............................................................126

D.2 Ki-67 staining protocol (ICC-IF) ........................................................................128

D.3 Skin processing protocol for histological analysis .............................................130
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>GFs</td>
<td>Growth Factors</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GT</td>
<td>Granulation Tissue</td>
</tr>
<tr>
<td>hBM-MSCs</td>
<td>Human Bone Marrow Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>HG</td>
<td>Hyperglycemia</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HUCPVCs</td>
<td>Human Umbilical Cord Perivascular Cells</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like Growth Factor</td>
</tr>
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<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte Growth Factor</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>MSCs</td>
<td>Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>NOD</td>
<td>Non Obese Diabetes</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD-scid-gamma</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>RBG</td>
<td>Random Blood Glucose</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>SM</td>
<td>Supplemented Medium</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WH</td>
<td>Wound Healing</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1 Cutaneous wound healing

1.1.1 Overview

Humans and other mammals are endowed with a natural repair mechanism, when injury of any kind happens, known as wound healing (WH). The healing concept is not new; in fact, it is well documented in historical records dating thousands of years ago [1]. The present understanding, however, of this physiological phenomenon and its components has evolved, although still incomplete, through assimilating the extensive research data generated over the years, especially, from the field of dermal wound healing [2, 3]. Currently, it is evident that injury would initiate progressive events at the affected site which are highly regulated, both cellularly and molecularly, to heal the damage. Tissue response, however, may vary depending on the host intrinsic factors, environmental factors, and the nature of injury itself [4, 5]. In general, 4 healing outcomes can be recognized: regenerative, acute, excessive, and chronic.

Regenerative healing, as the name indicates, results in perfect replacement of the injured tissue, both anatomically and functionally. It is usually seen in human fetus (early in gestation) and some organisms such as salamanders, planarians, and crabs. This regenerative ability is lost in human adults except for the liver, epidermis, and, to a certain extent, nerves. Acute healing is what humans experience starting at the third trimester of pregnancy and throughout their adult life which results in the formation of a scar tissue that is less morphologically and functionally similar to the original, i.e. repair not regeneration [2, 4]. In some cases, however, excessive healing might occur when regulatory signals, responsible for terminating the healing process, are lost through a mechanism which is, yet, not well understood. This is commonly manifested in either excessive deposition of the extracellular matrix (ECM) proteins by resident fibroblasts, resulting in the formation of fibrotic scars such as keloids and hypertrophic scars or in excessive tissue contraction resulting in the formation of contractures such as those seen in burn cases [4, 5, 6]. On the other hand, impaired healing may arise when disruption occurs early in the healing process.
Non-healing dermal wounds are usually manifested as chronic ulcers which can be broadly grouped into four major categories: diabetic ulcers, pressure sores, venous ulcers, and ischemic ulcers. Determining the exact causative agent(s) for different types of ulcers remains a complex issue in the medical practice. However, the accumulated preclinical and clinical observations have been used to set general criteria to characterize such wounds which include: the pathological profile of the host and the presence of complicating factors such as age, infection, hypoxia, pressure, medication, and nutritional status [7]. Ulcers due to metabolic disorders are very common. Diabetic foot ulcers still stand as one of the serious clinical complications of diabetes. This condition is discussed in more details in the next section.

1.1.2 Diabetic foot ulcers: Clinical significance

A recent release by the International Diabetes Federation (IDF) indicates that by 2030, 1 in every 10 adults in North America will have diabetes, a pancreatic disease affecting glucose homeostasis [8]. In Canada, there are more than 2.3 million people with diabetes. Among those cases, 90% have type II, insufficient or resistant insulin action, while type I, deficient insulin production, accounts for only 9%, and the rest have a different type of diabetes that is either gestational, drug, or surgery related [9, 10, 11]. The reported figures, however, may underestimate the scope of this clinical problem as current statistical surveys mainly involve diagnosed and/or treated cases but not undiagnosed or misreported. The extent of this problem may also include ignored cases of high-risk ethnical/racial groups and those who live in remote areas [8, 12].

Diabetes is known to be associated with a wide range of complications, of micro- and macrovascular nature, that have devastating consequences; among which is developing foot ulcerations, a group of foot pathologies that affect no less than 15% of the diabetic population, regardless of type. The clinical significance of this problem is well recognized as it accounts for about 85% of all lower leg amputations. According to recent Canadian reports, life expectancy has not been found to be greatly enhanced after amputation, a supposedly life-saving measure, as survival rates ranged between only 1 year (30%) and 5 years (69%) [9, 10]. Definitely, the psychological and financial impact of having foot ulcerations or even amputations cannot be ignored.

Ulcers usually develop as a result of multiple systemic and local factors. Systemic factors may include: peripheral arterial disease (PAD), uncontrolled hyperglycemia and its metabolic consequences, and age. Locally, peripheral neuropathy, injury, infections, persistent pressures, and foot deformities may contribute to the condition [7, 13, 10, 14]. In diabetic ulcers, the degree of interaction of these risk factors is not uniform; two main types are usually recognized: neuropathic
and neuroischaemic, which vary considerably, clinically and histologically [13, 10, 15].

The literature is replete with experimental evidence on the presence of various abnormalities in the cells and mediators important for normal healing in such ulcers. The next few paragraphs are dedicated to describe the essential aspects of normal healing and to further address the reported abnormalities in diabetic wounds, from an experimental and clinical perspective.

1.1.3 The biology of dermal wound healing

A cross-talk between local cells and between cells and their surrounding (both matrix and mediators) simply summarizes the healing process which is ultimately governed by stimulatory and inhibitory signals of extreme regulatory nature. Despite the overlapped nature of the process, distinct events can be recognized following injury until complete healing is attained. A 4-phase division criterion has been commonly used in the majority of scientific materials which include: (1) Hemostasis, (2) Inflammation, (3) Granulation tissue formation, (4) Remodelling. The same division will, therefore, be adopted herein and a description of the main events of each phase under normal conditions and the major impairments observed in chronic wounds will be presented in the forthcoming paragraphs.

1.1.3.A Normal (acute) wound healing

1.1.3.A1 Hemostasis

Immediately after injury, blood vessels constrict to stop haemorrhage, a process which is locally governed by thromboxane, secreted by the endothelium, and systemically by epinephrine, released by the adrenal glands [5, 16]. Under normal conditions, the endothelium usually secretes factors to keep circulating blood cells dormant such as, nitric oxide (NO) and prostaglandin I2 (PGI2) [17]; however, in response to vascular injury, the production of coagulation and aggregation activators [e.g. von Willebrand factor (vWF), fibronectin, fibrinogen, and tissue thromboplastin] by the endothelium will be activated. Such factors in addition to the exposed proteins from the vascular basement membrane (e.g. collagen) will activate platelets through respective receptors to form a “platelet plug”. The aggregation is also enhanced by factors secreted from activated platelets themselves, particularly from the dense granules, such as adenosine diphosphate (ADP) and calcium [5, 16].

Constricted vessels and the emerged plug are probably adequate to control bleeding in small vascular injuries; however, a fibrin clot (comprising platelets, other blood cells, and fibrin) is usually formed in large defects [5, 16, 18]. Fibrin is the end product of a series of coagulatory events that are linked
to the platelets plasma membrane. During these events, pro-thrombin is converted to thrombin which in turn converts the monomeric soluble fibrinogen into insoluble fibrin [5, 16]. The action of thrombin, which is also recognized as a platelet activating factor, will also result in the formation of two short fibrinopeptides (FpA and FpB) of chemotactic nature important in later steps [7, 19]. The interaction of platelets with fibrin through GPIIβ–IIIα integrin receptor has been shown to be important for early healing events as the removal of the fibrin matrix have been shown to impede repair [23].

Moreover, the cytoplasmic α–granules of platelets have been shown to contain a wide range of proteins [20]. Active secretion of these factors has been reported to begin within 10 minutes of clotting and reaches around 95% by 1 hour [18]. Coppinger et al. [21] identified, through proteomic means, more than 300 different proteins in human platelet releasate upon activation with thrombin. The pre-stored proteins are highly variable in function and specificity; for instance, some have a coagulatory nature (e.g. factor V, fibrinogen, vitronectin, fibronectin, thrombospondin, and vWF), while others promote cell proliferation [e.g. platelet-derived growth factor (PDGF), transforming growth factor (TGF-β1 and 2), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factor (IGF)], cell adhesion (e.g. platelet endothelial cell adhesion molecule (PECAM), inflammation [e.g. interleukin 1 (IL-1)], or act as heparin antagonist [e.g. platelet factor 4 (PF4)] [16, 21, 22]. These factors have been shown to act not only on platelets themselves (autocrine), but also other cell types in later stages of healing (paracrine) [21].

1.1.3.A2 Inflammation

Constricted vessels in response to vaso-permeable factors, such as histamine, will dilate permitting inflammatory cells and other plasma proteins including by-products of the coagulation events (e.g. bradykinin) and complement activation events (e.g. C3a and C5a) to reach the wound site, i.e. edema formation. In response to these events, the body will experience a classical clinical signs of [Latin: calor (heat), rubor (redness), dolor (pain), tumor (edema), and functio laesa (loss of function)] previously described by J. Hunter in 1797 [2, 5, 23, 24]. Leukocyte extravasation is a multistep process that is facilitated by the expression of adhesion molecules on the surface of the endothelium. Selectins, light adhesion molecules, are the first to be expressed followed by integrins to provide a stronger adhesion enabling the cells to crawl and emigrate (diapedesis) extravascularly [3, 7, 25]. Such cell-cell interaction is pivotal for cell movement that if defective or dysregulated, multiple pathological conditions can arise, reviewed by McIntyre et. al., 2003 [26].
Polymorphonuclear neutrophils (PMN) are the early inflammatory cells attracted to the wound site, within the first 24 hours post-injury. These cells mainly function to fight infection and to remove cell and matrix debris via the production of various enzymes such as, myeloperoxidases and lysozyme (antimicrobial effect), Cathepsin B and D (acid hydrolases), phospholipase A$_2$ (Lipid hydrolase), collagenase, elastase, and plasminogen activator (proteases) [2, 3, 4, 23, 27, 28]. As such, the number of infiltrating neutrophils has been found to be higher in highly contaminated and infected wounds compared to cleaner ones reflecting their inherent functionality as wound cleansing means [5]. Notably, apoptosis and efferocytosis (macrophage-mediated phagocytosis of apoptotic cells) are important processes controlling the release of these enzymes as oxidative stress and tissue degradation might occur the release is excessive or uncontrolled [29, 30].

Later in the inflammatory phase, about 48–72 hours post-injury, circulating monocytes are attracted [2, 4, 5, 23] by early factors of the coagulation, platelet degranulation, and complement activation events, and later in response to locally produced chemotactic signals [30]. Binding of monocytes to specific extracellular matrix (ECM) proteins via their integrin receptor stimulates their differentiation into macrophages [27]. Like neutrophils, macrophages are also recognized for their phagocytic ability in removing dead host cells, damaged matrix, and foreign debris from the wound site [4, 66]. They are also known to secrete multiple cytokines, growth factors, and proteases and their inhibitors that are critical for later steps [5, 30]. Over 20 different cytokines and growth factors are known to be secreted by macrophages [5]; most importantly are: CSF-1, TNF-α, PDGF, TGF-β, IGF-1, bFGF, and VEGF [4, 23, 27, 30]. Recent in vitro reports indicated that these cells can serve as pro- and non-inflammatory cells [30], an observation which was corroborated by an in vivo observation made by Lucas et. al., 2010 [31] where they found that depleting macrophages at various time-points of healing resulted in different outcomes reflecting that they may acquire different functional phenotypes at various healing phases.

Although the role of inflammatory cells in healing is well documented, fetal wound healing studies (1st and 2nd trimesters) indicate the opposite as there is a minimal or absent inflammatory response in such wounds [30, 32]. Several recent studies using mice genetically deficient in specific immune cells and molecules also support the concept that inflammatory cells are not needed for efficient tissue repair, as long as microbial contamination is controlled [30].
1.1.3.A3 Granulation tissue (GT) formation

The biological activity of the factors produced in earlier steps is usually manifested in stimulating the migration and proliferation of various cell types, producing matrix proteins and promoting angiogenesis. This will lead to the formation of a stroma that replaces the fibrin matrix called a granulation tissue [2, 27]. Main processes important to this stage are described below.

- **Re-epithelisation**
  
  This is an important process to close the wounds in humans, and contrary to dermal wounds in animals (e.g. rodents, rabbits, cats, and dogs) which heal mainly by contraction facilitated by their hypodermal muscular layer, the paniculous carnosus [5]. Epithelial cells at the wound margin, after few hours post-injury, undergo morphological changes and form lamellipodia and a disruption to the hemi-desmosomes (cells anchoring points to the basement membrane) occurs [2, 7, 27]. The formation of this epithelial free edge, originally described by Singer and Clack, 1999 [27], and the production of proteases (e.g. collagenase) and plasminogen activator, along with other factors such as EGF, KGF, and TGF-β by these cells, act as possible biological stimuli mediating cell migration and proliferation which is also facilitated by the expression of integrins and other adhesion glycoproteins (e.g. tenascin and fibronectin) for ECM binding [5, 27].

  It has been noted that in cutaneous wounds, under normal conditions, migratory epithelial cells are continuously provided by the basal cells located near the wound edge. Migratory cells, however, don’t seem to divide and differentiate until complete re-epithelialization is achieved. Keratinocytes along with fibroblasts will then secrete components necessary for the formation of the basement membrane (laminin and type IV collagen). Keratinocytes then differentiate to re-establish normal epidermal layers [5].

- **Fibroblasts and ECM deposition**
  
  In response to tissue damage and the initiated inflammatory response, fibroblasts undertake a migratory, proliferative phenotype instead of the original quiescent one. Activated cells synthesize and deposit extracellular matrix components and secrete various signaling molecules [27, 33, 34]. The deposited matrix is composed of mainly structural proteins (e.g. collagen and elastin) and also, adhesive glycoproteins (e.g. fibronectin and vitronectin), proteoglycans (e.g. hyaluronan), and matricellular proteins (e.g. thrombospondins, tenascins, osteonectin) [34]. These different families of macro-molecules, aside from being scaffolds for cell migration, also convey signals that modulate cellular activities either directly via cell-matrix contact or indirectly via matrix-growth factor-cell contact. Therefore, the ultrastructural properties of the three dimensional matrix is important for
proper healing [25].

Fibroblasts, and other wound cells, also secrete degrading enzymes (e.g. metalloproteinases MMPs) and integrins which facilitate their movement and other biological activities [7, 27]. Currently, there are 25 MMPs identified in mice and 24 in humans which have a wide-range of substrate specificities [25, 29, 35]. Based on their substrate preference, MMPs are grouped into collagenases, gelatinases, stromelysins, and matrilysins. It has been shown that the expression of these enzymes is up-regulated by multiple pro-inflammatory cytokines, growth factors, and hormones, and inhibited by tissue inhibitors of metalloproteinases (TIMPs) and serum α2-macroglobulin [25, 36]. Wound area innervation and the presence of nerve growth factor (NGF) have been also noted to be important for healing [1]. Neuropeptides have been found to modulate the function of various wound cells. Moreover, collagen synthesis is also dependent on co-factors such as O2, vitamin C and iron [1].

Fibroblast-to-myofibroblast differentiation is believed to play a pivotal role in wound closure by promoting the contraction of the granulation tissue as they express the contractile protein, α-smooth muscle actin (α-SMA) [15]. The origin of these cells has been reported to be very diverse, including differentiation of activated resident fibroblasts, recruited fibrocytes, and perivascular cells including mesenchymal stem cells [34, 37].

- **Neovascularization**

The dynamic process of neo-vascularization is critical not only for embryological growth, but also for adult tissue development, wound healing, and tumor metastasis [38, 39]. It is well established that, for full thickness cutaneous wounds in adults, new blood vessel formation is essential for normal healing and new tissue growth. Normally, endothelial cells of mature vessels produce low levels of pro-angiogenic molecules and high levels of angiogenic inhibitors; however, upon injury, these cells will shift producing high levels of angiogenic factors to re-establish tissue vasculature [29]. To do so, two distinctive processes are usually recognized, angiogenesis and vasculogenesis [29, 39].

Angiogenesis can be derived from endothelial cell proliferation and migration from damaged vessels [2]. The hypoxic nature of the wound environment, resulting from damaged blood vessels, is a potent stimulator not only for endothelial cells to re-establish the vascular network of the newly forming tissue, but also for keratinocytes and fibroblast migration and proliferation and for the up-regulation of various growth factors and cytokines [7, 27]. The process is positively regulated by mostly VEGF-A and bFGF, with the former playing a more prominent role [2]. This has been demonstrated by the presence of higher mRNA levels of these growth factors in the wounds of
normal mice during GT formation and the absence of angiogenesis when blocking antibodies were used against them [13]. Moreover, the up-regulation of various integrins and ECM receptors, especially for fibronectin, and the production of degrading enzymes by endothelial cells have been shown to contribute to angiogenesis progression [27].

Vasculogenesis, on the other hand, is the de novo generation of the vascular network through the differentiation of BM-derived endothelial progenitor cells (EPCs) [2, 39]. The presence of these cells in the peripheral blood of adult humans was identified in 1997 by Asahara et al. Since then, EPCs have been widely studied as biomarkers to assess the risk of cardiovascular disease and as a potential therapeutic agent for vascular regeneration [40]. Generally, the magnitude of the contribution of such a process to the overall vascular growth in wounds has been reported to be relatively smaller than the former [2].

Notably, the presence of healthy granulation tissue has been utilized clinically to indicate the suitability of the tissue for skin engraftment due to the presence of the vascular network for graft support [5].

1.1.3.A4 Remodelling

Around 2-3 weeks post-injury, active modification and re-organization of the newly formed matrix along with wound contraction will dominate. Remodelling the acellular matrix, which involves the conversion of type III collagen into type I, is usually a slow process taking, under normal conditions, between a few months to one year. The literature agrees on the proposition that a balance in ECM synthesis, deposition, and degradation is critical for proper remodelling; nevertheless, mechanisms regulating these processes are not completely understood [5, 28]. It has been suggested, however, that these processes are in part regulated by the action of MMPs and their inhibitors (tissue inhibitor of matrix metalloproteinases TIMPs) which are secreted not only by fibroblasts but also by other cell types including, keratinocytes, macrophages, and endothelial cells [2, 5, 27].

Myofibroblasts are considered the main contributing cell type to wound contraction and scar maturation. Wound strength is enhanced as more cross-linked collagen bundles are formed; however, only 70-80% of the original tissue strength is restored by the end of the repair process [2, 4, 5, 27]. Scar tissue is usually less elastic than normal skin as dermal collagen bundles are densely packed with the lack of the woven morphology of normal skin. The function of the wounded skin, particularly extensive wounds, is also altered as epidermal appendages (e.g. hair follicles or sweat glands) do not regenerate [5]. Myofibroblast senescence has been suggested as a self-control mechanism on fibrogenesis as they switch from ECM production to degradation [37]. Under certain
clinical conditions, excessive scar formation occurs such as skin keloids and hypertrophic scars. Fibrosis can also be seen in other conditions such as, scleroderma, Crohn’s disease, liver cirrhosis, and atherosclerosis [4, 27].

1.1.3.B Impaired Wound Healing in diabetes

Although the negative consequences of hyperglycemia on various healing events are well documented in the literature, a complete pathophysiological picture has not, yet, been attained [1, 3, 4]. The major locally-bound alterations evidenced from preclinical/clinical reports are described below.

1.1.3.B1 Cellular impairments

Increasing in vitro and in vivo evidence suggest that short- and long-term exposure to hyperglycemia and the resulting pathologies adversely affect various biological activates of different cells types involved in healing [2, 15]. In vitro studies have shown that platelets are hyper-responsive to aggregating factors such as, thrombin, epinephrine, and ADP; however, they are hypo-responsive to anti-aggregating factors such as, NO and PGI₂; a condition that make them irresponsive to anti-platelet treatment such as, aspirin. This change in activity has been linked to vascular diseases, e.g. atherosclerosis, common to people with diabetes (especially type 2) and other diseases [16, 41]. Poor glycemic control has been also reported as a complicating factor for hyper-activity [42].

Altered inflammatory cell functions in diabetes are also reported. Neutrophils, for instance, exhibit a prolonged infiltration which leads to an overproduction of elastase, reactive oxygen species (ROS), and reactive nitrogen species (RNS). The overproduction of ROS has been regarded as an important contributor in perpetuating the pro-inflammatory response characteristic to chronic wounds [15]. Additionally, impairment in neutrophil adhesion to the endothelium, migration to the site of inflammation, reduction in cytokines and prostaglandin production, and increased apoptosis have been also reported [43]. Lymphocyte infiltration in chronic wounds is also altered: Loots and colleagues, 2002 [44] reported a lower ratio of CD4+/CD8+ T lymphocytes in chronic wounds compared with acute healing wounds.

Excessive inflammation present in chronic wounds can also be caused by macrophages. Koh et. al., 2011 [30] indicated that macrophages derived from excisional wounds from diabetic db/db mice, a model of type II diabetes, failed to transition from a pro-inflammatory to a pro-healing phenotype which is required for new tissue build-up [30]. Moreover, animals deprived of macrophages showed impaired healing while neutrophil-deprived animals did not [29]. These observations might not be an
indication of the superiority of one cell type over another in healing as fetal wounds in early gestation heal with minimal or no inflammatory cell involvement [29]. The exact role and importance of these cells is yet to be further explored. Diabetic wound macrophages also produce higher levels of pro-inflammatory cytokines (e.g. TNF, IL-1) [7, 45]. Experimental and clinical diabetic wounds are rich in apoptotic cells which reflect, in part, the impaired efferocytosis capacity of macrophages [15, 45]. It has been shown that successful removal of apoptotic cells by wound macrophages reduces the expression of inflammatory cytokines. As such, manual debridement has been long clinically recognized as a key measure to remove dead, damaged, or infected tissue [45].

On the other hand, keratinocytes have been found to be hyperproliferative, hyperkeratotic, and parakeratotic (i.e. persistence of the nuclei in the stratum corneum) at the margins of diabetic ulcers [15, 46], giving the wound a thick rolled edge known as the “edge effect” [47]. Using immunohistochemistry, Usui et al, 2008 [48] showed that keratinocytes, at the ulcer edge, expressed higher levels of Ki-67, a proliferation marker, and reduced expression of LM-3A32, epithelial migration marker, compared to normal keratinocytes from acute wounds. An inverse relationship between the expression of c-myc and the nuclear localization of β-catenin at the basal epidermal layer and keratinocyte migration was also reported [36, 46]. Microarray data of biopsies taken from patients with vascular ulcers revealed a marked reduction in the expression of EGF receptor (EGFR) in keratinocytes while cells derived from the adjacent normal skin showed a normal expression of this receptor [46]. In vitro hyperglycemic conditions were also found to be toxic for keratinocytes affecting their proliferation, migration, and enhancing apoptosis [15].

Cell response to their microenvironment is well documented. The drastic effect of chronic wound fluid on various cellular activities of fibroblasts in vitro has been widely reported. For instance, Bucalo et al, 1993 [49] showed that venous ulcer fluid (n=6), collected from under a polyurethane occlusive dressing, had an inhibitory effect on human dermal fibroblast proliferation (p=0.008) in vitro. Also, the exposure of normal cultured fibroblasts to chronic wound fluid resulted in cell senescence [33]. Light microscopy observations showed that human diabetic ulcer fibroblasts were larger and more widespread compared to age-matched control fibroblasts which showed a spindle-shaped structure [33, 44]. Ultrastructural changes including a large dilated endoplasmic reticulum, lack of micro-tubular structures and multiple lamellar and vesicular bodies have been also reported [15]. Archer and Kaye, 1989 [50], studied skin fibroblasts form patients with Type I diabetes with controlled HbAlc (blood glycated hemoglobin) levels and no history of complications and age-matched controls. A decreasing trend in mean population doubling with increasing duration of
diabetes and with increasing passage number in vitro was found. Furthermore, Morocutti et al., 1996 [51] showed that skin fibroblasts isolated from a patient with diabetic nephropathy exhibited slower proliferation and premature aging with a tetraploid tendency compared to fibroblasts from a diabetic without renal diseases or non-diabetic controls.

In 2003, Lerman et al. [52] demonstrated that fibroblasts isolated from diabetic mice (db/db) showed 75% reduction in migration, failed hypoxia (1% O₂) stimulation, produced higher levels of MMP-9 and less VEGF compared to the wild-type cells. However, the authors could not detect significant differences in cell proliferation and senescence, an observation reported by other authors [15, 44]. Using Northern analysis and affinity labeling methods, Kim et al, 2003 [53] were able to show that venous ulcer fibroblasts have a decreased expression of TGF-β1 Type II receptor and thus had slower proliferative rate in response to stimulation with TGF-β1. Other studies have shown a higher expression SA-β-Gal, a senescence marker, in these cells [33]. Although the senescence phenotype of fibroblasts has been suggested as a control mechanism for tissue remodelling, Harding et al, 2005 [54] indicated that it might also contribute to wound chronicity. They stated that the presence of >15% senescent fibroblasts in the wound makes it hard to heal as those cells produce elevated levels of proteolytic enzymes and decreased levels of the MMPs inhibitors, especially TIMP-1 and TIMP-3.

1.1.3.B2 Defective extracellular matrix (ECM)

As mentioned earlier, components of the ECM, aside from being a platform for cell migration, work synergistically with growth factors to produce signals regulating various cellular activities necessary for healing. It is generally accepted that the local environment possesses a control over the extent and quality of matrix deposition and the magnitude of tissue degradation, mechanical stimuli transmitted, and GF released [34]. It is well documented that ECM production is less optimal in chronic wounds due to altered biochemical microenvironment [35, 36]. Dermal collagen and GAG content in the skin of animals and humans with diabetes was found to be less than their non-diabetic counterparts. Diminished ECM synthesis by fibroblasts, reduced NO levels, and increased protease activity have been linked to the condition [35]. MMPs and TIMPs are equally important in healing. Excessive and/or persistent MMP activity along with a lack of TIMPs would result in the destruction of new ECM and possibly growth factors, released to or harboured in the matrix, leading to delayed healing [33, 35]. It has been reported by Trengove et al, 1999 [55] that cultured fibroblasts exposed to pro-inflammatory cytokines (IL-1 and TNF-α) showed an enhanced expression of MMPs and a reduced expression of TIMPs. Increased MMP production in response to high glucose
conditions has been also reported in endothelial cells and macrophages [35].

Protease level and activity in wound fluid was tested in several studies. High levels of degraded proteins, such as fibrin, fibronectin, vitronectin, and tenascin, have been previously reported in ulcer fluid and cornea wounds [28]. For instance, Trengove et al., 1999 [55] compared fluids sampled from patients after mastectomy (acute wound) and those who have chronic wounds including mixed vascular diseases, diabetic ulcers, and pressure ulcers. They found ~60 times increase in protease levels in chronic wound fluids compared to the acute wound fluids (p<0.001). Chronic fluid samples were also found to produce more degradation of $^{125}$I labelled EGF in vitro compared to the acute ones. Additionally, it was reported that that fibronectin (Fn) was found intact in acute fluid samples, while it was partially or completely degraded in chronic fluid samples. In vitro fragmentation of intact Fn was faster under various chronic fluid samples confirming the presence of proteases in the fluids [56]. Topical application of anti-protease dressings such as Promogran® (collagen and oxidised regenerated cellulose) has been found to re-establish a balanced MMP/TIMPs ratio [44].

1.1.3.B3 Signalling factors abnormalities

The diabetic wound environment is deficient in GFs, cytokines, and chemokines, while rich in proinflammatory cytokines which is linked to the prolonged inflammatory state in such wounds [29, 45, 56]. A balanced production of inflammatory/anti-inflammatory mediators is fundamental for physiological healing. In type 2 diabetics, high serum levels of inflammatory cytokines, such as TNF-α, has been found. This cytokine has been found to stimulate IL-1β production leading to a persistent inflammatory phase. TNF-α imbalance also appears to contribute to apoptosis. The application of TNF-α inhibitor in experimental diabetic wounds resulted in reducing fibroblast apoptosis and enhancing ECM formation. Experimental diabetic wound studies in rodents also showed that the up-regulation of other pro-inflammatory cytokines such as macrophage inflammatory protein-2 (MIP-2) and macrophage chemo-attractant protein-1 (MCP-1) result in an increased and prolonged infiltration of neutrophils and macrophages into the wound [15].

Decreased levels of several growth factors (e.g. EGF, KGF, PDGF, and IGF) have been established in non-healing wounds [33]. For example, a delay in IGF-1 and IGF-2 mRNA expression and an overall decrease in their protein content were reported in wounds of genetically diabetic mice. A reduced expression of IGF receptor 1 was also found in wound fluid of diabetic rats. Reduced IGF-1 levels were also found in the basal epidermal layer, fibroblasts, and ulcer margin of diabetic subjects [35]. Proteomic means also verified that diabetic wounds are deficient in IGF-1, TGF-β1, and PDGF. The altered expression results in signalling abnormalities affecting various wound cells.
including keratinocytes, fibroblasts, endothelial cells, and infiltrating leukocytes [57].

1.1.3.B4 Defective neovascularization

Micro-vascular diseases such as, retinopathy, nephropathy, and neuropathy, and macro-vascular diseases such as coronary artery disease (CAD), peripheral vascular disease (PAD), and cerebrovascular disease, all 3 related to accelerated atherosclerosis, are well clinically recognized vasculopathies associated diabetes [29, 58]. Characteristic to diabetic wounds is the impaired state of angiogenesis and vasculogenesis [15, 38]. The direct effect of hyperglycemia on angiogenesis has been demonstrated when high concentration of glucose topically applied to non-diabetic rats resulted in angiogenesis inhibition [35]. Thickening of the capillary basement membrane is the most consistently viewed structural abnormality in micro-vasculopathies affecting various tissues including the skin which compromises healing [29, 58]. Reportedly, around 20% of diabetic ulcers suffer from insufficient arterial flow [39] due to the lack or inadequate vessel growth which affects oxygen and nutrient transport to the wound site [35]. Low oxygen tension is a strong stimulator to hypoxia inducible factor-1α (HIF-1α) which up-regulates various genes involved in angiogenesis, cell proliferation and survival. Such a response is altered in diabetes [15, 57]. In experimental and human diabetic wounds, lower levels of HIF-1α were reported [15]. The chronic wound environment is also deficient in GFs and other molecules involved in vessel growth and stability, most notably VEGF and FGF [29, 38]. For comprehensive review on endothelial dysfunction in diabetes see ref. [38].

The prominent role of EPC in adult neo-vasculogenesis is also affected in diabetes [29, 59]. Defective mobilization from the BM, recruitment to the wound site, and function have been shown in both type I and II diabetes which suggest their possible role in the pathogenesis of diabetic vascular diseases [29, 39]. An inverse correlation between circulatory EPC frequency and diabetes and the severity of its micro- and macro-vascular complications was also reported [40, 59]. A study by Fadini et al, 2006 [59] sampled (n=127) diabetics with and without PAD showed a significant reduction (around 53%) in EPC number in diabetics with PAD compared to non-PAD subjects. The decrease was found to be negatively related to PAD severity. A recent study by Albiero el al, 2010 [60] also demonstrated the defective nature of BM-EPC recruitment, survival and proliferation in a diabetes hind limb skin wound model in mice but with no observable difference in circulatory EPC number between diabetic and control mice which was attributable to the short duration of diabetes (4 weeks) after which the experiment was conducted. It can be concluded therefore, that a combination of interrelated factors seems to underlie vessel growth in diabetes.
1.1.3. B5 Advanced glycation end-products (AGEs)

A parallel relationship between glucose concentrations and the amount of AGEs produced has been previously demonstrated in experimental and clinical diabetes studies. High blood glucose causes non-enzymatic glycosylation or glycoxidation of various proteins and lipids which leads to the generation of AGEs. The structure and function of such molecules will be altered in return which, under favorable circumstances, can undergo even further rearrangement generating more complex AGEs leading to aggregate formation that are linked to diabetic complications [15, 43, 35]. Notably, different AGE receptors have been found to be up-regulated in response to diabetes [15] which was linked to altered signaling events [36]. In response to the formed AGEs and also NOS and arginase activity, higher levels of oxygen free radicals (or reactive oxygen species ROS) are generated [33, 43, 61]. The damaging cumulative effect of ROS on various cell types and its relation to aging and disease pathogenesis has been well established [40, 58]. Overall, AGE accumulation along with the resulted oxidative damage and the lack of anti-oxidant capacity are the most widely reported biochemical changes contributing to cell and tissue damage and the long-term micro- and macro-vascular complications in diabetes [15, 40, 43, 52, 61].

This biochemically altered environment also leads to defective VEGF signaling and inactivation of the VEGF receptor, FLK-1; a condition which was found to affect growth and migration of endothelial cells (ECs), and recruitment of monocytes, and EPCs [38]. Studies in diabetic animals have shown that modulation of oxidative damage or inhibition of the AGE receptors improves wound healing and was associated with the up-regulation of endogenous VEGF [57].

ECM components also undergo structural and functional changes in response to hyperglycemia [36]. Cross-linking of collagen, the major dermal constituent, has been previously described in humans and animals with diabetes. This altered stiffness and solubility properties of the matrix restrict cell migration, vessel growth, and wound closure [15, 35]. Cellular response (human fibroblasts) to a modified collagen matrix [cross-linked collagen- 1mM (3-deoxyglucosone 3DG)] was examined by Loughlin et al, 2009 [62]. It was found that fibroblast adherence to this substrate was stronger (using a jet wash adhesion assay at 3 and 24 hours) but migration was less efficient (scratch wound assay). Quantitative RT-PCR showed an increased expression of β1 integrin in those fibroblasts which was suggestive for their strong unexpected adhesion to the modified matrix. Increased expression of caspase-3, an apoptosis marker, and a decreased expression of collagen I and III, TGF-β1, and cell proliferation were also reported.
1.2 Treatment options for diabetic wounds

1.2.1 Conventional treatment modalities

Sumerian cuneiform clay tablets were the first to describe wound care in the so called “three healing gestures” that include: wound washing, dressing, and bandaging [1]. At present, these practices are still recognized as an important aspect of debridement, a measure used to enhance healing by removing hyperkeratotic and necrotic tissues and foreign objects from the wound bed [63, 64]. A DIME model— refers to debridement, infection control, moisture balance, and edge effect — has long been regarded as a standard, first choice approach for non-healing wounds [47]. Appropriate treatment, however, depends mainly on the initial diagnosis of the ulcer type, its severity, and other host-related factors such as age and disease profile. Therefore, routine wound care might not be limited to these 4 options but may further include: off-loading, glycemic control, life style changes, and surgical revascularization if indicated [63, 72]. Moreover, treating peripheral arterial diseases (PAD), which contribute to poor perfusion in lower limbs, has been noted to aid in ulcer healing.

However, if standard treatment did not produce a minimum of 30% reduction in wound size by the first month, advanced treatment choices are usually considered unless the chronicity of the wound, judged by the presence of uncontrolled infection, ischemia, and excessive tissue necrosis, would necessitate amputation [63, 47].

1.2.2 Advanced treatment modalities

New therapeutic approaches aim to compensate the deficient components of the chronic wound environment. Such modalities are very diverse in nature and have been reviewed extensively in the literature. In the context of diabetic wounds, examples of such products are described below.

1.2.2.A Growth factors

Since chronic wounds are deficient in a number of essential growth factors (GFs) that derive various cellular activities, it seems reasonable to supply the wound with such factors to accelerate healing. GF treatment has shown to improve healing in various experimental and human studies. Treating wounds of diabetic animals with IGF in combination with its binding protein IGFBP-1 enhanced healing [35]. PDGF also accelerated healing in chronic pressure sores and diabetic ulcers cases. bFGF has been also used to treat chronic pressure sores with some success [27]. The application of VEGF-A also stabilized healing in wounds of diabetic animals [2]. Among the variety of GF tested, FDA approval was granted only to PDGF, branded as (becaplermin, Regranex {rhPDGF-BB}, Johnson & Johnson, New Brunswick, NJ). This GF is a mitogenic and chemotactic agent for a
variety of cell types present in chronic wounds [72, 63]. It has been previously used in the treatment of diabetic neuropathic foot ulcers [65]. Randomized controlled clinical trials showed ~15% healing enhancement in neuropathic diabetic foot ulcers after PDGF treatment [66].

Studies have also indicated that routine wound care is needed with GF therapy to achieve acceptable outcome. That was first noted in a large prospective randomized trial (>1000 patients with diabetic foot ulcers) when Regranex treatment yielded only 10% healing enhancement. Results were attributed to the lack of standardized wound care during GF treatment [reviewed in 74]. This fact has also been accentuated when 4 randomized trials were compared in a meta-analysis study, reported by Woo et al, 2007 [47], to assess the differences between topical becaplermin gel and a placebo gel, with or without implementing standard care protocols, in a total of 874 patients with diabetic neuropathic foot ulcers. It was found that topical PDGF application was effective (83% wound closure) only when surgical debridement was used.

1.2.2.B Skin substitutes

Skin replacement models that can recapitulate the lost function, architecture, and aesthetic nature of damaged skin have not been yet reported [67, 68]. Autologous skin grafts (full thickness, split thickness, or cultured autologous skin) are considered one of the earliest conventional approaches used for such purpose [67, 68, 69]. Such grafts can also be derived from allogeneic (e.g. cadaver skin) [68, 70], syngeneic (e.g. monozygotic twins, rodents of the same inbred strain), and xenogeneic (e.g. porcine) sources [67]. Generally, they all have equal importance in restoring the biochemical balance and moisture of the wound and providing structural support for tissue repair. Allogeneic and xenogeneic grafts, however, are subject to rejection [65] which necessitates the use of immunosuppressive drugs, such as cyclosporine, FK506 and rapamycin [70]. The antigenicity of such sources can also be treated by removing the epidermis and solubilizing the cells leaving an acellular matrix that can be dry preserved for later use [67]. These products have been approved for venous and diabetic ulcers and diabetic neuropathic ulcers [65].

Tissue engineered skin replacements have been designed as an alternative for the former modalities to treat severe burns or chronic ulcers that require a fast procurement [71, 69]. These skin substitutes can be either cellular or acellular with an overall aim to aid in wound closure [68]. The idea of using cells has been developed by the notion that many diseases are linked to an abnormal cell number, function, or phenotype. The power of cells to replace, repair, and restore tissue functions over other therapeutic agents such as, assisted mechanical device, proteins, or chemical compounds, is profound due to their metabolic and physiologic activities [70]. Commercially
available cellular products commonly fall under 3 categories: epidermal substitutes [e.g. Epicel® (Genzyme Tissue repair Corporation), Laserskin® (Fidia Advanced Biopolmers), EpiDex® (Modex Therapeutiques), MySkin (CellTran)], dermal substitutes [e.g. Dermagraft® (Advanced Biohealing, Inc.), Integra® (Johnson & Johnson), AlloDerm® (Life Cell Corporation)], and composite substitutes [e.g. Apligraf® (Organogenesis), OrCel® (Ortec International)] [47, 67, 69, 71].

Evidence of skin replacement effectiveness has been demonstrated in several animal and human studies. In one retrospective review, the benefit of Epicel®, autologous keratinocyte, for extensive burn injury has been monitored over 5 years (n=30 adults). It was found that Epicel provided stable coverage similar to conventional grafts (26±15 % of total body surface area) with no need for re-grafting in up to 69±23 % of the cases [47]. Dermagraft® (human neonatal foreskin fibroblast, ECM, and a bio-absorbable scaffold) accelerated WH in neuropathic and non-neuropathic diabetic ulcers [72]. According to a large-scale multi-center randomized prospective clinical trial conducted by Veves et al., 2001 [63], Apligraft® (allogeneic neonatal foreskin keratinocytes, dermal fibroblasts, and ECM) was found to enhance healing better than conventional therapies. Seemingly, no immunosuppression is needed when using this product possibly due to tissue processing [70].

Temporary substitutes, such as porcine xenografts, have been also used for chronic wounds and neurological disorders. Although engraftment was reported to be successful, no pronounced effect on healing was found [65]. Composite synthetic or biological dressings are often used in chronic or burn wounds to speed and improve healing. Although effective, they are not permanent treatment. Autograft or allograft may be required to achieve complete healing [71].

Acellular skin substitutes, on the other hand, can be either natural or synthetic, with the required physical and chemical structure for optimal healing [73]. Natural polymers used include: collagen, chitosan, alginates, fibrin, elastin, fibronectin, glycosaminoglycans (GAGs), gelatin, and hyaluronic acid (hyaluronan) [67, 73]. Such materials are advantageous due to their low toxicity which imposes little concern on provoking an inflammatory response in the host. Synthetic materials used include, polyglycolide, polylactide, polylactide coglycolide, polytetrafluoroethylene, and polyethylene terephthalate. Matrices used routinely in therapeutic applications are made from polymers that are often resorbed or degraded in the body [67]. A number of collagen-based dressings have been marketed in the form of gel, sheet, lattice, or sponge such as Promogram® (Johnson & Johnson, New Brunswick, NJ) and Puraply® (Royce Medical) [73]. For example, Promogram, a mix of bovine collagen and oxidized cellulose, has been shown to decrease elastase activity in treated samples relative to the control when wound fluid was examined after 24 hr of treatment. This product has
been also tested in 276 patients with diabetic foot ulcers where no significant differences were found in healing between treated and the control (moistened gauze) group after 12 weeks of treatment. However, Vin et al. (2002) reported a significant reduction in the wound surface area in Promogran® treated wounds compared to petrolatum gauze treated wounds in a sample of 73 patients with venous leg ulcers randomly allocated to receive either treatment [47]. Fibrin Glue (Tissel, Tissucol, Fibrin Sealant FS, Tachosil) has been regarded as a “smart matrix” for wound healing. Its current usage exceeded its original intention as a tissue adhesive during surgery. Now, it is recognized as a scaffold for tissue migration and delivery material for cells and other bioactive molecules [47, 212].

1.2.2.C Other treatment options
Currently, other complementary approaches are being investigated which include hyperbaric oxygen, negative pressure therapy, electrotherapy, laser therapy, ultrasound, and ultraviolet light [47].

1.2.2.D Limitations of current treatment
Several issues have been raised with the previously described modalities. Growth factor treatment, for instance, requires large quantities and multiple applications, due to their short half-life and degradation by protease, which has been shown to be not only costly but also could lead to unanticipated side effects [71, 72]. Reportedly, most growth factor and skin grafts used for chronic wounds demonstrated only 25% increase in healing rates [63, 71]. Although various types of skin grafts did show some clinical success, product commercialization was not optimal [69]. Generally, vascularization is a key issue that is not always optimal when skin grafts are applied. Keratinocytes for example, were the first type of cultured cells used to treat burn cases around 30 years ago. Without a well-vascularized dermal wound bed, cultured keratinocytes alone are of limited value in treating full-thickness burns [69]. Mechanical issues and high cost are also reported [67, 73]. Moreover, autologous tissue harvest means creating a second defect which, under favorable circumstances, may cause infection and other complications especially when a larger piece is required. The procedure is also limited in extreme burn cases which necessitate the use of artificial skin substitutes or other modalities [67, 73]. Culture expansion of adult primary cells also bears practical and clinical limitations represented by the inability to produce large number of cells as the cells exhibit replicative senescence [70, 67]. It has been reported that aged cells have less proliferative capacity and faster senescence in vitro. Therefore, autologous cells are of less importance [74]. Allografts also have similar problems to the autologous ones with the addition of biocompatibility and immune rejection concerns [67, 70].
The lack of a vascular network is a key problem for currently available artificial dermal matrices [65]. Poor stability and low mechanical support have been reported with collagen-based scaffolds or dressings [73]. Limitations concerning the animal models available to test the effectiveness of the new drugs have been also raised as results obtained from such models may lack clinical efficiency when applied to humans [74].

1.3 Mesenchymal stem cells (MSCs) for impaired wounds

Unlike somatic cells, MSCs represent a population of undifferentiated multipotent cells that can self-renew and differentiate, under appropriate conditions, into other mesenchymal and non-mesenchymal lineages. They seem to play a supportive role in tissue homeostasis and repair (in case of injury or disease) by replenishing cellular and acellular components through various, incompletely understood, mechanisms [65, 75, 76].

1.3.1 The perivascular niche

Compelling experimental evidence indicates the universal presence of MSCs in various adult, fetal, and neonatal tissues in addition to bone marrow, including peripheral blood, cord blood, Wharton’s jelly, amniotic fluid, compact bone, periosteum, synovial membrane and fluid, adipose tissue, fetal tissues [76], brain, teeth [77, 79], muscles [77], placenta [78], skin, hair follicles, pancreas, and intestine [79], and possibly others.

Of particular interest to the subject is the establishment of the perivascular niche of these cells in various tissues [75, 79]. A recent study by Crisan et al., 2008 [80] provided in vivo and in vitro evidence linking adult human MSCs and perivascular cells (or pericytes). In 2009, preliminary in vivo evidence of a direct positive correlation between MSCs frequency and the extent of tissue vascularity in equine adipose tissue comprising various vessels densities was provided [81]. With age, MSCs titer was found to decrease in the BM as estimated by CFU-F assay [82]. Notably, vascular density in the aged population decreases by one or two orders of magnitude; this might explain the marked decrease in MSCs or pericyte availability with age [83]. Although these observations corroborate the MSCs perivascular niche proposition previously suggested, the presence of a cell population with MSC properties in avascular tissues, such as the cartilage, and the absence of direct evidence of pericyte differentiation in vivo highlights the possibility of an alternative physiological MSC niche [76].
Pericytes are branched mural (or Rouget) cells covering the endothelium of small and large vessels. Although specific surface markers are still lacking, their identification has relied mostly on their location, morphology, and specific gene/protein expression pattern. Their role in physiological and pathological repair processes is well documented. Essentially, they provide stability to blood vessels, synthesize matrix proteins, possess a similar role as macrophages, participate in immunologic protection, and serve as a potential source of progenitor and mesenchymal stem cells for tissue repair [76, 84, 85, 86, 87]. Pericyte implication in a number of pathological conditions has been also long been recognized [88]. A direct link between pericytes and diabetic angiopathies, such as retinopathy, is widely documented. Microvascular changes including pericyte apoptosis, thickening of the basement membrane, and altered blood flow in the retinal capillaries usually result in capillary leakage (macular edema) and vessel occlusion [reviewed in 84 and 85]. Another example is their role in fibrosis where pericytes exhibit altered surface markers, detach from the basement membrane leading to increased vessel permeability, trans-differentiate into myofibroblasts, and excessively deposit ECM leading to scar formation and organ failure [85]. This is indicative of a possible role of MSCs in such conditions.

1.3.2 Characteristics of MSCs

For research purposes, minimal criteria have been set by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy to identify MSCs using in vitro functional protocols, Dominici et al. (2006) [89]. Under normal culture conditions, MSCs should be characterized as fibroblastic cells, capable of adhering to tissue culture plates, differentiate into the three classical lineages, osteogenesis (verified by Alizarin Red or von Kossa staining), adipogenesis (verified by Oil Red O stain), and chondrogenesis (verified by Alcian blue or immune-histo-chemical staining for collagen type II), express ≥95% of certain surface markers (CD90, CD105 and CD73) and lack the expression of other markers (CD34, CD45, CD14, CD11b, CD79α, CD19, and HLA-DR (HLA II), as measured by flow cytometry [76, 89].

Heterogeneity also exists between MSCs isolated from various sources in terms of phenotype, growth kinetics, cytokine profiles [76], and frequency [78]. In vitro phenotype and functionality of cells may also differ from their in vivo ones as the natural cues are replaced with artificial ones in culture media which might influence and/or alter their characteristics [76, 87]. In addition, the lack of standardized protocols for cell isolation and expansion among different research laboratories makes the comparison of the published work difficult and indeed less informative [89].
1.3.3 Suggested mechanisms of action of MSCs in wound healing

1.3.3.A Paracrine effect and immunomodulation

The majority of the current in vivo evidence suggests that the many factors secreted by MSCs, such as GFs, cytokines, and chemokines, is the main contributor to their biological activity. Such factors have a wide-range of paracrine effects including, mitosis, angiogenesis, anti-apoptosis, chemotaxis, immune-modulation, and reduce scarring (anti-fibrotic) [87, 90]. The notion that similar effects on wound repair was achieved using MSCs and also their conditioned medium re-enforces the involvement of the paracrine pathway in healing. Direct and in-direct co-culture studies also verified the beneficial effect of MSC secreted factors on various biological activities of different cultured cell types [91]. Liu et al., 2006 [92] verified that un-stimulated cultures of rat BM-MSCs constitutively express a number of growth factors such as, TGF-β, VEGF, FGF, EGF, and KGF, and significant levels of VEGF protein. Upon culture stimulation with LPS, IL-1α, and TNF-α, differential expression of growth factors resulted which is also suggestive that a similar effect could be gained under the pro-inflammatory rich wound environment.

Re-establishing the vascular supply is important for repair particularly for compromised wounds [84, 90]. MSCs secreted factors such as VEGF, IGF-1, PIGF, MCP-1, bFGF and IL-6, have been shown to stimulate angiogenesis and granulation tissue build up [83]. Kagiwada et al., 2008 [93] demonstrated that VEGF was highly expressed in hBM-MSCs cultures and the expression was maintained up to passage 10. However, comparative analysis of the expression pattern of 20 angiogenic cytokines of hMSCs (n=2) and fibroblasts cultures showed that both cultures are very similar except for VEGF which was detectable only in MSCs cultures. They also noted that these cells can engraft in immunodeficient mice and produce human VEGF. The effect of mesenchymal stem cells on vascular tubule formation by human umbilical vein endothelial cells HUVEC in vitro was tested by Sorrell et al., 2009 [94]. BM-MSCs contributed to enhanced tubule formation and stability compared to fibroblasts co-cultures; however, cell alignment with the formed tubules was noted only in MSC cultures reflecting a possible beneficial cross talk. A recent study by Schlosser et al., 2012 [95] showed, utilizing an ischemic mouse skin flap model, that systematically injected GFP+BM-MSCs (Lin−CD105+) can home to perivascular areas at the defect site as early as day 4 post treatment while there was no GFP signal detected in the control group (GFP+ fibroblasts). Paracrine VEGF expression in the MSC group was also reported.
Cell engraftment and survival is a critical issue for sustained release of the bioactive mediators and also for cell differentiation [91]. Evidence extrapolated from in vitro studies indicated that MSCs possess immunomodulatory and immunosuppressive qualities. This concept suggests that such cells will not activate the host’s innate immune response nor provoke the adaptive one (cellular or humoral) allowing a state of host tolerance to the transplanted cells [83, 90, 96].

These capacities have been clinically appreciated in a wide range of conditions such as acute myocardial ischemia, stroke, kidney injury, inflammatory bowel disease, graft-versus-host disease (GVHD), multiple sclerosis, diabetes mellitus, and organ transplantation. Additionally, xenogeneic hMSCs transplantation in various immunocompetent mouse models of human diseases has been reported to be applicable corroborating in vitro observations [reviewed by Singer and Caplan, 2011 [83]. Human MSCs/CD34+ cells, and non-MSCs/CD34- cells showed successful engraftment in an injured rat liver model [97]. No rejection was also reported when cultured hBM-mononuclear cells were xenotransplanted, locally and systemically, into skin defects in immunocompetent mice [98]. Similar results were reported when hBM CD34+ and CD34- cells were injected into rat coccygeal discs [99].

However, inconsistent results were reported in large-scale clinical studies and several xenogeneic transplantation studies where allo- and xeno-immune recognition has been shown [91, 96]. Injection of hBM-MSCs to the myocardium of immunocompetent Sprague-Dawley rats resulted in significant infiltration of, mostly, rat macrophages [100]. A recent work by Rossignol et al., 2009 [101] also suggested that both allo- and xeno-MSCs cause a weak immune response without affecting their survival as they were detected for up to 63 days post treatment. Also, when genetically modified hMSCs were used to treat critical-sized calvarial bone defect in immunocompetent rats, host immune response was reported but the cells survived for up to 4 weeks and survival was enhanced when immunosuppressive drugs were used [102].

1.3.3.B Differentiation/Trans-differentiation

Within the context of dermal wound healing, few studies have reported that MSCs differentiate into keratinocytes, endothelial cells, pericytes, myofibroblasts, and sebocytes, and enhance healing with no current evidence of full skin regeneration [87, 91, 92]. A study by Sasaki et al., 2008 [203] revealed that female mice received a systemic dose of male GFP+ BM-MSCs showed cells positive for GFP and other markers specific for keratinocytes, endothelial cells, monocytes, and pericytes at the wound site. Healing was improved after treatment but a direct link to trans-differentiation could not be established. No cell fusion was reported as examined by sex-chromosomal analysis. Full-
thickness skin wounds in rats receiving BrdU-labeled MSCs previously co-cultured with heat-shocked sweat gland cells (SGCs) showed the presence of such cells in hair follicles, sebaceous glands, blood vessels, and the dermis [103].

Overall, MSCs differentiation/trans-differentiation into various skin cellular components was not evident in the majority of the published work. This capacity has been remotely considered as the main functionality of MSCs in vivo.

1.3.4 BM-MSCs for impaired wounds

MSCs isolated from the BM, originally characterized by Friedenstein and colleagues [104], have been extensively employed, experimentally and clinically, as a healing agent for various conditions due to their multi-potency, immune tolerance and modulation and secreted factors [88, 92], properties which earned them the title of being a gold standard cell source for therapy [78].

1.3.4.A Preclinical/clinical data

Experimental evidence of the healing potential of BM-MSCs in the context of impaired wounds due to diabetes has been provided in several studies. Despite the variability of the animal models used and BM cell subsets and their isolation and application methods, an overall agreement on their potential on healing has been reported [105-107, 108, 109]. Due to the significance of the experimental design to our thesis project, the work that has been completed over the past years involving BM-isolated cells (BM aspirate, BM-MSCs, BM-stromal cells) in various diabetic animal models of wound healing is summarized in Table 1.1.

It can be noted that these studies have attempted to investigate the overall contribution of MSCs to healing under the pathophysiological hyperglycemic wound environment, to suggest a possible healing mechanism, to assess the potency of various subsets of the BM-cells in healing, and to compare local and systemic routes of cell application on healing. There was an agreement in the majority of studies on their role in angiogenesis through the paracrine route as enhanced production of VEGF, EGF, HIF-α1 was reported [105, 106, 107]. BM-MSCs seem to be more potent than BM stroma in enhancing angiogenesis [107, 108]. MSCs are also better than BM stromal cells in enhancing other wound parameters including re-epithelialization, new tissue formation, and wound closure [105, 108, 109]. Cell dosage seems also to play a role as administering MSCs twice has been shown to be better than once on wound closure [105]. Enhanced wound strength and collagen production was also indicated [106]. Paradoxically, bone formation after treatment [108] and differentiation into keratinocytes and dermal appendages [109] were also reported which could be
related to the studied cell source and its homogeneity.

Clinically, BM-MSCs have been mostly used in the autologous setting. Badiavas and Falanga, 2003 [110] applied autologous bone marrow cells, BM aspirate and cultured BM cells, to non-diabetic chronic wounds with duration of more than 1-year that were irrespnsive to standard and advanced therapies, including bioengineered skin application and grafting with autologous skin, n=3. Cell engraftment and clinical and histologic improvement (especially in the dermis) were reported. Engraftment was judged based on increased wound cellularity and the presence of cells of different morphological features. A highly cited study by Falanga et al., 2007 [111] showed a significant correlation between the number of cells applied and the decrease in wound size (P= 0.0058) when chronic wound of n=8 subjects were treated with autologous BM aspirate followed by 4 injections of cultured autologous BM-MSCs using fibrin spray. GFP+ BM-MSCs (isolated from syngeneic strains) injected to db/db mice showed the presence of GFP+ blood vessels but did not confirm the state of possible differentiation or cell fusion. It has been also suggested that the healing effect of BM aspirate or cultured BM cells seems to be angiogenic in nature, i.e. through a paracrine action [112]. Dash et al., 2009 [113] applied autologous BM-MSCs with standard wound dressing to patients with non-healing ulcers (diabetic foot ulcers, n=6 and Buerger disease, n=18). Clinical improvement in foot pain, significant decrease in ulcer size by 12 weeks in both groups, and morphological improvement in the dermis was noted in treated groups compared to the untreated control groups.

Despite the beneficial clinical outcome reported with the use of MSCs, larger-scale studies and randomized controlled clinical trials are still needed to establish the merit of MSCs in the context of impaired wounds due to hyperglycemia [210, 211, 213].

1.3.4.B Limitations of adult MSCs in therapy

BM-MSCs, in fact, represent as a small fraction of the BM and their number, indicated by Caplan 2009 [114], declines with age (from 1:10⁴ in newborns to 1:10⁶ in older individuals, ratio reflects MSC number: nucleated marrow cells). This dramatic decrease also reflects the decrease in vascular density with age widely reported. As far as tissue/organ maintenance is concerned, the decrease in MSCs number with age is not the only factor; a decrease/loss of MSC function has been also noted. Stolzing et al., 2006 [115] showed, using a rat model, that BM-MSCs exhibit decreased levels of antioxidative enzymes, decreased proliferation and differentiation potential, and increased apoptosis. The accumulation of oxidative damage in such cells has been linked to age-related pathologies such as arthritis and osteoporosis. Such observations were also reported by others [116, 117].
Such low frequency of hMSCs in the BM makes their in vitro expansion a necessity for clinical use. The effect of long-term cultured hMSCs on various biological properties of such cells and their overall safety for clinical use has been examined by Kim et al., 2008 [118]. They noted a decrease in telomere length with no differences in telomerase activity and a reduction in growth rate in later passages with no chromosomal abnormalities. In vivo, long-term cultured hMSCs did not result in tumor formation when transplanted in nude mice which suggests their safety. The combined effect of increased in vitro passaging and donor age on hBM-MSC differentiation was examined by Stenderup et al., 2003 [119]. They found decreased osteogenic and adipogenic differentiation with increased number of passages for cells from young and old donors. The decrease in the trilineage differentiation capacity with increasing animal age and passage number was also noted in another study in murine MSCs, Kretlow et al., 2008 [120].

Schatteman and Ma, 2006 [121] stated that cells from older patients could be counterproductive both in autologous and allogeneic manners. This claim was based on their observation that treating non-diabetic and diabetic mice wounds with (20–24 month old) murine HSC-enriched BM cells resulted in a decrease in vessel size and density; on the contrary, cells of younger mice (2–4 month old) enhanced vascularization of the diabetic mice wounds. The constitutive secretory function of MSCs has also been noted to be dictated by age [114] and local environment [122]. The effect of the disease state of the host on MSCs activities has been also regarded as another limiting factor. Autologous BM-MSCs have been widely used clinically; however, under diabetes, for instance, native MSCs are compromised due to DNA damage and apoptosis which limit their potential use in treatment [122]. Stolzing et al., 2010 [214] demonstrated a marked reduction in CFU-F size and number of MSCs isolated from STZ-induced rats compared to the control which was proposed to be mediated by apoptosis and senescence resulted from the advanced glycation end products (AGEs) of the hyperglycemic milieu. In addition, Phadnis and Ghaskadbi, 2009 [215] indicated that only 57% of BM-MSCs isolated from elderly subjects (30-80yr) with chronic diabetes could be expanded in vitro for approximately 3 times while MSCs retrieved from young diabetics (15-30 yr) could be passaged up to 15 times. An inverse correlation between proliferation and the duration of diabetes was also noted as a marked reduction in MSC proliferation was associated with the state of chronic and uncontrolled hyperglycemia.

Accumulated evidence is therefore suggestive that MSCs isolated from adult tissues, such as the bone marrow, could compromise treatment success in terms of engraftment and potency. A younger source of MSCs could therefore serve a better choice for cell therapy.
**1.3.5 Neonatal versus adult mesenchymal stem cells**

MSCs have been successfully isolated from various neonatal tissues including the placenta, amnion, cord blood, umbilical cord, and Wharton’s jelly [78]. Neonatal MSCs can be easily retrieved from such non-controversial sources in higher numbers. They can be also cryopreserved after harvest for later use without significant loss of potential or function [123]. Adverse effect of age on adult MSCs has been widely reported. Neonatal and adult mouse BM-MSCs have been found to share the same differentiation potential and surface markers; however, faster in vitro proliferation was noted in neonatal MSCs [124]. This latter observation was noted by other researchers which is, indeed, a desired quality for clinical use [78]. Moreover, in a rat model of acute myocardial infarction, BM-MSCs from young animals (8–12 weeks) seem to be more tolerant to apoptotic stimuli and, in response to stress stimuli (anoxia), they exhibited higher angiogenic growth factors expression in comparison to older (24–26 weeks) rat MSCs, Jiang et al., 2008 [116].

From a therapeutic point of view it therefore seems that the younger the tissue or MSC age, the better for therapy.

**1.4 Human umbilical cord perivascular cells (HUCPVCs)**

MSCs have been previously isolated from different regions of the human umbilical cord (hUC) including the UC lining, the Wharton’s jelly, the sub-endothelial layer, the perivascular zone, and the whole UC, comprehensively reviewed by Conconi et al., 2011 [125]. A rich source of mesenchymal cells has been previously isolated in our lab from the perivascular region of the UC, called human umbilical cord perivascular cells (HUCPVCs). These cells were found to have a colony forming unit-fibroblast (CFU-F) frequency of about 1:300, a ratio that is higher than that of neonatal BM-MSCs (1:10000) [126]. They also have a shorter population doubling (PD) time compared to hBM-MSCs resulting in their rapid expansion in vitro. This is continued by multi-layering unlike the latter which showed contact inhibition growth upon confluence. Such continuous growth has not been associated to tumor activation when analyzed with gene array [123]. Similar to BM-MSCs, the multi-differentiation capacity of HUCPVCs has been clonally verified. Fibroblasts seem to be the default lineage of their differentiation pathway [127]. Interestingly, they have been shown to exhibit higher gene expression profiles of factors that are important for angiogenesis and wound healing compared to adult hBM-MSCs; suggestive of more pronounced effect in vivo through the paracrine pathway (unpublished data).
Interestingly, these cells are immunoprivileged and immunomodulatory as verified by in vitro assays. They lack the expression of MHC II, but express low levels of MHC I which was found to decrease with subsequent passaging. They also lack the expression of co-stimulatory molecules (CD80 and CD86). This, in addition to their secreted factors, may play a synergistic role in their evading and alleviating an active immune system of the host making them suitable for allogeneic use [128]. It is evident, therefore, that HUCPVC are biologically equivalent to BM-MSC in terms of their immune-phenotype, differentiation, and allogeneic use.

It has been hypothesized that the prime responsibility of HUCPVCs is to sustain their niche (i.e. the UC stroma) by differentiating into myofibroblasts [127]. HUCPVCs survival and synthetic activity have been tested in an intra-femoral defect model in NOD scid mice. Their survival was verified for up to 6 weeks and significant bone and cartilage repair was achieved at 2 weeks post implantation compared to sham controls, verified by \( \mu \)CT analysis and Masson's Trichrome staining of longitudinal histosections. Expression of human-specific collagen II and osteocalcin were found in the treated group which proves their in vivo functionality [127]. HUCPVC-Diffusion chamber implantation in the peritoneal cavity of normal rats showed that these cells are synthetically active in-vivo depositing fibrous ECM as examined in day 4 and 7 postoperative samples using SEM [129]. This is suggestive that their functional capacity is retained following implantation.

The wound healing potential of HUCPVCs has been investigated in full-thickness dermal wounds of non-hyperglycemic balb/c nude mice where accelerated healing, higher wound tensile strength and vascularity have been shown in HUCPVC-treated wounds compared to fibrin-treated controls, Zebardast et al., 2010 [129]. These cells were found localized to the dermis with no signs of differentiation. HUCPVCs have been also investigated in collagenase tendon injury model in nude rats. An increase in tendon tensile strength and stiffness by 30 days post-treatment was reported in the test group. Expression of human genes for collagen-1 and \( \beta \)-actin were also found. The presence of HUCPVCs at the injury site seemed to facilitate collagen bundle re-organization from disorganized to linear [130].
1.5 Cell delivery approaches

For skin wounds, cells can be either injected locally (intradermal) or systematically (intravenous), or topically applied in a gel or cream form. The latter methodology has been shown to be more reliable in delivering the cells specifically to the affected site with considerable ease and convenience especially for large wounds [131]. Various cell delivery materials have been used over the years including PBS, normal saline, collagen, matrigel, and fibrin. It has been shown that cell engraftment varies with the application method and the delivery material used [91].

1.5.1 Hydrogels for in vivo cell delivery

Hydrogels are generally preferred for soft tissue injuries. Their close resemblance to the gel-like-dermal matrix, biocompatibility, and their flexibility (from a technical point of view) have long made them useful as bio-adhesives, providing a 3-D matrix for mechanical stability and cell growth, and as a delivery material for bioactive agents such as drugs and cells [132, 133]. These insoluble polymeric matrices can be formed from natural, synthetic, or hybrid sources. Some of the natural sources previously used include agarose, alginate, chitosan, hyaluronan, fibrin, and collagen. On the other hand, synthetic materials classically used include poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), and polyacrylates such as poly(2-hydroxyethyl methacrylate) (PHEMA), among others [133].

Materials that show rheological features of in situ gelation in response to environmental changes in pH, temperature, electric field, or solvent composition are even more preferred due to their ease of use and convenience [132, 134]. Such hydrogels have been used to deliver various molecules such as steroids, proteins, and chemotherapeutics [132].

1.5.2 Methylcellulose (MC)

MC, a water soluble polymer, also referred to as methyl ether of cellulose and cellulose methyl ether, is a chemically modified form of cellulose, a non-water soluble material. MC is an example of a thermoreversible hydrogel that undergoes a “sol-gel” transition from viscous fluid at RT to a gel form (clear to opalescent viscous solution) at physiologic temperatures [135, 136, 137, 138]. Its chemical structure is shown in Fig.1.1. Since it is derived from natural sources that are universally available and low-cost, in addition to its biocompatibility and biodegradability properties [204], MC has found various applications in the food, drug, and cosmetic industries as an emulsifying agent [136, 138, 139]. MC solution seems also to foster nutrients and GF diffusion for cell survival within in vitro culture systems. Clinically, MC has been previously used during intraocular surgery as a corneal protectant [139].
Rogers and Wallick, 2011 [140] indexed 379 references mentioning MC as a microencapsulation agent; however, MC was only mentioned in 11 of them including those considering the use of MC as an additive or alternative material to others. Despite the paucity of the published work on using MC to deliver cells and other molecules, its use appears promising. Published studies indicating the use of MC in experimental and clinical settings are summarized in Table 1.2.

Figure 1.1 The chemical structure of methylcellulose (MC).

1.6 Animal models of impaired wound healing

1.6.1 Overview

The pathophysiological complexity of chronic wounds in humans has rendered the production of animal models of such wounds challenging [141, 142, 143]. Mice are widely used experimentally in wound healing studies. Non-negligible anatomical and physiological dissimilarities exist between the mice and humans with regard to skin layers (epidermis, dermis, and hypodermis) [142]. Mouse skin has also a distinctive thin muscular layer called the “paniculosus carnosus”, which contributes to wound contraction (main method of healing in loose-skinned animals). This is absent in humans, except for the neck (platysma), which explains why human wounds heal mainly via re-epithelialization and granulation tissue formation [142, 143]. Interestingly, Azzi et al. (2005) [144] found gender differences in the skin of C57BL6 mice (13–15 wk of age) where the dermis was thicker in males compared to females. The epidermis and the hypodermis, on the other hand, were thicker in females. Although this observation is not widely reported in the literature, it can certainly impact the study design.

Impaired dermal WH state can be generated experimentally using various settings. Such models have been shown to reflect, to a certain extent, some of the pathophysiological abnormalities related to the chronic state. Differences between wild type and diseased animals have also been demonstrated which re-enforce the presence of underlying abnormalities that can be targeted experimentally [143].
1.6.2 Hyperglycemic animal models

Hyperglycemic animal models represent a useful tool for impaired wound healing studies. Various models are currently available which can be produced using chemical, surgical, or genetic means. It should be mentioned, however, that such models usually lack a true representation of macro- and micro-vascular diseases usually associated with human diabetes and contribute to the delayed healing status [141]. Chemically-induced animal models are still, by far, the most prevalent type [reviewed in 146]. Hyperglycemia has been shown to be easily induced and, to a large extent, maintained in different animal species such as mice, rats, guinea pigs, hamsters, dogs, and monkeys [141]. Two chemical substances have been widely used in inducing hyperglycemia, alloxan and streptozotocin [141, 145]. Other toxins have been also mentioned in the literature but rarely used such as vacor, 8-hydroxyquinolone [145], dithizone [145, 146], and ferric nitritriacetate [146]. The agents can be administered using various methods such as intra-peritoneal, intra-venous, or subcutaneous; however, the first route is most popular in rodents [146].

Streptozotocin (STZ) is the most widely used agent in diabetes research and wound healing [143, 149] Rakieten et al. were the first to describe its diabetogenic properties in the early 1960s [147]. STZ is a toxic glucose equivalent (nitrosurea derivative) that was originally isolated from Streptomyces achromogenes var. streptozoticus, a gram-positive bacterium. The chemical structures of STZ and D-glucose are shown in Fig. 1.2. It has been widely used as an antibiotic and chemotherapeutic agent for pancreatic islet cells and some neuroendocrine tumors [145, 146, 148, 149]. This drug is often used to induce a clinical condition in animals similar to human type I diabetes [150]. However, induction of type II diabetes-like condition has been reported in rats using a single dose of STZ (60 mg/kg) combined with nicotinamide (120 mg/kg) [146, 150].

Three induction protocols have been previously described. First, a single high treatment dose which is usually higher in mice (~150 mg/kg) compared to rats (~45 mg/kg) [149]. This method is fast, causing β-cell death within 24 hrs post-induction [149, 151]. However, examining freeze-fractured pancreatic samples using ultrastructural means showed that changes in the plasma membrane of the β-cells appear as early as 2-4 hrs post-induction with evident necrosis. There is also a congruent rise in blood glucose values 1-2 days post-induction which usually remains elevated when using the appropriate doses [151]. The second method is to deliver the drug on multiple low doses, ~40 mg/kg for 5 successive days [149]. This method is slower, causing insulitis that involves cell-mediated immunity [141, 145, 149]. The autoimmune basis of β-cell destruction was first described by Like and Rossini, 1976 [151]. It has been suggested, not yet precisely verified, that the yield of
this method is more consistent than the first one [149]. The last method is rarely used which involves a single low dose (half the high dose) which will cause a gradual onset of diabetes (10–60 days) [141].

In animals, STZ specifically attacks the pancreatic (insulin-producing) \( \beta \)-cells, through the low affinity GLUT2 glucose transporters [147]. Upon uptake, STZ splits into glucose and methylnitrosourea. The latter moiety has been linked to its alkylating properties as it modifies macromolecules [147], fragments DNA [146, 147, 149], and interferes with glucose transport and glucokinase activity [145]. Disrupted \( \beta \)-cell function is usually reflected by glucose and insulin homeostasis [147, 150]. Noteworthy, the presence of the GLUT2 transporters in other organs such as the kidney and the liver might explain the damage reported in such organs after STZ application [147]. It has been also found that a reduced expression of GLUT2 prevents the diabetogenic action of STZ [150]. Human \( \beta \)-cells have been shown to lack a significant expression of GLUT2 which results in their insensitivity to this toxin, a reason why it is not considered diabetogenic to humans when it is used in chemotherapy, specifically for pancreatic cancer [147, 152].

![Chemical structure of streptozotocin and D-glucose](image)

**Figure 1.2** The chemical structure of streptozotocin and D-glucose.
1.6.3 Dermal wound models
The full-thickness excisional wound model, resulting in full removal of the epidermis, dermis, and the panniculus carnosus, is the most widely used surgical procedure for creating dermal defects in animals and can be accomplished using various devices such as, a biopsy punch, scalpel, and a dermatome [142, 143]. As such, these wounds lack a wound base and healing progresses, therefore, from the wound margins with contraction as the main mechanism of closure [143]. In some dermal WH studies, a splinted wound model, using sutured or glued rings (made of silicone or other materials) fixed around the wound, have been described as a better option to annul the role of contraction in wound closure and to better observe the elaboration of various healing parameters, similar to human WH [142, 143]. Galiano et al., 2004 [153] indicated that granulation tissue deposition was increased in silicone splinted wounds (glued and sutured) compared to un-splinted ones while the rate of re-epithelialization was not affected. Indeed, adopting this approach might be reasonable; however, for small animals, such as mice, applying such devices is stressful and could impose a new variable affecting healing dynamics.

1.6.4 Immunodeficient mice in hyperglycemic wound healing studies

1.6.4.A NSG mice: Strain properties
Generally, for xenotransplantation purposes, immunodeficient mice are preferred. A comprehensive overview of such models currently in use has been recently reviewed by J. E. Belizário, 2009 [154]. NSG mice, a new strain that results from crossing NOD scid mice and IL2rg−/− mice has been regarded as a better choice for human cell/tissue engraftment compared to standard SCID models. Mice of this strain are homozygous for targeted mutations in the interleukin-2 receptor gamma chain, also known as the common cytokine-receptor gamma chain, which intermediate different cytokines signalling including, IL-2, 4, 7, 9, 15 and 21 [154]. Defective cytokine signaling pathways usually result in severe impairments in T- and B-cell development and function and the absence of NK-cell [154, 205]. Collectively, these abnormalities contribute to the enhanced engraftment state reported. Ito et al., 2002 [155] found that human hematopoietic cell engraftment in NOD scid γcnull (NSG) mice is better than other immunodeficient mice strains tested (NOD scid β2mnull and NOD/Shi-scid mice injected with anti-NK cell antibody).
1.6.4.B  Hyperglycemia induction in immunodeficient mouse strains

Various hyperglycemic immunodeficient animal models have been previously reported in the literature [148, 156, 157]. Reports on induction success varied among the published work and appears to be related to the animal strain studied and induction drug type, quality, dosing, and administration protocol used. In one study, where a multiple low-doses STZ induction protocol was used, hyperglycemia was induced in only 55% of CB.17 Scid mice [148]. De la Garza-Rodea et al., 2010 [149] found a significant difference in mortality rate between freshly prepared and equilibrated solutions of STZ when injected in Nod scid mice, 36% and 7% respectively, stressing on the quality of the administered drug may also play a role. Pearson et al., 2008 [156] reported that hyperglycemia can also be induced in NSG mice, both male and females, using doses ranging from 120 to 160 mg/kg; the percentage of mice who failed to become hyperglycaemic, however, did not correlate with STZ dose administered or gender.

1.6.4.C  WH is impaired in hyperglycemic immunodeficient mouse strains

Wound healing studies involving immunodeficient hyperglycemic murine strains are scarce. A study conducted by Crow et al., 2000 [158] showed that wound healing was delayed in immunodeficient TGF-β1 knockout mice (Tgf-β1−/− Scid−/−, lack B and T cells) by around 1 week compared to Scid−/− mice with a wild-type Tgf-β1. They attributed this delay to the lack of both lymphocytes and TGF-β1 expression. Histologically, they noted a delay in inflammatory cell infiltration until day 14 post wounding. The granulation tissue was thin by day 21 with less organized matrix compared to the control. A recent study by Kim et al., 2010 [159] showed that STZ- induced Balb/c nude mice had a marked decrease in healing compared to normal athymic nude mice where the former achieved ~50% wound closure by day 12, while the latter reached this percentage by day 7.
1.7 Thesis rationale

Mesenchymal stem cell therapy seems to support impaired wound healing resulting from hyperglycemia through different mechanistic facets. This has been previously established using adult BM mesenchymal stroma/stem cells and MSCs from other adult sources in various preclinical and clinical models of chronic/impaired wounds under a hyperglycemic state. Ample evidence generated from such studies is also indicative that mesenchymal cells from younger and healthier subjects is better for optimal results compared to cells isolated from adults or aged subjects especially those with chronic illnesses, such as diabetes and its complications, as cell frequency and functionality have been shown to be altered. Findings that MSCs isolated from neonatal sources, such as HUCPVCs from the umbilical cord, are biologically similar to adult MSCs in their stemness, differentiation and immunoprivileged capacities; yet with a higher MSC frequency at harvest and better proliferation potential, seem to be, therapeutically, a better choice particularly for recalcitrant clinical conditions.

1.8 Thesis hypothesis and objectives

We hypothesized that when administered to dermal wounds in hyperglycemic subjects, neonatal mesenchymal stem cells (MSCs) would be more effective than adult MSCs in accelerating healing.

This project was initiated to (i); examine the in vitro impact of various glucose culture conditions on proliferation and senescence states of HUCPVCs, a neonatal MSC source, and hBM-MSCs, an adult MSC source; and (ii) to investigate, using histomorphometry and planimetry, the healing potential of HUCPVCs and hBM-MSCs in full-thickness dermal wounds of streptozotocin induced immunocompromised mice reflecting an impaired wound healing state.
Table 1.1 Publications summary referencing the pre-clinical use of BM-MSCs in diabetic wound healing models.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>HG animal model</th>
<th>WH model</th>
<th>Cell type/ Treatment protocol</th>
<th>Data analysis</th>
<th>Findings/conclusions</th>
</tr>
</thead>
</table>
| Kuo et al., 2011 [105] | STZ-induced  Wistar rats (4-month-old) | Dorsal skin defect (6x5 cm) | ISOGENIC rat BM-MSCs (P3)- i.e. same strain 1x10^7/dose, day 7 PW | IHC: Ki-67, CD45, prolyl 4-hydroxylase, EGF, VEGF | Cell treatment:  
  • Sig. ↓ wound size compared to C. The decrease is more sig. in wounds treated twice compared to once  
  • Sig.↓ in inflammation (↓CD45), ↑ (F, K) proliferation  
  • ↑Epithelialization and neovascularization  
  • ↑ GF expression { EGF & VEGF} |
|            | STZ dose: 50 mg/kg, IP                               | Margins sutured                   | 1x10^7/dose, day 7 and 10 PW                      | H&E                 | Cell treatment:  
  • Sig. ↓ wound size compared to C. The decrease is more sig. in wounds treated twice compared to once  
  • Sig.↓ in inflammation (↓CD45), ↑ (F, K) proliferation  
  • ↑Epithelialization and neovascularization  
  • ↑ GF expression { EGF & VEGF} |
|            | n=10/group x 4 groups                                | Transparent Tegaderm used         | Follow up: day 3 & 7 PT                           |                     | Cell treatment:  
  • Sig. ↓ wound size compared to C. The decrease is more sig. in wounds treated twice compared to once  
  • Sig.↓ in inflammation (↓CD45), ↑ (F, K) proliferation  
  • ↑Epithelialization and neovascularization  
  • ↑ GF expression { EGF & VEGF} |
| Kwon et al., 2008 [106] | STZ-induced  Sprague-Dawley rats (5 wk) | 5-cm abdominal fascial incision along midline Sutured together | ISOGENIC-BM-MSCs (Cognate BioService Inc)/ (P2-6) | | Cell treatment:  
  • Both routes : ↑ collagen & ↑ GF expression  
  • Systemic:↑ tensile strength compared to control  
  • Local: Sig.↑ tensile strength compared to control |
|            | STZ in 0.1 mM SC buffer                               |                                   | Systemic (start 1 day PW): Tail IV (1.5x 10^6 cells in PBS/for 4 days; Local (right PW): 6x10^6 (50 μl) in PBS (0.5 ml)/ 10 injections, 1 mm along the entire length |                     | Cell treatment:  
  • Both routes : ↑ collagen & ↑ GF expression  
  • Systemic:↑ tensile strength compared to control  
  • Local: Sig.↑ tensile strength compared to control |
|            | STZ dose: 64 mg/kg, fasted rats for 16 hr.           |                                   |                                                                 |                     | Cell treatment:  
  • Both routes : ↑ collagen & ↑ GF expression  
  • Systemic:↑ tensile strength compared to control  
  • Local: Sig.↑ tensile strength compared to control |
|            | PZI, U40 insulin (0.5–2.0 U) used Systemic: n=7, Local: n=6 | | |                     | Cell treatment:  
  • Both routes : ↑ collagen & ↑ GF expression  
  • Systemic:↑ tensile strength compared to control  
  • Local: Sig.↑ tensile strength compared to control |
| Inoue et al., 2008 [107] | STZ-induced  LEW rats | full-thickness scalp defects (2x2 cm) | BMCs & MSCs from: LacZ-Tg LEW rats & luc-Tg LEW rats | In vivo luminescence | Cell treatment:  
  • BM-MSCs: ↑ VEGF, HIF-α  
  • At d21: BM-MSCs retention >BMCs  
  • ↑ angiogenesis (MSCs>BMC) |
|            | STZ dose: 60 mg/kg, IV, penile vein (0.1 M citrate buffer) | | Artificial dermis loaded with BMCs (1x10^7)/ PBS (200 μl) | | Cell treatment:  
  • BM-MSCs: ↑ VEGF, HIF-α  
  • At d21: BM-MSCs retention >BMCs  
  • ↑ angiogenesis (MSCs>BMC) |
Abbreviations: mHPCs: Mouse hematopoietic progenitor cells (mHPCs), enriched in Sca-1 cells. They were 28.8-33.6 % positive for sca-1, PT: Post-treatment; PW: Post-wounding; Am-SPCs: Adult murine stromal progenitor cells; SubC: Sub-cutaneous; SC: Sodium citrate buffer; C: Citrate buffer; LEW: Lewis; MBC: Modified boyden chamber; CM: Conditioned media; Protamine zinc insulin (PZI); Sig: Significant; PC: Panniculous carnosus; STZ: Streptozotocin; F: Fibroblast; K: Keratinocytes; IHC: Immunohistochemistry; H&E: Hematoxylin and eosin; IF: Immunofluorescence; PBS: Phosphate buffered saline; WH: Wound healing.

| **Wu et al., 2007** [109] | female BALB/c mice (8 wk) | 2 (6 mm) full-thickness dorsal wounds | GFP+BM-MSCs (allogeneic), Neonatal fibroblasts 1x10^6 each | Planimetry Histomorphometry | Cell treatment: 
> • ↑ wound closure, re-epithelialization, cellularity, and angiogenesis. 
> • Differentiated into K and appendages 
> • ↑ VEGF and angiopoietin-1 proteins expression in wounds 
> • BM-MSC-conditioned medium ↑ endothelial cell tube formation. 

| **Javason et al., 2006** [108] | db/db mice (C57) control mice | 8 mm excisional dorsal wound (PC intact) | Am-SPCs from BM & ficoll separated BM cells 30 mL (7.5x10^5 P33 AmSPCs, BM cells, or only PBS) injected beneath the transparent dressing onto the wound bed using Hamilton syringe 7 & 28 days PW | Histomorphometry | SPCs treatment: 
> • Sig ↑ re-epithelialization, granulation tissue formation, and neovascularization compared BM or PBS treated wounds. 
> • Day 7: Higher retention of SPCs compared with BM cells 
> • Bone formation in Am-SPCs-treated wounds was found 

| **Stepanovic et al., 2003** [200] | male congenic-db/db mice, (8-10 wk) | 2 (6 mm) full-thickness dorsal wounds | Fresh BM cells enriched for mHPCs (from control or diabetic mice) Injections, 3 days P.W. 2.5x10^5 cells in 25 μl 0.9 % NaCl 
C: 25 μl 0.9% NaCl 11 days PIn (14 days PW) | IHC:CD31 H&E | Non-diabetic bone marrow-derived cells ↑ vascularization and WH but little effect on the control 
> • diabetic-derived marrow cells ↓-vascularization but promote wound healing in diabetic mice
Table 1.2 Publications summary referencing the in vitro/in vivo use of methylcellulose.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Study type</th>
<th>Model</th>
<th>MC conc. tested</th>
<th>Cells (Y/N)</th>
<th>Findings/conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shouman et al., 2003 [160]</td>
<td>In vivo</td>
<td>Albino rabbits, 2 groups (n=5 each). 1st group injected with saline, the 2nd group with MC</td>
<td>2% injected</td>
<td>N</td>
<td>• A reduction in intraocular pressure was noted in MC treated group</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• No inflammatory cellular infiltrate (MC is tolerable)</td>
</tr>
<tr>
<td>Gallik et al., 1993 [139]</td>
<td>In vitro</td>
<td>Cell culture (DMEM+F12 media + various MC concentrations)</td>
<td>1%</td>
<td>Y</td>
<td>• MC (1-2 %) stimulate bovine aortic endothelial cell and rat mesothelial cell proliferation in vitro and have no effect on cell morphology or viability.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.75%</td>
<td></td>
<td>• No sig. differences between the 3 concentrations on cell proliferation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wells et al., 1997 [161]</td>
<td>In vivo</td>
<td>Nerve gap injury model in rat sciatic nerve. Comparison of laminin, collagen, MC with/or without PDGF-BB &amp;IGF-I</td>
<td>2%</td>
<td>N</td>
<td>• MC with and without GFs showed better nerve regeneration and functional outcome compared to other matrices, with or without GFs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Significant improvement was noted when MC and collagen matrices combined with GFs were used</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total volume injected (25 μl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tate et al., 2001 [162]</td>
<td>In vitro</td>
<td>Cortical impact injury (Rats) MC (30 μl) microinjected into the brain after 1 wk post-injury</td>
<td>In vitro: 4, 5, &amp; 8 % in 1X D-PBS; 2 &amp; 8 % in 5X PBS; 5 % in H2O, 1X, 2X, 5X, and 10X D-PBS</td>
<td>In vitro: Y (Astrocytes and primary neurons) In vivo: N</td>
<td>• Conc. up to 8% did not elicit cell death in primary rat astrocytes or neurons at 1 or 7 days</td>
</tr>
<tr>
<td></td>
<td>In vivo</td>
<td></td>
<td></td>
<td></td>
<td>• MC did not alter the size of the injury cavity or change the patterns of gliosis as compared to injured vehicle-injected rats</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• MC can be used as a biocompatible injectable scaffold for brain injury</td>
</tr>
</tbody>
</table>
| **Petal et al., 2010** | **In vivo** Moderate thoracic contusion injury. | **5% in D-PBS** Y: GFP labeled rat Schwan cells (SC), $2 \times 10^6$ | **At 12 weeks post implantation:**
- ECM (laminin:collagen) & Matrigel matrices enhanced cell survival better than media or MC
- Lowest cell survival was with MC
- ECM (laminin:collagen) & Matrigel matrices enhanced axonal growth, revascularization, and functional outcome better than MC and media. |

**Abbreviations:** MC: Methylcellulose; ECM: Extracellular matrix; Conc.: Concentration.
Chapter 2

Materials and Methods

2.1 Cells

2.1.1 Source

All cells used in this study were kindly provided by Tissue Regeneration Therapeutics Inc. (TRT), Toronto, ON, Canada. Human Umbilical Cord Perivascular Cells (HUCPVCs) and Human Bone Marrow Mesenchymal Stem cells (hBM-MSCs), either at passage 1 or 2, were received as (1ml) frozen aliquots in cryogenic vials and stored in a Liquid Nitrogen (LN$_2$) freezer (isothermal v-1500 series, CBS, SANYO, Bensenville, IL, USA) until needed.

2.1.2 Culture

Cells were first thawed from LN$_2$ and plated to recover from the effect of cryopreservation before using them in all in vitro and in vivo studies performed. This process, in short, started by the removal of cryovials from LN$_2$ and their quick submergence in a water bath set to 37-40 °C. After thawing, 1ml of LG-DMEM (Low Glucose-Dulbecco's Modified Eagle Medium, 1X, GIBCO®, Invitrogen, Grand Island, NY, USA) was aseptically added very slowly to each cryovial and left for 2 minutes at room temperature (RT), inside a biosafety cabinet. Afterwards, the whole content of the vials were transferred to 15mL polypyrrole conical centrifuge tubes (BD Falcon™, Franklin Lakes, NJ, USA) where another 2 mL of LG-DMEM was added. After another 2 minutes, a final 6 mL of LG-DMEM was added and the tubes were centrifuged at 1150 rpm in (Sorvail® Legand RT, Kendro SORVAIL, Asheville, NC, USA) centrifuge for 5 minutes at 4 °C.

After centrifugation, the supernatant was removed and the pellet was re-suspended in a supplemented medium SM [80% LG-DMEM, 10% antibiotic stock solution (10x){167 units/ml Penicillin G (Sigma-Aldrich, St. Louis, MO, USA); 50 µg/ml Gentamicin (Sigma-Aldrich, St. Louis, MO, USA); 0.3 µg/ml Amphotericin B (Fungizone) (Sigma-Aldrich, St. Louis, MO, USA), and 10% FBS (Fetal Bovine Serum-Qualified, GIBCO®, Grand Island, NY, USA)]. Cells were then counted using a cell viability analyzer (Vi-Cell™ XR, Beckman Coulter Inc., Brea, California, USA). Cells were then plated in T-75 flasks (BD Falcon™ 75 cm$^2$ Cell Culture Flask, Franklin Lakes, NJ, USA), incubated at 37 °C in a humidified environment of 95% air and 5% CO$_2$ (Binder CO$_2$ incubator CB...
53 series, Tuttlingen, Germany), and the medium was changed every other day. Upon reaching 80-90% confluence, examined under phase contrast microscope (Nikon Diaphot, Nikon Instruments Inc., Melville, New York, USA), cells were washed first with D-PBS (−/−) [Dulbecco’s Phosphate Buffered Saline (without calcium chloride and magnesium chloride), GIBCO®, Grand Island, NY, USA], trypsinized using 0.08% trypsin (0.25% Trypsin-EDTA,1 X, GIBCO®, Grand Island, NY, USA), and eventually used for various in vitro and in vivo studies (described in the following sections).

2.2 In vitro studies

2.2.1 MSCs proliferation under various glucose conditions

2.2.1.A Ki-67 proliferation marker

Ki-67 is a nuclear protein that is expressed during all phases of the cell cycle (i.e. G1, S, G2, and M phases) except for non-diving cells (i.e. G0). This protein has been widely used as a proliferation marker to assess the percentage of cell growth of a certain cell population when exposed to various treatment conditions [113]. Due to these properties, Ki-67 was chosen to analyze the proportion of proliferating cells in both HUCPVC and hBM-MSC populations as a function of glucose concentration.

2.2.1.B Proliferation assay: Experimental design

Plated HUCPVCs and hBM-MSCs (Passage 3, n=4 donor each) were trypsinized and counted when they reached 80-90% confluence as mentioned above (Section 2.1.2). The number of viable cells generated was used to calculate the required number of cells for seeding. Cells were then cultured on sterile 13mm round glass coverslips (VWR, West Chester, PA, USA) placed into 24-well plates (BD Falcon™, Franklin Lakes, NJ, USA). Prior to seeding, the glass coverslips were dipped in 70% ethanol for 15 min then allowed to dry inside the biosafety cabinet. Afterwards, they were carefully placed into the 24-well plate and UV sterilized for 1 hr.

We have found out from a pilot study performed prior to conducting this study that, under normal culture conditions, there was a difference in proliferation capacity between HUCPVCs and hBM-MSCs, an observation previously reported in the literature [123]. Therefore, cells were seeded on sterilized coverslips at different densities (HUCPVCs: 6000 cells/well and hBM-MSCs: 8000 cells/well). This measure allowed both cell types to reach around 70-80% confluence by day 7 needed to ensure the validity of the assay. Confluence has been previously reported to decrease the expression of Ki-67 due to the increase in the number of cells in G0 [169].
DMEM media supplemented with 5% FBS, 10% antibiotic solution and 3 different concentrations of glucose: 2 mM, 5.5 mM, and 25 mM were used for cell cultivation. These 3 concentrations were chosen to reflect hypoglycemic, normoglycemic, and hyperglycemic in vitro conditions respectively. Commercially available media, LG-DMEM (Dulbecco's Modified Eagle Medium 1X, contains 1000 mg/L D-glucose, GIBCO®, Grand Island, NY, USA) and HG-DMEM (Dulbecco's Modified Eagle Medium, 1X, contains 4500 mg/L D-glucose, GIBCO®, Grand Island, NY, USA), served as our 5.5 mM, and 25 mM glucose culture conditions. On the other hand, the 2 mM condition was prepared by adding D(+)glucose (Sigma-Aldrich, St. Louis, MO, USA) to the commercially available NO-glucose-DMEM (Dulbecco's Modified Eagle Medium, 1X, without glucose, GIBCO®, Grand Island, NY, USA) then filter sterilized (0.22 µm filters, Millex® GP syringe filters, Millipore, Billerica, MA, USA) before use. Plates were incubated at 37 °C in a humidified environment of 95% air and 5% CO₂ for 7 days and media was changed every other day.

2.2.1. C Ki-67 Immunocytochemistry/immunofluorescence protocol

At day 7, cells were first washed with PBS (+/+ ) [Dulbecco’s Phosphate Buffered Saline (with calcium chloride and magnesium chloride), GIBCO®, Grand Island, NY, USA] twice, 3 minutes each, then fixed with 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes at RT. Cells were then washed with PBS (+/+ ) 3 times, 3 minutes each, and were then permeabilized using 0.05% Triton X-100 (BioShop Canada Inc., Burlington, ON, Canada) for 10 minutes at RT, on a shaker. Afterwards, cells were washed again with PBS (+/+ ) 3 times, 3 minutes each, then incubated with a blocking buffer (1% BSA in PBS) [Albumin Bovine Fraction V. heat, Biotechnology Grade, Bioshop Canada Inc., Burlington, ON, Canada] for 1 hr at RT.

After another washing round, Ki-67 primary antibody (mouse monoclonal Ki67 antibody IgG, ab8191, cross-reacts with human, 0.15 mg/ml, Abcam®, Cambridge, MA, USA) was diluted (1:300) in the blocking buffer and 50 µl aliquots of the antibody were placed on a sheet of parafilm (Parafilm® M, Structure Probe Inc., West Chester, PA, USA) inside a humidity chamber. Coverslips [with cell monolayer grown on the surface examined under a phase contrast microscope (Nikon Diaphot)] were carefully lifted from the wells and placed upside down on top of the diluted antibody droplets (cells facing down). A positive control was included in the experiments in which (HeLa cells) were used and also incubated with the primary antibody. As a negative control, we used either HUCPVCs or hBM-MSCs but without the incubation with the primary antibody; blocking buffer was used instead. The chamber was then incubated at 4 °C overnight.
The next day, coverslips were carefully lifted up and returned to the 24-well plates and washed twice with PBS (+/+) as indicated above. A secondary antibody (Alexa Fluor® 488 goat anti-mouse IgG (H+L), A-11001, 2 mg/ml, Invitrogen, Molecular Probes Inc., Eugene, OR, USA) was diluted (1:500) with the blocking buffer and 50 µl aliquots were placed on a sheet of parafilm inside a humidity chamber. Coverslips were lifted and placed again on the diluted antibody droplets the same way mentioned above and incubated in the dark for 1hr at RT. Positive and negative control cells were also processed the same way. After the incubation period, coverslips were returned to the 24-well plates and washed with PBS (+/+) then (-/-), 1 time each. They were then counterstained with Hoechst 33342 (stock 10 mg/ml, Invitrogen, Molecular Probes Inc., Eugene, OR, USA) at a dilution of 1:5000, also by placing coverslips on the dye droplets placed inside a chamber protected from light for 5min at RT. Coverslips were finally rinsed twice with PBS (-/-) and stored at 4 °C protected from light until imaged.

### 2.2.1.D Imaging and data analysis

Fluorescently labeled cells were viewed, under appropriate filters, using a live cell imaging microscope (Olympus IX81, Centre Valley, PA, USA) under a (10x) objective lens and seven random fields of view from each coverslip were imaged using a CoolSNAP _HQ^2_ camera (Photometrics, Tucson, Arizona, USA) and Metamorph software (Meta imaging series, version7, Molecular Devices, Downingtown, PA, USA). The total number of cell nuclei expressing the Ki-67 marker (bright green) and the total number of nuclei present in each image (blue) were counted and data was entered into an Excel™ spreadsheet. Proliferation index of each cell type under various culture conditions was calculated according to the following formula:

\[
\text{Ki-67 proliferation index} = \frac{\text{Total no. of Ki-67 positive nuclei (green)}}{\text{Total no. of nuclei (blue)}} \times 100
\]

### 2.2.2 Mesenchymal cells senescence under various glucose conditions

#### 2.2.2.A Senescence associated-β-galactosidase assay (SA-β-Gal)

This assay is designed to cytochemically detect the expression of senescence associated-β-galactosidase (SA-β-Gal) activity in senescent cells (not in pre-senescent, quiescent or cancer cells) at pH6 using 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-Gal stain). Other lysosomal β-galactosidases are usually expressed at pH 4 [201].
2.2.2.B  Experimental design
HUCVCs and hBM-MSCs (Passage 3, n=2 donors each) were cultured following the same experimental design mentioned for the proliferation assay (Section 2.2.1.B) except that cells were directly cultured into 24-well plates.

2.2.2.C  SA-β-Gal labeling procedure
Senescence Detection Kit (Biovision, Cat#K320-250, Mountain View, CA, USA) was used as per the manufacturer’s instruction to determine the expression of SA-β-Gal activity in our cell cultures. Briefly, at day 7, cells cultured under different culture conditions were washed with PBS (+/+ ) then fixed with the fixative provided in the kit for 10 min at RT. Then, wells were washed twice with PBS (-/- ) and X-Gal labeling solution (pH 6) was prepared and added to all wells. As a positive control: same cells used were stained with X-Gal staining solution adjusted to (pH 4) to label lysosomal β-galactosidases present in all cells. Also, aged cells [HUCPVCs (P 15) and hBM-MSCs (P 14)] were labeled with X-Gal solution (pH 6) and served as a positive control to show the expression of SA-β-Gal. As a negative control: early passage embryonic human fibroblasts were used and stained with a pH 6 staining solution. Plates were then incubated at 37 °C incubator (without CO₂) (Sanyo, Wood Dale, IL, USA) for 16-18 hr. The next day, the labeling solution was removed and wells were washed twice with PBS (-/- ) and 70% glycerol (Caledon Laboratories Ltd., Georgetown, ON, Canada) was added to the wells for long-term storage. Plates were kept at 4 °C until viewed.

2.2.2.D  Imaging and data analysis
Cultures were viewed under an inverted microscope (Leitz® Diavert, Wetzlar, Germany) and 6-10 random fields of view for each well were taken using a QImaging camera (QImaging micropublisher 5-RTV, Surrey, BC, Canada) and Q-Capture software (version 2.90.1, Surrey, BC, Canada). The total number of blue-stained cells (positive for the senescence marker) and the total number of cells present in each image were counted and data was entered into an Excel™ spreadsheet. The percentage of the SA-β-Gal positive cells of each cell type under various culture conditions was calculated according to the following formula:

\[
\text{Senescence \%} = \frac{\text{Total no. of senescent cells (blue)}}{\text{Total no. of cells}} \times 100
\]
2.3 In vivo study

2.3.1 Ethical approval

All animal work was approved by the University of Toronto Animal Care Committee (Protocol #20008875) and was conducted in accordance to the guidelines set by the Department of the Comparative Medicine (DCM)/University of Toronto (UofT). All animal procedures and handling were done inside a biosafety cabinet.

2.3.2 Animals

A total of 20 male NOD-scid-gamma (NSG) mice strain (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, stock no: 005557) were employed in this study. Mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) at 7 weeks of age, housed individually for 1 week, acclimatization period, at the DCM facility/UofT in a sterile condition under a temperature and humidity-controlled environment with free access to food and water.

2.3.3 Pilot studies

2.3.3.A Scope

NSG mice lack mature T and B cells, functional NK cells, and have defective signaling through several cytokines which make them a better choice than other immunodeficient mice strains for xenotransplantation studies. This animal strain is fairly recent and, to our knowledge, has been only used in diabetes and transplantation studies [157, 202] but not wound healing. Therefore, conducting pilot studies was imperative before commencing our project in order to tackle the following issues:

1. To establish a reproducible protocol for STZ-induction
2. To establish a working procedure for full-thickness dermal defects creation
3. To evaluate whether wound healing is delayed in STZ-induced mice compared to non-STZ induced controls
4. To establish a working method to deliver the cells
5. To monitor animals’ response to surgery and wound coverage with Tegaderm™ dressing
6. To study and optimize skin harvest techniques for histology

Discrete pilot studies were performed to work on the issues mentioned above. The total number of animals used was (8) divided into 4 studies (n=2 each). We found that by day 7, untreated wounds (n=4) in one control mouse showed >50% closure compared to ~30% closure in (n=4) wounds of one hyperglycemic mouse (see Results chapter). Therefore, tracking wounds for 7 days post-
wounding seemed to be a reasonable time to detect differences in wound healing between treated and untreated wounds. The time-line used for all in vivo studies except the 1st pilot study (run for 14 days) is shown below, Fig. 2.1.

<table>
<thead>
<tr>
<th>STZ</th>
<th>Verify ↑ RBG</th>
<th>Surgery / Treatment</th>
<th>Sacrifice/Tissue harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Day 2</td>
<td>Day 5</td>
<td>Day 12</td>
</tr>
<tr>
<td>✔ G/Wt</td>
<td>✔ G/Wt</td>
<td>✔ G/Wt</td>
<td>✔ G/Wt</td>
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<tr>
<td></td>
<td></td>
<td>✔ Imaging (d0)</td>
<td>✔ Imaging (d7)</td>
</tr>
</tbody>
</table>

Figure 2.1 In vivo study timeline. Abbreviations: STZ: Streptozotocin; G: Glucose measurement; Wt: Weight measurement; d: Day; RBG: Random blood glucose.

2.3.3.B Developing a hyperglycemic animal model

The total number of animals used for the evaluation of this step was 8 and data were collected and analyzed from 4 separate studies. During the acclimatization period, mice were handled regularly where they were subjected to mock, later to be used, procedures including restraining, tail pricking, and body weighing.

After the one week adaptation period, hyperglycemia was induced in NSG mice, Fig. 2.3, using a single intraperitoneal (IP) injection of Streptozotocin (STZ) [Streptozotocin mixed anomers (≥75% α-anomer basis, ≥98% HPLC, powder), Sigma-Aldrich, S0130-1G, St. Louis, MO, USA], at 150 mg/kg body weight in Sodium-Citrate buffer pH 4.6 [prepared by mixing 0.1 M Sodium citrate (Fisher) and 0.1 M Citric acid (Sigma) then filter sterilize (0.2 µm filter- Millipore)]. The process started by measuring animals' random blood glucose (RBG) and weight before induction (baseline reading- day 0); then fasting them by just removing food from their cages- not water, for 4-6 hours until induction time. A mouse restrainer (Restrainer Rodent BR 1 INDX3.3, Fisher Scientific, Ottawa, ON, Canada) was used to capture the animals for blood sampling. Blood samples were taken by pricking the tail vein with a 25G 5/8 needle (BD, Franklin Lakes, NJ, USA) and RBG was measured (in mmol/L values) using a glucometer (Free Style Lite®, Abbott Diabetes Care ltd, Alameda, CA, USA). Animals’ weight (in gram values) was measured simultaneously using a balance where animals were placed in a closed container.
Based on the weight measured, STZ dose was prepared. Dose calculation was further adjusted to compensate the volume of solution lost in the syringe needle. STZ was weighed in the lab and individual doses were kept in eppendorf tubes on ice, protected from light. Sodium-citrate buffer (0.1 M, pH 4.6) was prepared fresh that day and kept in a 15 ml polypropylene tube (BD, Franklin Lakes, NJ, USA) also on ice. See Appendix A for STZ dosing chart and Appendix D.1 for the induction protocol.

At the induction time, STZ was mixed with the buffer right before injection and administered intraperitoneally within 2 min after dissolving using 1cc syringe and 25G 5/8 needle (both from BD). Random blood glucose measurement was taken after 48 hr. to confirm induction success where a value of >10 mmol/L is considered hyperglycemic. The choice of this cut-off value is explained in (Chapter 3: Section 3.2.3.A).

2.3.3.3 Developing a wound healing surgical model

The total number of animals used for the evaluation of this step was 8, from 4 separate studies. At day 5 post-induction, RBG was measured to further confirm hyperglycemia state in the induced mice before surgery conduction; weight measurements were also taken. Surgery started by anesthetizing the animals, one at a time, in accordance with the regulations of anaesthesia induction in mice recommended by the DCM/UofT, by inhalation of isofluorane in O₂ (5% induction, 1.5-2.5% maintenance). Then, the analgesic (buprenorphine, 0.05-0.1 mg/kg) was subcutaneously given to reduce pain after surgery. Dorsal hair was removed using an electric clipper and, only for 2 mice, a hair removal product (Nair, www.nairpretty.ca, ON, Canada) was later applied to remove remaining fine hairs. The skin was finally swabbed with 70% alcohol and 10% betadine.

The mouse was then placed on a solid surface and dorsal skin was folded along the midline; the animal was flipped on his side in order to rest the folded skin on a solid surface. While holding the skin between two fingers with subtle stretching, two full-thickness dermal wounds, including the Paniculus carnosus muscle layer, parallel to the dorsal midline, were created using a sterile (4 mm in diameter) biopsy punch (AcuPunch, Acuderm Inc., Ft. Lauderdale, FL, USA). This resulted in 4 circular dorsal wounds that span around (0.5 cm) from the midline and around (1 cm) from each other, Fig. 2.3 and Fig. 2.4.

Wounds were then imaged using a digital camera (Sony Cyber-Shot, 12.1MP, JAPAN) fixed to a tripod with an approximate height of 8 cm (images represent day 0 readings). A tag (mouse number and surgery date) in addition to a ruler (mm) were placed in the field of view before capturing the
image. For some animals, surface view images were taken, but due to the natural dorsal curvature of the mice, the border of most wounds was not very clear; therefore, mice were slightly flipped to their left and right sides and two wounds were imaged at each time to decrease wound area calculation errors.

For animals who did not receive treatment, wounds were covered right after taking the pictures using a transparent film dressing (Tegaderm™, 3M, #1622W, 4.4×4.4 cm, MN, USA). For some animals, two sterile Elastoplast adhesive bandages (Beiersdorf Canada Inc., St-Laurent, Québec, Canada) were placed on top of the Tegaderm dressing and wrapped in opposite directions around the animal trunk. Animals who received treatment were processed as described in Section 2.3.3.D.4. After surgery, animals were left to recover from anesthesia under a heat lamp for 15-30 min before they were returned to their respective space in the holding room at the animal facility; in most cases, however, mice were left on a heat pad overnight due to their weak body condition after surgery.

2.3.3.D Evaluating wound healing in normal and STZ-induced NSG mice
The percentage decrease in wound area at day 7 compared to day 0 was evaluated from the digital images taken at these 2 time-points from untreated wounds (n=4) of one hyperglycemic and one normal control mouse. Skin samples obtained were processed as mentioned below but results obtained from only (2.3.3.D3) were used for the evaluation.

2.3.3.D1 Sacrifice and tissue harvest
To evaluate tissue harvest techniques used, n=6 animals were used to compare results obtained for further optimization of this process.

At day 7 post-wounding, animals were euthanized by CO₂ inhalation followed by cervical dislocation at the animal facility. The dressing was carefully removed (if it was still attached) and wounds were imaged the same way mentioned above (day 7 reading). For some animals, the 4 dorsal wounds were excised as a big square and then each wound was individually cut to produce a smaller square with an ample tissue surrounding each wound. Some wounds were cut through the center (of the wounds’ largest diameter) before fixation while others left intact and bisected through the center before paraffin embedding. All samples were pinned to a flat piece of cork to prevent skin rolling and were fixed in 10% neutral buffered formalin (Sigma) for 24 hour at 4°C. Other animals were left intact (whole body) in the fixative and were processed as described in Sections 2.3.4.C and 2.3.4.D.
2.3.3.D2 Skin samples processing for histology

The following day, skin samples were washed with D-PBS (−/−) on a shaker for 1 hr at RT. They were then dehydrated using increasing grades of ethanol (70, 95, and 100 %), 1 hr each at RT also on a shaker, to remove any traces of water. Then, they were left in methyl benzoate (Sigma, # M29908) overnight at 4 °C. The next day, samples were cleared with xylene (Caledon Laboratories Ltd.) for 1 hr at RT on a shaker then infiltrated with melted paraffin (POLYFIN™ Embedding and Infiltration Paraffin, Triangle Biomedical Sciences®, Durham, NC, USA) in a vacuum oven (Fisher Scientific, model 280) set at 60 °C for 1 hr. The infiltration was repeated 3 times, last step was done under pressure (20"Hg). An off-center cut was made for each wound (Fig. 2.5) and the largest part was embedded vertically in paraffin (cut surface facing the bottom of the metal mold) using (Leica EG 1160 microsystems embedding station, Nussloch, Germany). After solidification, samples were kept at 4 °C for 24 hr. The next day, samples were trimmed using a Leica microtome (American Optical 820 Rotary Microtome, Richmond, Illinois, USA) and left in tissue softener (Molifex, EMD, Gibbstown, NJ, USA) for a few days before sectioning. Paraffin blocks were sectioned at 6-7 µm thickness. Slides were then processed for routine H&E staining (see Appendix D.3). Stained slides were viewed using a (Leitz Aristoplan, Germany) microscope and imaged using (Q-imaging micropublisher 5 camera) and Q-Capture software (version 2.90.1, Surrey, BC, Canada).

2.3.3.D3 Wound Image analysis

Digital images of wounds without the dressing were taken at day 0 and 7, and were analyzed using Image Pro analysis software 6.2 (Media Cybernetics Inc., Bethesda, MD, USA) where the wound surface area was measured (in mm²) at these 2 time-points. Each image was calibrated in (mm) length units according to the ruler that was placed in the field of view of that image. Explanatory steps to this process are shown in Fig. 2.2. The percentage of wound closure was calculated according to the following formula:

\[
\text{Wound closure %} = \frac{\text{Wound area (day0)} - \text{Wound area (day7)}}{\text{Wound area (day0)}} \times 100
\]
2.3.3.D4 Establish a wound treatment protocol

The application of treatment interventions: sham (cell delivery material only-methylcellulose MC), HUCPVCs in MC, and hBM-MSCs in MC, were studied in (n=4) mice in order to evaluate the ease of treatment application using methylcellulose (MC) and animals’ response to treatment.

- **Cell culture and dose preparation**

  HUCPVCs and hBM-MSC (each from 4 different donors and at 2 different passage numbers, P2 and P3) were cultured 4-5 days before surgery in LG-DMEM SM (10% FBS, 10% antibiotics). At the surgery day, both cell types were trypsinized, pooled, centrifuged, and resuspended in PBS and counted using Vi-Cell® (Beckman Coulter) as mentioned before. The viable cell number generated was then used to prepare the required dose for each cell type. Methylcellulose, the cell delivery material, was kindly prepared and provided by Limin Guan, TRT, at a concentration of 2% in sterile PBS and was further filtered using 0.22 µm filter (Millipore). A final volume of 5µl was chosen to adequately fill the 4 mm size wounds created according to skin thickness (epidermis + dermis) measurements obtained from histological sections.

  Cells were applied at 2 different densities: 0.05x10^6 cells/5µl/wound (n=2) and 0.1x10^6 cells/5µl/wound (n=2), and all cell doses were prepared by mixing equal volumes (1:1) of cell suspension with 2% MC in 0.5 ml eppendorf tube (VWR, West Chester, PA, USA) (for example, to prepare a dose of 0.1x10^6 cells/5µl, 10^6 cells were suspended in 25 µl PBS then added to 25 µl MC in 0.5 ml eppendorf tubes). Sham treatment solution was prepared by mixing 2% MC with PBS (1:1). Eppendorf tubes containing our 3 treatment types (HUCPVCs in 1% MC, hBM-MSCs in 1% MC, and 1% MC in PBS) were kept on ice and taken to surgery right after preparation.

- **Treatment regimen**

  Wound treatment was carried out right after wounding where 5 µl of each treatment type was topically applied to their respective wounds using an automatic pipette (Gilson, # M218006, Middleton, WI, USA) and yellow tips (Axygen, T-200-Y, Union City, California, USA). In each animal, three wounds received the treatment mentioned above and one wound was left without treatment to serve as an internal control. Wounds were then covered with the dressing.
2.3.4 Main studies

After the challenges associated with each step of the in vivo study were identified and protocols were optimized, we started the main study using \((n=10)\) mice.

2.3.4.A Experimental outline

The study started by inducing hyperglycemia in study animals using STZ according to the protocol described earlier in section 2.3.3.B. After 48 hr, hyperglycemia was checked and surgery started after 5 days post-induction (as explained in section 2.3.3.C). Treatment preparation was done as mentioned earlier using a cell dose of \(0.1 \times 10^6\) cells/5µl/wound. A Latin square design (Fig. 2.3-E) was utilized in the application of various treatment types to eliminate anatomical location variations in the obtained results. Wounds were then imaged as mentioned above and covered with only transparent film dressing (Tegaderm\textsuperscript{TM}, 4,4 x 4,4 cm, # 1622W, 3M, MN, USA). Animals were left to recover from anesthesia as mentioned previously. An illustration summarizing the in vivo experimental design is given in Fig. 2.3 and a pictorial guide to the surgical procedure is shown in Fig. 2.4.

2.3.4.B Mice health monitoring

Throughout the entire course of the study, mice overall health and behavioral changes were closely monitored. Interventions were undertaken when necessary.

2.3.4.C Sacrifice and tissue harvest

At day 7 post-wounding, animals were euthanized by CO\textsubscript{2} inhalation followed by cervical dislocation at the animal facility. The dressing was carefully removed and wounds were imaged the same way mentioned earlier (day 7 reading). The whole animals’ body was fixed in 10% neutral buffered formalin for 24 hr at 4 °C. Small pins were used to slightly stretch the skin around the wounds to reduce skin folding while in the fixative. The next day, the 4 wounds were excised as a big square along with the underlying spine, ribs, and dorsal musculature and pinned to flat piece of cork and returned back in the fixative and left for another day. Then, the excised skin was cut in half along the spine and carefully freed from the backbone and the ribs using a small surgical scissor and a blade under a dissecting microscope (Heerbrugg Switzerland Wild M3Z, Type B, Germany). The freed pieces of skin were then pinned again to the piece of cork and returned to the fixative and left for another 24 hr at 4 °C before tissue processing for histology. More details on tissue harvest and processing are provided in Fig. 2.5 and Appendix D.3.
2.3.4.D  Skin samples processing for histology

The following day, the skin samples were first imaged under the dissecting microscope (Heerbrugg Switzerland Wild M3Z, Type B, Germany) for further documentation and then washed, dehydrated, cleared, and embedded (see Appendix D.3). Prior to embedding, each individual wound was cut as a small square with enough surrounding tissue, a final cut was then made across the widest wound diameter far from the centre (closer to the wound margin). According to this method, the size of the wound starts small and then gets wider as sectioning continues towards the center of the wound and then gets smaller again. All slides obtained from each wound were compared to determine the exact center of that specific wound. Slides were then processed for routine H&E staining. Stained slides were viewed using a (Leitz Aristoplan, Germany) microscope and imaged using Q-imaging camera and Q-Capture software (version 2.90.1, Surrey, BC, Canada).

2.3.4.E  Wound analysis

2.3.4.E1  Wound image analysis

Digital images of wounds without the dressing were taken at day 0 and 7, and were processed for planimetric analysis (wound surface area - mm$^2$) as described earlier using Image Pro analysis software (6.2).

2.3.4.E2  Histomorphometry

Digital micrographs of H&E stained sections of wounds received various treatment types from all animals were analyzed using Image Pro analysis software (6.2). Parameters selected for measurements are shown in Fig. 2.6 and they include:

1. Epithelial gap: measured as the (mm) distance of the horizontal line drawn between the lateral edges of the newly formed epithelium.

2. Wound size (width): measured as the (mm) distance of the horizontal line drawn between the lateral margins of the dermal layer (adjacent to the newly formed granulation tissue) starting from the last hair follicle seen from both sides. Original dermal layer is distinguished from the newly formed granulation tissue by the unique appearance of collagen fibers and the presence of hair follicles and sebaceous glands.

3. Neo-epithelial layer length: measured as the (mm) distance of the newly formed epithelium (recognized as a hypertrophic layer extended on top of the granulation tissue) from both sides. Measurements started from the last hair follicle seen on each side.
4. Neo-epithelial layer thickness: three straight lines, across the entire newly formed epithelium on each side, were drawn and measured in (mm) and averaged.

Calculations made include the following:

a) The percentage of re-epithelialization was calculated from values obtained from steps 2 and 3 previously mentioned according to the formula:

\[
\text{Re-epithelialization } \% = \frac{\text{Neo-epithelium length (mm) from both lateral sides}}{\text{Wound width (mm)}} \times 100
\]

b) The percentage decrease in wound size was calculated from values obtained from steps 1 and 2 previously mentioned and according to the formula used for image analysis.
2.4  Statistical analysis

Microsoft Excel™ 2010 was used for data calculation and both Excel™ and PASW 18 software were interchangeably used for statistical analysis. A p-value of (<0.05) was considered statistically significant.

2.4.1  In vitro studies

Ki-67 proliferation indices generated from all experimental groups of both cell types were averaged and recorded as (mean±SD). Differences between experimental groups were analyzed using unpaired t-test. Senescence % for both cell types under various conditions were averaged and recorded as (mean±SD) but no statistical analysis was performed due to small sample size.

2.4.2  In vivo studies

Data was recorded as (mean±SD) except for raw wound area values (mm$^2$) at day 0 and 7 that were left without manipulation for box plot graphing. Paired-t-Test was used to compare wound areas of various treatment groups at day 0 and 7. One-Way-ANOVA was used to compare various treatment groups and wound closure. Repeated Measures ANOVA was used to compare animals’ glucose and weight at various time-points post-induction.

Figure 2.2 Wound planimetric analysis. Digital images taken at day 0 and 7 were both processed using image pro analyzer (6.2) software. (A) Representative image of a day 0 wound; (B) Wound’s margin was manually traced using the “wand tool”; (C) the count size function was used to measure the area “mm$^2$”.
Figure 2.3 Schematic illustration of the in vivo study design. (A) STZ induction in NSG mice; (B) Checking blood glucose level after 48 hr post-induction to verify hyperglycemia (successful if >10 mmol/L); (C) Treatment preparation: Cells (HUCPVCs and hBM-MSCs suspensions mixed separately with MC, 1:1); Sham: MC in PBS (1:1); (D) Creating 4 full-thickness dermal defects on the mouse dorsum using the punch technique; (E) Diagram showing the Latin Square model used to administer our treatment types; (F) Data analysis. Abbreviations: HUC: HUCPVCs in MC; BM: hBM-MSCs in MC; Sham: Methylcellulose (MC) in PBS (delivery material); X: No cell or delivery material; d: Day.
Figure 2.4 Summary of the surgical procedure. (A) General view of the surgical area set-up; (B) Mouse dorsum was shaved with an electric clipper; (C) Nair hair removal product was used to remove fine hair left from previous step; (D) Mouse dorsal skin was swabbed with 70% EtOH and Betadine and then the mouse was laid on a solid surface; (E) The dorsal skin was folded by the midline; (F) Two full-thickness dermal defects were made using a (4mm) punch; (G) Treatments were topically applied using a pipette according to our Latin-square model mentioned before; (H) Tegaderm™ transparent dressing was placed on the treated wounds.
Figure 2.5 Wound harvest procedure. (A) Whole mouse body was fixed in 10% neutral buffered formalin for 24 hr, at 4 °C; (B) The wounded area along with the underlying spine and dorsal musculature were cut with ample tissue around all wounds to give a square-shaped piece; (C) The excised tissue was then pinned to a flat piece of cork; (D) The sample was returned to the fixative (tissue facing down) and left at 4 °C, 24 hr; (E) Skin samples were freed from the spine and the ribs, pinned back to the cork, and left in the fixative for another 24 hr, at 4 °C; (F) Image showing the cut position of a wound sample before embedding in paraffin.
Figure 2.6 Histomorphometric measurements. Representative H&E stained histological wound section (center) showing histomorphometric measurements procedure. (1) Epithelial gap (horizontal distance between neo-epithelium edges); (2) Wound size (horizontal line between the lateral wound margins); (3 & 4) Blue lines represent the length of the newly formed epithelium on both sides; (5 & 6) The thickness of the newly formed epithelium (an average of 3 measurements on each side was taken). (7) The thickness of the wound bed (an average of 3 measurements was taken); Dotted lines (…) : Guidelines denoting lateral wound margins; Dashed lines (- - -): Guideline denoting the lateral neo-epithelial edges.
Chapter 3

Results

3.1 In vitro studies

3.1.1 Ki-67 Proliferation index

The fraction of proliferating cells, both HUCPVCs and hBM-MSCs, under various glucose culture conditions was quantified as described earlier (Chapter 2: Section 2.2.1.D). Representative images of both cell types cultured under the 2, 5.5, and 25 mM glucose culture media are shown in Fig. 3.1-A and their proliferation index under each condition is given in Fig. 3.1-B. Cells stained with only the secondary antibody (negative control) did not show any positive Ki-67 staining (image not shown).

It can be noted that HUCPVCs exhibited a higher proliferation index under the normal (5.5 mM) condition and lower indices under both the hypo- (2 mM) and the hyperglycemic (25 mM) conditions. Significant differences were found between the 2mM and the 5.5mM conditions (14.32±5.58 vs. 55.96±8.68 %, P<0.001) and between the 2mM and the 25mM conditions (14.32±5.58 vs. 49.61±10.56 %, P<0.01).

A similar expression trend was detected in in hBM-MSCs cultures where their proliferation index was higher under the normal condition and lower under both the hypo- and the hyperglycemic conditions (32.31±13.49 vs. 19.22±6.38 and 27.49±14.56 % respectively); however, these differences did not reach statistical significance. Interestingly, HUCPVCs had significantly higher proliferation index under normal and hyperglycemic conditions compared to hBM-MSCs, but the difference was statistically significant only under the normal condition (55.96±8.68 vs. 32.31±13.49 %, P<0.05).
Figure 3.1 Proliferation of HUCPVCs and hBM-MSCs under various glucose culture conditions.

(A) Representative images of day 7 cultures of HUCPVCs (upper row) and hBM-MSCs (lower row) under 2, 5.5, and 25 mM glucose culture conditions. Ki-67+ve nuclei (bright pale green - white arrows) indicates proliferating cells; Hoechst 33342 (blue) used as a nuclear counterstain. Scale bar=50 µm.

(B) Proliferation index of HUCPVCs and hBM-MSCs under various glucose culture conditions. Values plotted represent (mean±SD) of (n=4) for HUCPVCs and (n=4) for hBM-MSCs for the 2 and 5.5 mM conditions and (n=3) for the 25 mM condition. *, P<0.05; **, P<0.01; ***: P<0.001.
3.1.2 Senescence: SA-β-Gal expression

The percentage of senescing cells of both cell types cultured for 7 days under various glucose conditions was calculated as described in (Chapter 2: Section 2.2.2.D). Representative images of day 7 cultures are shown in Fig. 3.2-A. At pH 6, a homogeneous perinuclear SA-β-Gal staining was detected in aged (senescent cells) of both cell types and a more punctate pattern was found at pH 4 due to lysosomal activity. Cells used as a negative control (early passage embryonic fibroblasts) did not show any staining at pH 6. Control images are shown in Fig. 3.2-C.

Quantitative image analysis showed that hypo- and hyperglycemic culture conditions has increased the expression of SA-β-Gal marker in HUCPVCs cultures (27.5±13.44 and 45.5±14.85 % respectively) compared to the normal (5.5 mM) condition (15±1.41 %), Fig. 3.2-B. In hBM-MSCs cultures, a similar trend, though less prominent, was found where the expression was higher under hypo-and hyperglycemic conditions (54.5±10.61 and 54±4.24 %) compared to normal conditions (52.5±4.95 %).

Notably, the expression of the senescence marker was higher in hBM-MSCs cultures under all culture conditions compared to HUCPVCs. The expression of the senescence marker was negatively correlated with proliferation of both cell types.
Figure 3.2 Senescence of HUCPVCs and hBM-MSCs under various glucose culture conditions. (A) Representative images of day 7 cultures of HUCPVCs and hBM-MSCs cultured under 2, 5.5, and 25 mM glucose culture conditions. Positive SA-β-Gal expressing cells appear blue (or light blue). (B) The percentage of β-Gal positive cells in HUCPVCs and hBM-MSCs cultures under the previous conditions. Values plotted represent (mean ± SD) of (n=2) for both cell types under all conditions. (C) Positive control samples: (1) Aged HUCPVCs (P15) at pH 6; (2) Aged hBM-MSCs (P14) at pH 6; (4 & 5) HUCPVCs and hBM-MSC stained with X-Gal (pH 4) to show the lysosomal activity present in all cells; (3) Negative control: Human embryonic fibroblasts at pH 6 (no SA-β-Gal expression). Field width for all images = 0.544 mm.
3.2 In vivo studies

3.2.1 Behavioral observations in NSG mice

Although mice handling was instituted on a regular basis during the acclimatization period in early pilot studies, we noticed that the majority of NSG mice showed a jumpy and fearful behavior even after the first week of regular handling. They also showed a tendency to turn their back and try to bite when captured by the tail. Since the adaptation level hoped for to our handling and restraining procedures could not be reached by the first week, the frequency of their handling was, therefore, reduced in later experiments as little effect on glucose and weight measurements was found.

3.2.2 Clinical observations in STZ-induced NSG mice

Most hyperglycemic animals involved in the study showed prominent clinical signs of weight loss, dehydration, lethargy, a pinched face appearance with rough fur, and in some cases a hunched posture. The onset of these symptoms varied among mice where some developed them after few days post-induction while others after surgery (i.e. after 5 days post-induction). Therefore, Lactated Ringer’s solution was administered subcutaneously to all mice (done by assigned DCM technician), once or twice a day, after or 1 day before surgery, to restore body fluids and reduce dehydration. This intervention lasted until sacrifice (day 7 post-surgery). The overall health of mice was also monitored by DCM technicians who were instrumental in suggesting appropriate intervention methods to be used.

Physiological response to the STZ drug and the stress that followed the surgical procedure varied among NSG mice tested. Fig. 3.3 shows a case of 2 NSG mice that had a close body weight at the induction day; however, they manifested different outcomes in terms of their overall health, weight loss, and wound healing. The mouse in Fig. 3.3-A lost 31.42% of his body weight by day 7 while the other mouse in Fig. 3.3-B lost only 19.1%. The first mouse was excluded from the study due to poor wound healing and the histological outcome that resulted from his poor health condition. Also, nine STZ-induced mice were originally included in the main study; however, one mouse was excluded before surgery due to his weak body condition; another died during surgery, and yet another was excluded after study completion due to his poor healing and histological outcome. Therefore, data obtained from only 6 animals were analyzed for the main wound healing study.
Figure 3.3 Differences in the physiological response to hyperglycemia in NSG mice. The upper panel shows 2 mice after sacrifice with the dressing still attached. The lower panel is showing standardized images of both mice without the dressing. Both mice (A & B) started with almost the same weight at the induction day (day 0) but mouse (A) exhibited poor wound healing and lost >25% of his weight by day 12 post-induction compared to (B) who lost only 19.1% of his weight. Mouse (A) was excluded from the study due to his poor healing and histological outcome.
3.2.3 Random blood glucose (RBG) and body weight profiles

3.2.3.A Initial observations

Hyperglycemia was induced in fasting NSG mice using a single IP injection of (150 mg/kg) STZ in sodium citrate buffer pH 4.6. A cut-off value to determine a positive state of hyperglycemia was initially determined by testing random blood glucose levels in NSG mice (8 weeks of age, n=8) before and 48 hours post-injection with either STZ (hyperglycemic group, n=4) or sodium citrate buffer (normal control group, n=4). By day 2 post-induction, there was a significant increase in blood glucose levels in STZ-induced mice compared to their baseline values (15.35±3.73 vs. 5.1±0.96 mmol/L, P<0.05), while blood glucose levels in normal control mice remained fairly constant (5±1.1 vs. 5.28±0.68 mmol/L, P>0.05). Average blood glucose levels of the hyperglycemic group at day 2 post-induction was also significantly different from that of the normal control group at that time-point (15.35±3.73 vs. 5±1.1 mmol/L, P<0.01). A comparison of average blood glucose levels for the 2 groups at these 2 time-points is shown in Fig. 3.4.

From this group of induced mice, the minimum glucose value detected at day 2 post-induction was (10.6 mmol/L) and the maximum was (19.6 mmol/L) and all induced mice showed a progressive increase in their blood glucose until the sacrifice time-point. Since the minimum glucose value detected at day 2 was (10.6 mmol/L) which is almost double the average normal baseline value (5.1±0.96 mmol/L, we chose a cut-off value of (>10 mmol/L) to confirm a state of hyperglycemia after STZ induction in NSG mice.

Moreover, body weight measurements conducted at the time of blood sampling for glucose analysis revealed that the STZ-induced mice had lost, on average, a significant amount of weight by day 2 post-induction compared to the baseline level (23.8±1.56 vs. 25.8±0.98 g, P<0.05, weight loss %: 7.81±3.03). On the contrary, control mice showed a slight increase in weight by day 2 compared to day 0 (25.85±0.66 vs. 25.63±0.87 g, P>0.05); a characteristic of healthy animals. However, no significant differences in the weight of both groups were found at day 2 post-induction (hyperglycemic group: 23.8±1.56 vs. control group: 25.85±0.66 g, P>0.05), data not shown.
Figure 3.4 A comparison of mean random blood glucose (RBG) levels between STZ-induced and non-STZ induced control NSG mice (n=4 each) at 2 time-points. At day 2 post-induction, mean blood glucose level of the hyperglycemic mice was significantly different from their baseline level (* P<0.05) and from the control group (** P<0.01). From these data a cut off value of (>10 mmol/L) was chosen to judge STZ induction success. Values plotted represent (mean±SD).

Figure 3.5 A Pie chart showing the percentage of STZ induction success in NSG mice (n=16). Induction success was judged by having a random blood glucose level of (>10 mmol/L) at day 2 post-induction.
3.2.3.B  Main study observations

A total of 10 mice were used for the main study. The normal control group (sodium citrate buffer injected) was canceled from the study due to reasons that will be explained later; therefore, all 10 mice were given STZ injections and those who developed hyperglycemia were included in the wound healing study. By day 2 post-induction, all mice, except 1, had significantly elevated blood glucose levels compared to day 0 (15.71±1.91 vs. 4.44±0.38 mmol/L, P<0.001, n=9) with values ranging between (12.7 to 18.2 mmol/L). Significant weight loss was also noted by day 2 in those mice compared to day 0 (22.52±2.79 vs. 25.32±2.8 g, P<0.001, weight loss %: 11.1±4.9), data not shown.

After STZ induction, some mice were in a better shape than others. This was reflected in their glucose and weight profiles where mean glucose levels were higher in the group with the poorest clinical signs (n=5) while average weight was higher in the group who had a better overall health (n=4). It should be mentioned that both groups lost weight after induction, as part of the progression of the hyperglycemic condition. However, healthier hyperglycemic mice started with an average weight that was higher at time-point 0 compared to the other mice and thus the loss continued to be constant compared to the other group at all time-points and never intersected, data not shown. No statistically significant differences were found in these 2 parameters (i.e. glucose and weight) between the 2 groups of mice at all time-points studied except for weight measurements at day 5 post-induction where the weight lost by the group with the poorest health was much lower than the other group [23.25±2.5 g (n=4), 19.0±2 g (n=5), P<0.05], data not shown.

3.2.3.C  Overall observations

Overall, 87.5% induction success was achieved in all the STZ induced NSG mice included in the in vivo study (n=16), Fig. 3.5. Out of the 16 animals, 2 mice (12.5%) failed to achieve a glucose level >10 mmol/L and thus were excluded from the study. A profile of mean glucose and weight values of all the STZ-induced and control mice, regardless whether they received treatment or not, at all time-points studied are shown in Fig. 3.6. A sharp increase in blood glucose at day 2 post-induction can be noted in the induced mice which then continued to rise until sacrifice. Mean glucose values were highly significant at all time-points post-induction compared to day 0 (P<0.0001). Also, significant differences in blood glucose levels were found between day 2 and 12 (P<0.05) and between day 5 and 12 (P<0.05). No significant difference was found between day 2 and 5 values. The increase in blood glucose was accompanied, as explained earlier, by a decrease in body weight where mice lost (10.1±4.55 %) of their weight by day 2 post-induction and that continued to
increase reaching (16.08±7.78 and 18.66±6.18 %) by day 5 and 12 post-induction respectively. The decrease in body weight was statistically significant at all time-points post-induction compared to the baseline level (P<0.0001). Significant differences in body weight were also found between day 2 and 5 (P<0.001), day 2 and 12 (P<0.0001), and between day 5 and 12 (P<0.05).

The trend of mean blood glucose in the control group (n=4) was quite different where it remained fairly constant, except between day 5 and 12 where there was a slight, but not statistically significant, increase between them. There was an accompanying increase in body weight until day 5 then a decrease to day 12. The increase in weight at day 5 was found to be significantly different from day 0 (26.175±0.74 vs. 25.625±0.87 g, P<0.05). The decrease in body weight noted does not reflect a healthy condition a normal control group is expected to manifest and the slight increase in body glucose during the same period (day 5-12) might reflect some physiological changes resulting from the stressful events following surgery and dressing application (explained in Section 3.2.4.B). The control group was, therefore, cancelled from the study and their glucose and weight values are presented herein as a reference for the sake of comparison with the hyperglycemic group.
Figure 3.6 Mean random blood glucose (RBG) and body weight profiles for all the STZ-induced and non-STZ induced control NSG mice over a course of 12 days post-induction. Values plotted represent (mean±SD) of (n=14 at day 0 & 5; n=13 at day 2; n=12 at day 12) for the hyperglycemic mice and (n=4) for the control ones. **In the hyperglycemic group**, mean glucose values at all time-points post-induction were all highly significant from day 0 value (P<0.0001). Also, significant differences in mean glucose levels between day 2 & 12 (P<0.05) and between day 5 & 12 (P<0.05) were found. The decrease in body weight at all time-points was also significantly different from the baseline (P<0.0001), between day 2 & 5 (P<0.001), and between day 5 & 12 (P<0.05). **In the normal control group**, no significant differences were found in mean glucose levels at all time-points. The same is true for mean weight values except between day 0 & 5 (P<0.05). Note a decrease in body weight between day 5 (surgery) and 12 (sacrifice) which could be the result of the stressful events followed surgery and wound dressing application. The control group was, therefore, cancelled from the study. Abbreviations: NC: normal control; HG: hyperglycemic
3.2.4 Wound healing (WH) study: Early observations

3.2.4.A Healing is delayed in STZ-induced NSG mice

In order to study the rate of wound healing in normal and STZ-induced NSG mice, four wounds were created on the back of the one hyperglycemic and one normal control mouse (as mentioned in Chapter 2). WH was monitored for 1 week where no treatment was applied at this point. At the day of sacrifice, wounds were imaged and skin samples were collected for histology.

Image planimetric analysis (surface area) of the (n=4) wounds in each mouse was performed for day 0 images (wounding) and day 7 (sacrifice) and the decrease in wound size at day 7 relative to day 0 was calculated for each wound and averaged. Fig. 3.7-A and -B shows the gross macroscopic appearance of 2 representative wound images from both mice at these 2 time-points. Area analysis showed that there was a significant delay in WH of the dermal wounds of the hyperglycemic mouse compared to those of the normal control (decrease % in wound size: 27.74±17.14 % in the former vs. 65.68±13 % in the latter; P<0.05), Fig. 3.7-C.

Histologically, there was a discernible visual difference in skin thickness between the hyperglycemic and the normal control mice. Four random skin height measurements (including: the epidermis, dermis, and the Paniculus carnosus) were taken from (n=4) H&E stained sections of both groups, the farthest intact skin region from the defected area, and then averaged and compared. It was found that the thickness of the hyperglycemic skin was significantly less than the control (0.35±0.08 vs. 0.5±0.05 mm, P<0.05), Fig. 3.8-B. In fact, the main difference lies in the dermal layer where less dense collagen fibers were noticed in the hyperglycemic skin compared to the control with the lack the basket-woven morphology recognized in the dermal layer of the control, Fig. 3.8-A. In addition, the wound bed was thicker in the control mouse with well-established blood vessels and extra cellular matrix (ECM) reflecting a healthy forming granulation tissue while the opposite was true for the hyperglycemic mouse, Fig. 3.9. The neo-epithelial layer of both mice was also morphologically different where it took a bulging shape in the hyperglycemic mouse reflecting a state of hyperplasia with little evidence of migration while it was more extended with recognizable cell layers in the control.
Figure 3.7 Wound healing is impaired in hyperglycemic NSG mice. Representative digital images of 2 wounds from normal control (A) and hyperglycemic (B) NSG mice wounds at day 0 and 7 (Upper 2 panels, front view); back view images of day 7 wounds from both mice (lower panels). At day 7, normal control wounds look smaller than the hyperglycemic ones. A difference in the vascular supply can be seen in the harvested wounded skin of both mice (back view). (C) Percentage decrease in wound size at day 7 post-wounding relative to day 0 in hyperglycemic and normal control NSG mice. The surface area of (n=4) wounds in hyperglycemic and normal control mice (n=1 each) was measured from digital images taken at day 0 and 7 using image pro analyzer (6.2). Percentage decrease in wound size of each of the 4 wounds was calculated and the average decrease is plotted above as (mean±SD). *: P<0.05).
Figure 3.8 Differences in skin thickness between hyperglycemic and normal control NSG mice. (A) Representative images of H&E stained histosections of day 7 wounds of control and hyperglycemic NSG mice showing differences in dorsal skin thickness. Note the difference in the thickness of the dermal layer of both mice. Collagen fibers in the hyperglycemic mouse are less dense and lack the basket-woven morphology seen in that of the control. Fields width (FW): Upper row=0.845 mm; Lower row=0.341 mm. (B) Average skin thickness in control and hyperglycemic NSG mice. Data plotted represent the average of 4 measurements in (n=4) histosections in each animal (mean±SD). *: P<0.05. Abbreviations: E: Epidermis; HF: Hair follicle; D: Dermis; SG: Sebaceous gland; PC: Panniculus carnosus muscle layer.
Figure 3.9 Histological appearance of dermal wounds of STZ-induced and non-STZ induced control NSG mice at day 7. Left panel: Low power field micrographs of H&E stained wound sections (off-centre images), FW=3.454 mm. Middle panel: Enlarged images of the wound bed showing differences in vascularity, thickness, ECM elaboration between normal control and STZ-induced NSG mice, FW=0.432 mm. More mature granulation tissue can be seen in the control mouse with well-formed blood vessels (arrow heads) while the opposite can be seen in the hyperglycemic mouse. Right panel: Neo-epithelial layer morphology in the two mice. More epithelial coverage is seen in the control compared to the hyperglycemic mouse. Abbreviations: GT: Granulation tissue; NE: Neo-epithelium; FW: Field width.

3.2.4.B  Wound coverage

Probably one of the most challenging aspects of this study was covering the wounds and keeping the dressing attached until sacrifice (day 7 post-wounding). From early trials, we have noted that normal control mice were able to remove the dressing faster than the hyperglycemic ones who, in most cases, were not capable of taking the dressing completely off by day 7. We have also noted that control animals covered with either Tegaderm dressing alone, or Tegaderm & Elastoplast, were able to remove both settings either immediately after surgery or approximately 2-3 days afterwards. Fig. 3.10 shows an example, from a trial study, of the appearance of the dressing in both hyperglycemic and control NSG mice after surgery. In that study, the control mouse was highly distressed and agitated and had ripped off the bandage from the abdominal side by day 2. This behavior was not evident in the hyperglycemic mouse.
As mentioned earlier, NSG mice are very active and jumpy. By inducing hyperglycemia, their overall health started to decline which made them less active and weaker compared to their original state. This contributed to them being less capable of interfering with their dressings. Control mice, on the other hand, remained active after surgery and started to show a more restless behavior which left them helpless trying to remove the dressing. That might also explain the loss in their body weight observed after surgery (shown previously in Fig 3.6).

Due to these problems and in order to avoid the waste in the experimental animals, we excluded the control mice from the wound healing study. Only STZ-induced mice were included where each animal served as his own control by leaving one of the wounds without treatment. Since Tegaderm\textsuperscript{TM} dressing was found to be quiet stable in the hyperglycemic group and in an effort to reduce animal stress, one piece of this dressing, slightly adjusted to adequately cover the wounded area, was used in the main study without the use of an extra bandage.

![Image](A) Hyperglycemic day 2  ![Image](B) Control day 2 day 3

**Figure 3.10 Dressing appearance in STZ-induced and non-STZ induced control NSG mice.** At day 2 post-surgery, the dressing (Elastoplast + Tegaderm) was stable in the hyperglycemic mouse (A) while the control mouse (B) was able to interfere with the dressing and almost removing it by day 3. No pictures were taken for the hyperglycemic mouse at day 3 but the bandage was still intact and in a good shape. The bandage and the dressing were removed from both mice at day 3 as the control mouse was highly distressed.

3.2.4.C Treatment protocol

The final cell dosage used in the main study was $0.1 \times 10^6$ cells in 5 µl of 1% methylcellulose (MC) for the 4mm (in diameter) wounds created. This dosage was adjusted from an earlier trial where $0.05 \times 10^6$ cells in 5 µl of 1.5% MC was used. This adjustment was made to make the application easier by reducing the concentration of the delivery material to give a less viscous solution which was easier to pipette, and also to see a more prominent effect on WH by increasing the number of applied cells.
3.2.4.D Wound tissue harvest

Several strategies were used in earlier trials to optimize the technique of tissue harvest. Initially, we attempted to excise the wounded dorsal skin as a single piece by carefully splitting it from the underlying musculature. The excised skin was then cut into 4 smaller squares; each contains one of the four wounds. For some wounds, a cut through the center of the wounds’ widest diameter was made immediately after harvest and these pieces were then pinned to a small piece of flat cork and fixed. Using this technique, the wound bed was lost from some of the histological sections, as shown in Fig. 3.11, indicating an artifact during skin processing which resulted in tissue damage or retraction before embedding (as the newly forming wound tissue was very delicate). Attempting to bisect the sample prior to embedding gave a better results; however, we noticed that by making the cut directly through the center, a true representation of the exact wound center was lost due to, initially, the thickness of the scalpel blade used (~400 µm) and, subsequently, block trimming before actual sectioning, which took some extra microns off the sample.

Therefore, to minimize these compromising factors, the whole animal was fixed immediately in formalin after sacrifice with his dorsal wounds intact. This way, a better preservation of the newly formed granulation tissue was assured as it remained attached to the underlying tissue. Also, an off-center cut through the wounds’ widest diameter was made prior to embedding. All samples were embedded vertically in the mold and serial sections were produced from these blocks, starting from the cut edge towards the other end of the wound, with a thickness that ranged between 6-7µm. Slides were then made from every 10th or 20th sections produced, depending on the size of the wound. All slides were compared to determine the wound center by carefully looking at the wound gap under a light microscope. For some sections, H&E staining was attempted at this stage to aid in visualizing the wound gap. The wound section with the widest gap was considered the center of that wound and was, therefore, processed for H&E staining if it was not already stained.

This method worked better in terms of exactly determining the center of each wound for a better histological comparison; yet, there were certain unfavorable results of some of the wounds that were closer to the ribs and the spine as they tended to curve while in the fixative. This could be avoided by making the wounds farther to the animal’s back or away from the spine on the lateral sides. However, this method preserved the overall morphology of the wounds and the granulation tissue and the aforementioned problem did not interfere with data analysis.
Figure 3.11 Effect of tissue harvest and processing technique on histological outcome (pilot study). (A) H&E stained paraffin section of a wound with disruptive wound bed. (B) H&E stained frozen section of another wound from the same animal with an intact granulation tissue.

3.2.5 Wound healing (WH) results: Main studies

As mentioned earlier, a total of (n=9) STZ-induced mice were originally included in the WH study; however, due to various factors such as failure to develop hyperglycemia, death, or poor health which necessitates cancelation, results described below are based on a total of only (n=6) mice.

3.2.5.A Image analysis results

The surface area (mm²) of day 0 and day 7 wounds were measured from digital images obtained at these 2 time-points as described in (Chapter 2: Section 2.3.3.D3). The distribution trend of raw area measurements of wounds received various treatment types at day 0 (Surgery) and 7 (sacrifice) are shown in Fig. 3.12-A. It can be noted that the area of all wounds, either treated or not, were significantly smaller at day 7 compared to day 0; however, the significance values obtained varied among all groups where HUCPVCs-treated wounds showed the highest significance level (P=0.00003) followed by hBM-MSCs (P=0.0023), then methylcellulose (P=0.0037), and finally non-treated wounds (P=0.0039).
As mentioned earlier, hyperglycemic mice showed variable tolerance to the toxic effect of the STZ drug and also to the stressful events following surgery; therefore, comparing wound healing between healthier and weaker hyperglycemic mice was initiated in order to correlate health and nutritional status and WH in different treatment groups. Not surprisingly, the distribution trend of wound area of all treatment groups at day 7 was different between healthier and weaker mice, Fig. 3.12- B and C respectively. In the healthier group, it can be noted that day 7 values of all treated wounds were smaller compared to those of the weaker one.

The percentage decrease in wound size at day 7 relative to day 0 was found to be higher in cell and sham-treated wounds compared to the control Fig. 3.13-A with significant differences only between sham and control wounds (55.47±21.56 vs. 30.47±16.4 % respectively, P<0.05). Wound closure percentages of all treated wounds also differed between healthier and weaker hyperglycemic mice, Fig. 3.13-B. Interestingly, in the weaker group, average wound closure percentage was highest in HUCPVC-treated wounds (46.4%) compared to hBM-MSCs (32.6%) and sham (39.5%), and was lowest in untreated wounds (34.2%). It can be also noted that wound closure percentages in HUCPVC-treated wounds in both healthier and weaker mice are closer than other treatment types used where values were more separated and were less in the weaker mice compared to the healthier ones. It can be inferred that HUCPVCs showed a better endurance to the harsh wound microenvironments of the weaker mice and enhanced healing.

It should be mentioned that no statistical analysis was performed on differences between healthier and weaker groups for various healing parameters due to small sample size, n=3 each.
Figure 3.12 Distribution trends of wound areas for all treatment groups in hyperglycemic NSG mice. 

(A) Distribution trend of wound surface areas (mm$^2$) at day 0 (wounding) and 7 (sacrifice) in $n=6$ mice. The box plot graph is showing the 25th and the 75th percentiles (lower and upper box borders), median (middle line), smallest and largest observations (whiskers), and outliers (stars). The area of all wounds were significantly smaller at day 7 compared to day 0. HUCPVCs treated wounds had the highest significance value while control wounds had the lowest. 

(B) Distribution trend of wound surface areas (mm$^2$) in healthier hyperglycemic mice ($n=3$) at day 0 and day 7. 

(C) Distribution trend of wound surface areas (mm$^2$) in weaker hyperglycemic mice ($n=3$) at day 0 and 7. Abbreviation: MC: Methylcellulose.
Figure 3.13 Distribution trends of the percentage decrease in wound size at day 7 relative to day 0 for all treatment groups in hyperglycemic NSG mice. (A) Distribution trends of all values in n=6 mice. The decrease percentage was higher in wounds received either cells or methylcellulose compared to untreated wounds. A significant difference (* P<0.05) was found between MC and control wounds. (B) Distribution trends of all values in healthier and weaker hyperglycemic mice (n=3 each).
3.2.5.B  Histologic and histomorphometric observations

3.2.5.B1  General observations

Despite the several immune dysfunctions associated with the NSG mice strain mentioned earlier, white blood cell infiltrate was observed in treated and untreated wounds with no undesirable inflammatory response. In exposed dry wounds, there was an intense accumulation of these cells in the scab layer above the wound bed. Generally, scab is formed in response to dehydration and it is usually composed of dried components of the early phase of healing (inflammation) including dried proteins (mainly fibrin), and dead red and white blood cells [186]. Dead inflammatory cells in this layer don't usually autolyze which make them recognizable using H&E stain as they stain dark blue/purple with haematoxylin due to their dense chromatin structure, Fig. 3.14.

Since all wounds of the main study mice were Tegaderm™ dressed, no scab formation was observed except for 3 wounds which were exposed due to animal interference with the dressing which was noted 2-3 days before sacrifice. Under the wet environment provided by the dressing, less inflammatory cell infiltrate, mostly dispersed within the granulation tissue, was observed in all wounds. These corroborate what has previously been reported about dry and wet wound healing [185]. The thickness of the granulation tissue was also different between dressed and exposed wounds where it was thinner in the former and thicker in the later.

![Figure 3.14 Dressed and exposed wounds.](image)

Representative micrographs of H&E stained histosections of day 7 wounds. **Upper row:** exposed wound where the scab is present covering the wound bed. **Lower row:** dressed wound where there is no scab formation. Notice also the wound bed thickness in both images. The right side panel is an enlarged image of the black square shown on the left. Abbreviations: Dashed line (---): Wound bed; DM: Dorsal musculature; GT: Granulation tissue; FW: Field width.
3.2.5.B2  **Wound histological width**

The width of all wounds was measured from micrographs of H&E stained paraffin histosections as the straight line (mm) extending between the two vertical hypothetical lines drawn at the last epidermal appendage seen in lateral wound margins denoting the original cut point. Wounds that received cell or sham treatment had a slightly, on average, smaller width compared to control wounds, but the difference between groups did not reach statistical significance, Fig.3.15. The distribution of these values was also noted to differ between healthier and weaker hyperglycemic mice. These data reflects, on average, results obtained from wound image analysis.

![Graph showing wound width](image)

**Figure 3.15 Wound width of all treatment groups in hyperglycemic NSG mice.** Width was measured from micrographs of H&E stained paraffin wound sections. Data plotted represent (mean±SD) of n=3 for both healthier and weaker hyperglycemic mice. (---) represents the total average (n=6).
3.2.5.B3 Re-epithelialization

From earlier pilot studies, we noticed that some of the exposed wounds of various types were completely epithelialized by day 7 post-surgery, Fig. 3.16. Since full epithelialization was noted even in the untreated wound, this might indicate that factors other than treatment might have played a role. Morphologically, the margins of day 7 untreated wounds, Fig. 3.16 (3), look very close to each other as if they were brought together due to either contraction and/or animal interference such as licking and scratching. Full epithelialization was not achieved, however, in treated and untreated wounds of main study animals. Pilot study observations were not taken into consideration as all wounds involved in the final analysis were dressed except for 3 ones that were exposed as the dressing was naturally removed by the animal 2-3 days before sacrifice. Moreover, wound healing kinetics varied considerably under dry and wet local conditions which made the comparison invalid [185, 186].

Morphologically, the neo-epithelial layer in main study mice looked hyperplastic with a well-defined cuboidal basal layer in the majority of the wounds, regardless of their type. Spinous cells (3-10 layers), granular cells (2-3 layers), and a keratinized layer were all easily recognizable in all wounds except for those of the weaker hyperglycemic group which received HUCPVCs treatment where few cell layers with less recognizable morphology were observed. Keratin was noted to cover the entire neo-epithelium except the leading edges (tips) which were thinner with a pointed appearance and without cell organization. Cell organization was most evident in the healthier mice and some wounds of the weaker ones with no observable preference to a certain treatment type. This layer was also found to be moving inwardly, at a lower plane than the original skin, and on top of the newly forming granulating tissue. Representative micrographs of the general morphology of this layer in wounds received various treatment types are shown in Fig. 3.17.

Histomorphometrically, no significant differences were found between treatment groups in terms of their effect on the thickness of this layer; however, cell-treated wounds had, slightly, a thinner neo-epithelium in comparison to sham (which scored the highest) and control wounds. Comparing this parameter between the healthier and weaker mice also showed a difference in the distribution pattern; however, it was not consistent where the thickness was less in the weaker group compared to the healthier one in HUCPVC- and sham-treated wounds while it was almost similar in hBM-MSC-treated wounds and higher in the untreated wounds of the weaker mice, Fig. 3.18-A.
In terms of the length of the newly formed epithelium, there were also no significant differences between groups; however, the length was found to be higher in the sham and control wounds compared to cell treated ones which was unexpected. This length was also found to be less in treated and untreated wounds of the weaker group in comparison to the healthier one, Fig. 3.18-B.

The percentage of re-epithelialization for each wound was calculated from the histologically measured neo-epithelial length and wound width. Results also showed no significant differences between groups; however, sham treated wounds had the highest percentage among the rest, Fig. 3.20-A. The percentage of epithelial coverage was also found to be less in the weaker group compared to the healthier one, Fig. 3.18-C.

Figure 3.16 Full epithelialization is seen in exposed wounds of different types (Pilot studies). (A) Dissecting microscope images of day 7 wounds. 1: HUCPVC-treated; 2: hBM-MSC-treated; 3: Control. FW for all images=24.35 mm (B) Micrographs of H&E stained histosections of the center of day 7 wounds. FW for images 1 and 2=4.06 mm; FW for image 3=2.74 mm. Arrow heads indicate wound margins.
Figure 3.17 Morphological comparison of the neo-epithelial layer of all treated wounds in healthier and weaker hyperglycemic NSG mice. FW for all images=0.864 mm. Abbreviations: NE: Neo-epithelium; D: Original dermis; GT: Granulation tissue; DM: Dorsal musculature.
Figure 3.18 Neo-epithelium thickness, length, and re-epithelialization % for all treatment groups in hyperglycemic NSG mice. Data plotted in all three figures represent (mean±SD) of n=3 for both healthier and weaker mice. (●●●●) represents the total average (n=6).

(A) Neo-epithelium thickness. The thickness was measured from histosections, both left and right epithelial tongues, 3-4 measurements each. Average reading was taken for each tongue and then a final score was obtained by averaging both left and right sides values.

(B) Neo-epithelial length. The length was measured as the distance traveled by the newly formed epithelium from both left and right tongues. Left and right side values were then summed and the average reading from all groups was plotted.

(C) Re-epithelialization %. The percentage was calculated from histologically measured neo-epithelial length and wound width. No significant differences were found between groups.
Granulation tissue (GT) formation

By day 7 post-surgery, all wounds were covered with a layer of GT that varied in thickness and morphology not only among various treatment groups, but also between healthier and weaker mice. In all dressed wounds, the GT ranged from thin to medium in thickness and was found to contain fibroblastic cells, infiltrating WBCs, extracellular matrix ECM, and emerging blood vessels at various densities. In most cases, the GT was thicker near the wound margin and less thick under the un-epithelialized region at the center of the wounds. Also, it looked denser near the wound margins and under the neo-epithelial tongues in comparison to the rest of the tissue, in most cases, which reflects the presence of a more mature GT. That area was also rich in blood vessels scattered throughout the tissue. Wound healing and GT formation seem to be emerging mostly from wound margins and the adjacent areolar loose connective tissue found under the paniculus carnosus muscle layer surrounding the defected area, Fig. 3.19.

Histomorphometric analysis has shown that the GT was, as expected, thicker in the healthier group compared to the weaker one. However, in the weaker group, cell-treated wounds had slightly a thicker GT compared to sham and untreated wounds, Fig. 3.20. Morphologically, blood vessels of the wound bed were mostly found clumped at the center of the wounds or near the neo-epithelial tongues and were surrounded by white blood cells and fibroblasts laying matrix while the rest of the tissue was merely a loose connective tissue with few scattered inflammatory and fibroblastic cells. Generally, blood vessels looked denser in hBM-MSC-treated wounds followed by HCPVCs and, unexpectedly, untreated wounds while sham-treated wounds had few blood vessels emerging especially in the weaker hyperglycemic mice. Representative micrographs of the general morphology of this layer in wounds received various treatment types are shown in Fig. 3.21.

Moreover, in 2 wounds, HUCPVC- and sham-treated, a strip of adipose connective tissue was noted running along with blood vessels in the wound bed close to the dorsal musculature. It was found to be originating from the areolar connective tissue beneath the Paniculus carnosus surrounding the wound margins, Fig. 3.22. This observation could be of less importance to our study but mentioned herein for case reporting purposes.
Figure 3.19 Granulation tissue formation. GT was found to develop from wound margins and the loose connective tissue found under the PC muscle layer. The GT under the NE layer was found to be denser and more mature compared to the rest of the tissue in most wounds. Notice the neo-epithelium moving in an inward direction. Arrows indicate the origin of cells and blood vessels invading the wound bed and forming the GT. Dashed line mark the wound margin. **Abbreviations:** NE: Neo-epithelium; D: Original dermis; GT: Granulation tissue; DM: Dorsal musculature, E: Epithelium; PC: Paniculus carnosus muscle, FW: Field width.

Figure 3.20 Wound bed thickness of all treatment groups in hyperglycemic NSG mice. Data plotted represent (mean±SD) of n=3 for both healthier and weaker hyperglycemic mice. ( ) represents the total average (n=6).
Figure 3.21 Representative micrographs of the center of the wound bed of all treatment groups in healthier and weaker hyperglycemic NSG mice. FW for all images=0.864 mm. Arrow heads indicate blood vessels; Stars indicate: clumps of fibroblastic cells laying matrix. Abbreviation: DM: Dorsal musculature.
Figure 3.2 Fat connective tissue in the wound bed of some samples. A strip of fat tissue was seen emerging from the adjacent areolar connective tissue under the PC muscle layer. Black arrows show the strip of the fat cells seen in HUCPVCs and sham treated wounds (n=1 each). Abbreviations: NE: neo-epithelium; D: Original dermis; GT: Granulation tissue; DM: Dorsal musculature, E: Epithelium; PC: Paniculus carnosus muscle; FT: Fat tissue; FW: Field width.
Chapter 4

Discussion

4.1 In vitro results: Hypo- and hyper-glycemic culture conditions decrease cell proliferation and enhance senescence

For cells to proliferate, they have to replicate their cellular structures prior to mitosis. In vitro, glucose in culture media is utilized to generate energy and the required biomass for cell division by supporting nucleotides, lipids and proteins biosynthesis [164, 165, 166]. Accordingly, abnormal glucose concentrations, both excessive and deficient states, lead to a state of metabolic stress which, in return, reflects not only on proliferation, but other cellular activities. For instance, it has been shown that imbalanced glucose metabolism generates oxidative byproducts such as, dicarbonyls glyoxal (GO), methylglyoxal (MGO), 3-deoxyglucosone (3-DG) and glucosone, collectively known as reactive carbonyl species (RCS). Such metabolites are toxic to the cells as they cause DNA and proteins damage and lead to premature in vitro senescence [167]. The accumulation of the advanced glycation end products AGEs generated has also been shown to be implicated in several age-related pathologies including Alzheimer’s, atherosclerosis, kidney failure, cataracts and diabetes [167].

The effect of in vivo and in vitro hyperglycemic state on mesenchymal stem cell proliferation and senescence has been previously investigated. In vitro, a 2-D monolayer cell culture system is widely used to test the effect of different glucose culture conditions on various cellular activities mimicking, to a certain extent, the main characteristic of a diabetic state. In this study, we utilized the same system to examine proliferation and senescence behavior of HUCPVCs and hBM-MSCs under three different glucose culture conditions [2, 5.5, and 25 mM]. Several bio-markers have been reported in the literature to assay cell proliferation among which is the Ki-67 nuclear protein. This marker has been appreciated for its presence in all phases of the cell cycle except the G0. Thus, the fraction of proliferating cells can be easily calculated from the total by, for example, immunofluorescence using appropriate anti-Ki-67 antibodies, as we did in this work [168, 169].

In our cultures, we have noted that both cell types exhibited a higher proliferation index under the normal (5.5 mM) condition and lower indices under both the hypo- (2 mM) and the hyperglycemic (25 mM) conditions. However, HUCPVCs showed a higher proliferation index compared to hBM-
MSCs under normal and hyperglycemic conditions. Less efficient proliferation under hypo- and hyper-glycemic conditions have been previously reported in the literature. Lo et al, 2011 [170] examined the effect of glucose reduction in culture media on hBM-MSCs proliferation and other activities utilizing slightly a different experimental set-up than ours where cells were cultured under high glucose media (25 mM) for 14 population doublings (PD) and then switched to media containing 2.5, 7.5 or kept in 25 mM glucose. After 35 days in culture, it was found that cells kept under high glucose conditions were morphologically larger and had a decreased cumulative PD. Glucose reduction was associated with metabolic improvement where decreased lactate production, increased oxygen consumption, unexpected decreased ROS, and enhanced anti-oxidant enzyme expression were noted. Interestingly, they did not observe adverse events with the hypoglycemic media (2.5 mM). Similarly, in an earlier study by Stolzing et al., 2006 [171], lowering glucose concentrations in culture media also enhanced cell growth and differentiation capacities of rat BM-MSCs, tracked for 36 days in culture. They noted that low glucose concentrations, even those technically considered hypoglycemic (1.4 and 2.8 mM), led to decreased apoptosis and increased CFU-F number and size.

On the other hand, Weil et al., 2009 [172] did not observe any change in the proliferation of hMSCs cultured under 5.5, 20, and 30 mM glucose conditions after 48 hours. They also noted that such glucose conditions did not affect growth factors production particularly (VEGF, HGF, and FGF2) at 24 and 48 h in both stimulated (TNF-α, LPS, or hypoxia) and un-stimulated cultures. It seems that short-term exposure to high glucose environment does not have a strong impact on cell proliferation and their GF production capacity. Similar observations were reported by Li et al., 2007 [173] in their 4-day cultures exposed to 5.6, 11, and 25 mM glucose concentrations where no effect of such conditions on proliferation status was found. However, in their 4-week cultures a significant decrease in proliferation was observed under the 25mM condition. Paradoxically, they noted that even very high levels of glucose (40 mM) did not inhibit cell growth at both time-points. One should reflect on their observation as no reference has been provided to the purity of their BM isolated “MSCs”. Also, data presented reflects the average of 3 independent experiments a factor that could also have contributed to these results.

A recent study by Deorosan and Nauman, 2011 [174] showed that under a 3-D collagen culture system, glucose concentrations and collagen density were the most significant factors affecting murine BM-MSCs metabolism and growth.
It is well documented that cell senescence, under normal culture conditions, is an inevitable phenomenon where cells become irresponsive to mitogenic signals, also referred to as a “Hayflick limit” initially observed by Hayflick and Moorhead in human fibroblasts [175, 176]. Cell senescence has been viewed as an important process for tumor suppression, tumor advancement, aging, and tissue repair [177]. Although senescent cells retain their viability and metabolic activity, their gene expression patterns are atypical [176]. For instance, down-regulation of genes associated with cell cycle progression, DNA replication, and DNA repair and up-regulation of cell-cycle inhibitors, pro-inflammatory cytokines (e.g. IL-6), and anti-fibrynolytic agents (e.g. plasminogen activator inhibitor-1 PAI-1) have been observed [175]. Senescent cells in culture, regardless of their source, show classical features of an enlarged morphology, granular cytoplasm, excessive actin fibers expression, decreased expression of certain surface markers, and reduced differentiation potential [175, 176].

In our cultures, we noted that hyper- and hypo-glycemic conditions were associated with a higher expression of the senescence marker, SA-β-Gal, in day-7 HUCPVCs cultures compared to the normoglycemic condition. In hBM-MSCs cultures, the variability of the values recorded under all three conditions was less prominent; however, values under the normoglycemic condition were slightly less than the other two conditions similar to HUCPVCs. Interestingly, such expression trend was almost opposite to the proliferation patterns reported earlier where lower expression of the senescence marker was associated with a higher proliferation and vice versa. Importantly, the percentage of senescence was higher in hBM-MSCs cultures under all three conditions compared to HUCPVCs. Despite the small experimental replicates used, our results seem to agree with previous work. Non-optimal culture conditions have been shown to force the cells to show advanced senescence. In fibroblast cultures, high glucose concentrations (25 mM) enhanced apoptosis and senescence; the latter was suggested to be irreversible especially when cells cultured under this condition reached >20 PD [176]. A similar increase in senescence was observed by Lo et al, 2011 [170] in hBM-MSCs exposed to high glucose (25 mM) for 35 days.
4.2 In vivo results

4.2.1 Hyperglycemia can be induced in NSG mice

STZ induction using a single high-dose is a widely used protocol for hyperglycemia induction in laboratory animals. We have employed this protocol and administered STZ intra-peritoneally to NSG mice using a single dose of (150 mg/kg) dissolved in sodium citrate buffer pH 4.6 following the instructions provided by the Animal Models of Diabetic Complications Consortium (AMDCC Protocols, version 1, edited by: The University of Michigan Medical Center, Frank Brosius, 04/03/09) with slight modifications. In that protocol, it was stated that mice need to be fasted for 4-6 hours before induction, a measure that we followed; however, in other studies, where this step was eliminated, induction was also possible in various murine models. It has been suggested that this short fasting state is less likely to influence STZ action [149]. It would have been valuable, however, if linkage was established in the published work between fasting state in various animal models used and induction success or mortality rate.

Moreover, it was indicated not only in that protocol, but also in the majority of the published work, that the buffer solution should be mixed prior to injection and administered immediately after dissolution due to rapid drug degradation (~15-20 minutes). Streptozotocin is composed of 2 anomers α and β, with α being more toxic. A recent study by de la Garza-Rodea et al., 2010 [149] showed, through HPLC analysis, that the composition of the dissolved STZ started to change during the first 30 minutes of dissolution and equilibrium, between α and β anomers, was reached around 60-90 min afterwards. It should be mentioned that this is dependent on the proportion of the α anomer in the STZ lot which, in their case, was ≥84%. They further showed that STZ solutions left at 4 °C to equilibrate for 2-3 hours do not lose potency as induction was also possible when a high dose of both freshly prepared and equilibrated STZ solutions were injected in NOD scid mice. Interestingly, mice received fresh preparations showed 20% higher blood glucose values and significantly higher mortality rate compared to mice received equilibrated solutions. A minimal weight loss was reported in animals received the later solution compared to the former. This difference was also supported histologically where fresh solutions seem to cause more pancreatic tissue damage compared to the other type. Anomer-equilibrated STZ solution was, therefore, suggested for reproducible induction.

In our study where STZ (Sigma, S0130) with α-anomer basis of ≥75% was used, an overall 87.5% induction success was achieved. Comparing this percentage to the literature is rather difficult bearing in mind the circumstances associated with the animal strain studied and drug type used, its quality
and dosing. Induction and mortality percentages are often not reported in the published work which, we believe, is important to indicate for the sake of comparison in future studies. Generally, induction success seems to be dictated by the module used for STZ application and the animal model itself. For example, Ventura-Sobrevilla et al., 2011 [178] indicated that the high-dose module (150 mg/kg STZ) caused a faster onset of hyperglycemia in male CD1 mice compared to the low-dose one; but with a higher body weight loss and mortality (50% death by day 21). Arora et al., 2009 [179] studied various doses of STZ in Swiss albino mice. Induction was effective with a high-dose of (180 mg/kg), but with a higher mortality (>20% by week 5), and with a multiple lower-doses were less mortality was reported. However, no effect of the sub-optimal dose of (100 mg/kg) was found.

In our animal model, we chose a cut-off blood glucose value of >10 mmol/L by day 2 post-induction to identify a positive hyperglycemia state. Selection criteria varied among the published work and ranged between >13.9 mmol/L [180] to >16.6 mmol/L [149, 156], with the majority inclining towards the later criterion, judged at various time-points post-induction. However, our observed glucose values in the induced mice fall within the reported range. In a study where NSG mice strain were used, the selection criterion was set to >25 mmol/L, a value that we identified, in our experiment, as high and possibly fatal. Moreover, this value could not be observed in the majority of the induced mice even at day 12 post induction. In another study, severe hyperglycemia, ≥33.3 mmol/L, was reported in male CD1 mice when high and multiple low-doses were used [178]. Indeed, mice strain, age, gender, and induction method are likely to influence such observations.

More recently, a comparative analysis on the effect of mice suppliers on induction success was examined by Graham et al., 2011 [181]. In their work, congenitally athymic nude mice from 3 different sources [Taconic Farms (TAC), Jackson Laboratories (JAX), and Charles River Laboratories (CRL)], similar in age, body weight, and glucose level before STZ induction, were injected with high doses of pharmaceutical-grade formulation of STZ (Zanosar Teva Pharmaceuticals, 160-240 mg/kg), a rather different form than what is normally used based on the claim that less lot variability is present. Induction was successful in all mice; however, differences were reported in mice sensitivity to STZ induction, morbidity, and mortality. Onset of hyperglycemia was faster in JAX mice and slower in CRL mice. Average blood glucose levels were also highest in JAX mice and lowest in CRL mice. JAX and TAC mice required more insulin injections and exhibited significant weight loss. However, better survival (30 days follow-up) was noted with CRL mice. Moreover, Pearson el. Al. 2010 [156] showed that various doses ranged from (145-160 mg/kg) generated hyperglycemia in both male and female NSG mice. However, induction
success did not correlate with the dose administered or gender. However, it was noted that average blood glucose values measured at day 10 post-induction was higher in male NSG mice that received higher doses (155-160 mg/kg) compared to female NSG mice, while both had a similar glucose value under the lower doses (145-150 mg/kg).

### 4.2.2 Behavioral observations in NSG mice

An important trait noted for NSG mice is that being jumpy, fearful, and, occasionally, aggressive. This comes with no surprise as genetically manipulated animals usually exhibit different behavioural patterns in comparison to the wild type which also seem to be modulated by the environment [182, 183]. Notably, the stressful events following surgery led to noticeable behavioural changes especially in the non-STZ induced control mice where they exhibited a restless agitated behaviour. Their good overall health enabled them to interfere with the dressing, and completely remove them, within 2-3 days. This was problematic especially for wounds receiving treatment. When we attempted to remove the dressing, they showed a behavioural switch and became calmer. It seemed, therefore, that wound coverage was the most stressful part for this group.

The stability of the dressing the entire experimental time was an important measure in our study. We have used transparent dressing (3M Tegaderm™), a semipermeable thin film that is designed to maintain moisture, allow gas exchange, and protect the wounds from the external environment [184, 185]. Studies have shown that a moist wound environment is better for healing than a dry environment as a decrease in dehydration, cell death, inflammatory response, and pain and an enhancement in re-epithelialization, matrix deposition, and angiogenesis were reported [184, 186]. This improvement has been attributed to the increase noted in GF and cytokine levels in the wound fluid and to the reduction of scab formation, which usually characterizes dry wounds, facilitating cell migration [186]. In human subjects, wound moisture achieved by the dressing has been found to enhance healing rates, give better aesthetic results, and reduce pain and infection rate [185]. Healing of full-thickness wounds created in nu/nu mice transplanted with human skin were found to be better when Op-Site dressing was used as compared to wounds left without dressing (dry). Scab was also noted in dry wounds [186]. One possible disadvantage to this dressing is that there is no absorption; excess fluid retention may adversely affect healing [184]. Exudate accumulation, resulted from a prolonged inflammation state that characterizes the majority of chronic wounds, can also affects the optimal moisture level needed for proper healing [185]. Moreover, it is well known that saliva contains several factors necessary for healing. The probability of animals licking the wounds is very high and unwarranted. Abbasian et al., 2010 [187] exemplified this concept in rats by creating...
longitudinal ventral and dorsal full-thickness sutured incisions. The ventral side was accessible to saliva through animals licking while the dorsal one was beyond their reach. As expected, better healing, assessed clinically and histologically, was noted in the ventral wound. Since we are applying cells to the wounds, the use of dressing was, thus, imperative to prevent animal interference and desiccation. The non-STZ induced control group was, therefore, canceled from the experimental design due to the problems mentioned above.

4.2.3 Clinical observations in STZ-induced NSG mice

In our experiment, animal health was compromised after induction and diabetes-like clinical signs were noted similar to what have been previously reported in various experimental hyperglycemic animal models. As previously mentioned in Chapter 3, we noted weight loss, dehydration, lethargy, loss of appetite, a pinched face appearance with a rough fur, and in some cases, a hunched posture. Other signs reported in the literature include, respiratory distress, poor body condition [181], polyuria, and polydipsia, [178, 181]. Polyphagia was also reported [178]; however, we did not observe this sign in our animal model. Also, in the study mentioned earlier by Graham et al., 2011 [181], clinical signs exhibited by the hyperglycemic nude mice varied across the suppliers as the signs were more frequent in JAX and TAC compared to CRL.

4.2.4 Wound healing is compromised after STZ induction

Wound closure was found to be significantly delayed by day 7 post-wounding in STZ-induced mice compared with non-STZ induced control mice, with wound closure percentages of 27.74±17.14 and 65.68±13 % respectively. Although these values represent the average measurements of n=4 wounds in solitary observations, replicates could not be made due to technical difficulties experienced in earlier pilot studies which resulted in canceling the control group from the experiment and thus could not be repeated. However, a similar healing response was noted from another solitary observation from an early pilot study. Unfortunately, data could not be included due to variances in wound size and shape originally created which was part of optimizing the punch biopsy technique. There is paucity in published work investigating wound healing in hyperglycemic immunodeficient animal models. One recent study by Kim et al., 2010 [159] showed a marked decrease in healing in STZ-induced Balb/c nude mice compared to normal athymic nude mice where the former achieved ~50% wound closure by day 12, while the latter reached this percentage by day 7. Their values are somewhat close to ours; a possible indication that our data could be close to a true observation.
In other non-STZ induced immunodeficient mice models, delayed healing was also reported; however, differences seem to be present among various immunodeficient mice strains. In 2006, Gawronska-Kozak et al. [188] compared healing of full-thickness dorsal incisional wounds (clipped for 5 days) among several immunocompromised mice with the C57BL/6J background [athymic nude-nu, SCID, Rag (lack B and T cells), athymic, and mice treated with cyclosporin A, an immunosuppressant, and wild-type controls]. They noted that only athymic nude-nu mice showed low levels of pro-scarring cytokines, PDGF-B, and TGF-β1, and collagen. However, all wounds exhibited complete closure by day 7 post-wounding.

Histologically, we found significant difference in skin thickness between hyperglycemic and control NSG mice where it was thinner in the former (0.35±0.08 vs. 0.5±0.05 mm respectively). Most notably are the thickness and the appearance of the dermal layer between the 2 mice. Less dense collagen fibers were noticed in the dermis of the hyperglycemic mouse skin compared to the control with the lack the basket-woven morphology recognized in normal dermis. In addition, the wound bed was thicker and more vascularized and more new-epithelial growth was noted in the non STZ-induced control mouse reflecting a healthy forming granulation tissue while the opposite was true for the hyperglycemic mouse. These observations corroborate previous reports.

4.2.5  No observable effect of cells on wound healing

4.2.5.A  Review of treatment results in relation to the literature

Results of wound area analysis suggest that wound closure was enhanced in cell- and sham-treated wounds in comparison to untreated ones by day 7 post-treatment. Unfortunately, the bona fide effect of using cells over MC or even using HUCPVCs over hBM-MSCs in such a model could not be established. Histologically, no significant signs of improvement in various healing parameters were observed in various treatments groups. In fact, results were highly variable and could not be attributed to one of the treatment type used. Theoretically, and based on the many qualities HUCPVCs have compared with BM-MSCs in addition to our own observations that proliferation capacity of HUCPVCs under hyperglycemic conditions is better than BM-MSCs, we expected better healing outcome after HUCPVCs treatment particularly to the quality and vascularity of the wound bed. Surprisingly, the morphology of the wound bed was found in some cases to be better in sham and untreated wounds compared to cell-treated ones. Our results, therefore, don’t coincide with the majority of the published work where enhanced healing parameters were reported when MSCs from the BM [105, 106, 107, 108, 109, 200], blood [159, 180, 189-191], adipose tissue [192, 193] were applied in different experimental hyperglycemic wound models. Having said that, we did not
confirm whether human cells and/or products are present in the wound, an important measure to confirm engraftment and possible functional contribution to healing. Certainly, the study needs to be repeated using a larger sample size.

We have previously explored in Chapter 1 the biological differences between HUCPVCs and adult hBM-MSCs derived from in vitro observations. Recent reports in the literature also highlight such differences between MSCs isolated from younger and older sources [77, 78]; however, studies on the beneficial effect of one type over another in an in vivo setting are not that common. A study published in 2009 by Markel et al. [194] showed that neonatal rat BM-MSCs did not show better recovery after an ischemic heart injury compared to adult MSCs. In fact they reported enhancement in certain myocardial functional parameters after adult MSC treatment. The same group in an earlier study did show biological differences between adult and neonatal mouse BM-MSCs in vitro [124] where neonatal cells showed faster proliferation and lower levels of IL-6 and VEGF and higher levels of IGF-1 under both basal conditions and after challenge with TNF, but not LPS. A more recent study by Dayan et al., 2011 [195] showed using an experimental acute myocardial infarction (MI) model in NSG mice that both hBM-MSCs and HUCPVCs increased infiltration frequency of monocytes/macrophages that have the anti-inflammatory phenotype to the injury site. This was believed to be due to, in part, IL-10 release by the MSCs. Improved cardiac function and a decrease in cardiac remodeling was also noted when either cell types were used. More comparative in vivo studies implicating MSCs from younger and older sources are certainly needed in various clinical contexts.

4.2.5.B Stress and malnutrition

As mentioned in Chapter 3, we noticed differences in wound healing capacity between the hyperglycemic mice, regardless of treatment type used. The only theme that seemed to be different among them was their overall health status. Criteria used to judge “healthier” from “weaker” hyperglycemic mice was based on behavioural observations made on their overall appearance, activity, weight loss, appetite, and also on wound healing parameters evaluated from digital images and histosections. We have previously mentioned that healing is governed by local and systemic factors. It seems that systemic factors related to the overall health and disease state are of great importance in our case. Among such factors are the degree of hyperglycemia, stress, and malnutrition [196]. In our study, we have shown that healing is impaired in hyperglycemic NSG mice compared to non-STZ-induced control mice reflecting, in part, the influence of hyperglycemia-related stress on the healing response. We also noted that the degree of hyperglycemia, per se, did
not significantly correlate with overall observations made on mice health and healing outcome. Indeed, glucose values were higher in the weaker hyperglycemic mice than the healthier ones at the day of surgery (18.4±2.5 vs. 14.5±2.4 mmol/L respectively); however, by the end of the experiment, they both reached almost similar values (18.5±0.5 mmol/L in the weaker vs. 17.9±3.1 mmol/L in the healther). It has been previously shown that the damaging effect of STZ can include other organs besides the pancreatic β-cells; therefore, individual tolerance and the extent of the toxic effect of STZ cannot be anticipated.

However, the nature of the experiment itself from hyperglycemia induction to wounding and dressing placement can be viewed as a major source stress to the animals. Generally, stress has great pathophysiological and behavioral impact which has been previously examined in both human and animal studies. Clinically, it has been previously implicated in various conditions including cardiovascular disease, cancer, diabetes, and impaired wound healing [196]. Stress has been found to up-regulate glucocorticoids (GCs), a class of steroid hormones, and reduce the expression of the pro-inflammatory cytokines such as IL-1α and β, IL-6, IL-8, and TNF-α at the wound site affecting, therefore, the initial phase of healing. In glucocorticoid-treated mice, the induction of pro-inflammatory cytokines was found to be significantly reduced after cutaneous injury. The impact of glucocorticoids on angiogenesis and matrix deposition has been also shown as down-regulation of VEGF and TGF-β was reported [197]. Stressors can also negatively impact behavior and appetite, which in turn influence health and, ultimately, healing [196, 197]. The effect of stress on wound healing is comprehensively reviewed by Guo and DiPietro, 2010 [196]. However, behavioral stress response in mice from different batches does not seem to be uniform. Since there is a mutual communication between the central nervous system, endocrine, and immune systems, it seems logical, therefore, that health is greatly impacted in our NSG mice as their immune system is greatly compromised compared to other immunodeficient strains. A larger number of animals need to be used in order to elucidate whether this strain serves as a good hyperglycemic model for impaired wound healing studies.

Moreover, one of the negative consequences of hyperglycemia and stress is poor nutrition. Energy is critical for various cellular activities at the wound site [166]. In 2004, Gupta et al. [165] profiled important energy metabolizing enzymes (glycolysis, Krebs cycle, and pentose phosphate pathway) and significant alterations in their activities in the granulation tissue of immunocompromised and aged Sprague-Dawley rats were noted. The negative influence of the prolonged inflammatory phase and malnutrition observed in chronic wounds has been shown to be enhanced when nutrients such
as arginine, glutamine, butyrate, and vitamins were used [166, 196].

In our study, wound image analysis has showed that wound closure in HUCPVC-treated wounds was very close in both healthier and weaker hyperglycemic mice unlike hBM-MSC- and sham-treated wounds where values were lower in the weaker groups and higher in the healthier ones. Although non-significant, this could possibly indicate that HUCPVCs performed better under the harsh microenvironment of hyperglycemic mice’ wounds and enhanced healing, an important quality warranted for cells intended to be used in similar circumstances. However, this observation needs to be supported by more evidence, a matter that we could not provide in this study.

4.2.5. C Other possible factors

There could be other factors that contributed to the lack of an observable beneficial effect of cell treatment on healing. Cell engraftment, the number of cells applied to the wounds, and the efficiency of MC as a cell delivery material, may all have contributed to the observed results.

For cells to produce the desired effect they have to successfully engraft in the injured tissue. A strong correlation between the degree of cell survival and vascularization at the implantation site has been also reported [163]. However, there seems to be strain and gender related efficiency to be considered among immunodeficient mice. NSG mice were found to be better than other immunocompromised strains for hematopoietic and cancer stem cell engraftment. However, females were found to be better than males [198, 199]. Generally, cell loss following implantation is often attributed to necrosis and apoptosis. This, in part, can be due to the unfavorable microenvironment of the injured site which is usually hypoxic, rich in oxidative by products and inflammatory cytokines. The lack of mitogenic signals or appropriate adherence to the ECM, anoikis, have also been suggested as other possible factors affecting cell survival [91, 163, 212].

It has been widely reported that only a small percentage of the total administered cells will successfully engraft. Wu et al., 2007 [109] indicated that by day 7 post-transplantation, only 27% of the $1 \times 10^6$ allogeneic GFP+ BM-MSCs administered into mice dermal wounds were present, assessed by FACS analysis of GFP+ cells in skin digests. This percentage further decreased with time and reached 2.5% by 28 days. In another study where $(1 \times 10^7)$ human CD34+ and CD34- BM cells were xenotransplanted in rat intervertebral coccygeal discs, CD34- cells were detected at all time-points tested up until 42 days while no CD34+ cells were detected by day 21. A decrease in the percentage of CD34- cells was also noted (78% and 67% by 21 and 42 days respectively), Wei et al., 2009 [99].

It appears, therefore, that engraftment and survival is also cell type specific within the context of a certain environment. Giannoni et al., 2010 [209] also demonstrated, using Nu/Nu immunodeficient
mouse model of ectopic bone formation, a decrease in engraftment (30% and 50% by 24 and 48 hours respectively) of Luc+ sheep BM-MSCs seeded on ceramic scaffolds and implanted subcutaneously. Detection of these cells were possible for 2 months, although preconditioning the cells with TNF-α enhanced long-term survival (from <5% to >8%). Undoubtedly, methods used to track implanted cells in vivo, critically reviewed by Terrovitis et al. 2010 [208], are also important to be considered as they have various sensitivities and, thus, would impact the results.

In addition to cell engraftment, the number of the applied cells is also important to be considered due to the critical influence of a particular microenvironment which probably requires a certain number of cells, or threshold, to provide the maximum effect [213]. This threshold number can be determined by conducting a cell-dose response study. Although such studies are infrequently conducted, they do provide useful information about the capacity of different types of cells under various pathological conditions. A recent study by Winkler et al., 2009 [206] provided experimental evidence showing the logarithmic association of the number of BM-MSCs transplanted and functional improvement in a rat skeletal muscle injury model. Doses ranged between 0 and 10x10^6 where the latter dose showed the most pronounced effect on muscle contraction forces measured.

However, the choice of cell dose reported in the literature is rather arbitrary with no justification provided for the number employed in a specific wound size. For example, for a 5mm punch wound, 1x10^6 cells were applied [159] while for 6mm punch wounds, 2.5x10^6 [200] and 0.5x10^6 [191] were used. For an 8-mm wound size both 0.75x10^6 [108] and 2x10^6 [189] were reported. Clearly, there is no logical correlation between dose and wound size in the published work. In our study, we applied a one-time cell dose of 0.1x10^6 to the 4mm wounds created which was calculated based on the final volume used to deliver the cells (5μl). Due to the harsh wound microenvironment of the hyperglycemic mice, especially those exhibiting poor health, a larger number of cells and/or multiple applications may have been better choices.

It has been reported that delivering cells in matrices that structurally resemble the ECM of the damaged tissue is better than normal saline for a better therapeutic outcome [161]. However, differences do exist between various natural and synthetic materials employed, which influence their reparative capacity and ultimate use for cell delivery [207]. In our study, MC at a final concentration of 1% was used to apply the cells to the wounded sites. In a previous study conducted by Wells et al., 1997 [161] where no cells were delivered, MC, at a concentration of 2%, was found to perform better, either alone or combined with GFs (PDGF-BB and IGF-I), than other matrices tested (collagen and laminin), also either alone or mixed with GFs. In another study of albino rabbits
undergoing trabeculectomy a procedure, 2% MC was found be better than normal saline in reducing intraocular pressure [160].

However, a recent study by Patel et al. 2010 [163], reporting the influence of various matrices (ECM “laminin:collagen”, Matrigel™, and MC) on long-term survival and functional outcome of Schwan cells in a rat model of thoracic spinal cord injury, showed that cell survival, assessed at 12 weeks following transplantation, was highest in cells transplanted in Matrigel and ECM, 36% and 27% respectively (evaluated using stereological quantification method for GFP labeled implanted cells), compared to MC (2%) and medium alone (DMEM/F12 – 14%). They ascribed the poor results obtained with MC to the lack of cell adherence to this material and/or its unsuitability to support angiogenesis and axon growth. They also highlighted that their MC formulation of 5%, which initially used to provide more mechanical support, might have restricted cells from functioning properly and prevented nutrient diffusion to the cells. Therefore, it would seem that material formulation impacts cell survival and function. Cell death due to the lack of attachment to MC, anoikis, has been also reported elsewhere. It was even suggested that MC should be modified with certain ECM adhesion molecules to enhance cell attachment. For example, when laminin was immobilized to MC, higher survival of cortical neurons was observed compared to MC alone [11].

In an earlier in vitro pilot study, we cultured HUCPVCs and hBM-MSCs in the presence of MC in culture medium, and even at concentrations of 3%, cells survived for several days (data not shown). The behavior of cells, however, when applied with MC in vivo, and their survival rate, has not been assessed. Importantly, the exact number of cells that has been successfully delivered to the wound site from the intended dose (i.e. 0.1x10⁶ cells) is also not known. Although cell-MC suspensions were well mixed before each application, we don’t exclude pipetting errors and, thus, the exact number of cells applied. Another factor is the loss of a certain percentage of cells while in the MC solution. Although cell-MC suspensions were kept on ice the entire surgery time, waiting time until transplanted varied, which ranged between 30 minutes and 3 hours. Undoubtedly, in such type of studies, tracking human cells and/or products at the defect site is an imperative measure to be implemented. Overall, MC biocompatibility has been previously assessed [161, 162]; however, reports of its potency in vivo, with or without cells, are not conclusive. More studies are, therefore, needed to evaluate its optimal formulation for cell delivery in various tissue repair models. MC, as a delivery system, possesses great advantages due to its availability, ease of preparation at different densities, and its accessibility to sterilization.
Conclusions

1. HUCPVCs have higher proliferation capacity under normal and hyperglycemic culture conditions, and lower expression of a senescence marker under all tested conditions, than hBM-MSCs.

2. Hyperglycemia can be induced in NSG mice and wound healing was found to be impaired in the STZ-induced animals compared to normal controls.

3. Tolerance to the toxic effect of STZ, and overall health status, varied among the induced mice and was reflected in wound healing.

4. The percentage of wound closure was higher in cell- and sham-treated wounds compared to untreated ones with no significant differences between treatment groups as tested at day 7 post-treatment.

5. While the healing potential of HUCPVCs and hBM-MSCs of full-thickness dermal wounds could not be established in this hyperglycemic animal model, results indicated that HUCPVCs had a greater healing effect in animals of poorest health status.
Future Directions

The project and the resultant outcomes provided evidence that the subject under study was genuinely complex and involved many different variables. This opens the door to a multitude of possibilities to address the shortcomings of the study and to optimize both in vitro and in vivo models; and make them more robust for testing desired treatment choices. To this end, the following options are suggested:

**In vitro:**

1. Due to the complex nature of the hyperglycemic wound microenvironment, testing a combination of biochemical factors (e.g. hyperglycemia, hypoxia, AGEs, etc.) on various cellular activities in vitro is warranted to better compare the metabolic and functional capacities of cells isolated from neonatal and adult sources.

2. The molecular changes induced by hyperglycemia, hypoxia, AGEs and others on cellular functions and growth factor secretion profiles are important to be considered. This would be necessary to understand their influence on the biological functions of the administered cell type, and possibly predict their in vivo potency.

3. Angiogenesis is an important part of wound healing which happens to be, as evidenced in the literature, to be less optimal in diabetes. Testing co-culture systems of HUCPVCs and hBM-MSCs with human endothelial cells (ECs) could provide comparative insight into their paracrine potency on the latter.

4. A 2-D in vitro system was used herein. It would be more informative to employ a 3-D configuration to assess the biochemical factors listed above.

**In vivo:**

1. The major limitation of the study was the sample size (n=6), especially given the great variability noticed in weight loss and overall health of hyperglycemic individuals.

2. The in vivo study did not include normal control (non-hyperglycemic) mice. Ideally, it is a preferred measure to include such a group in any work involving testing a treatment module in animals as this would establish an accurate baseline and a true representation for the differences seen, if there is any. However, the strain of mice chosen mitigated against this possibility. Thus, assessing other more compliant strains would be beneficial.
3. One of the study limitations was cell engraftment although not technically verified. However, the overall study outcome indicated that it was rather unsuccessful. Several options could be tested:

- Further analysis of the histosections to verify the presence of human cells and/or products in the wound bed. Immunofluorescence or immunohistochemistry could be utilized for this purpose using various human specific antibodies (e.g. anti-human nuclear antibody HuNu).
- A cell-dose response relationship between cell number introduced and engraftment success. This would eventually be reflected on wound healing.
- Testing 2 or more cell carriers beside MC like collagen sponge and fibrin, the 2 most widely cited in the literature.
- Timing of cell application to examine the effectiveness of applying cells at various time-points after wounding and healing rate. Allowing the animal to acclimatize to the dressing and for fluid to build-up under the dressing could positively affect engraftment.

4. The extent of the toxic effect of STZ on animal health was not tested. As noted in the study, animal health deteriorated very quickly after induction and commencement of surgery. This affected wound healing as other factors, such as nutrition and stress, were added. It would be interesting to compare various doses of STZ on induction success in NSG mice and hyperglycemia stability over a period of no less than 4 weeks. Another possibility in this regard is to test the low-dose protocol and compare it to the one used in this study.

5. A time-point study is desirable when designing wound healing studies as this would provide information about the specific contribution of the implanted cells at various stages of healing. This would also involve using larger number of animals to raise the power of the study and make time-points more sensible.

6. After establishing a working hyperglycemic animal model, it would be interesting to test the mechanisms used by implanted cells in wound healing as reports on this issue are still scarce.

7. Studying wound tissue extract to detect the cell release profile of various factors at different time points would also be important as part of assessing the in vivo synthetic activity. Western blotting, ELISA, and PCR can be used for this purpose.
References


95. S. Schlosser, C. Dennler, R. Schweizer, D. Eberli, J. V. Stein, V. Enzmann, P. Giovanoli, D. Erni, and J. A. Plock. Paracrine effects of mesenchymal stem cells enhance vascular


### Appendix A: Streptozotocin (STZ) dosing chart

Table A.1: Adjusted STZ dosage calculation chart.

<table>
<thead>
<tr>
<th>Mice weight (g)</th>
<th>STZ dose (mg)</th>
<th>Na citrate buffer volume (µl)</th>
<th>Adjusted STZ dose (mg)</th>
<th>Adjusted STZ dose (g)</th>
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Notes:

**Dose used:** 150 mg/kg (0.15 mg/g)

**Delivery concentration:** 22.5 mg/ml in sodium citrate buffer pH 4.6

**Application method:** Intra-peritoneal (IP)

- A correction criterion has been employed to accurately deliver the intended STZ dose. We, therefore, calculated the volume of the solution left in the syringe tip plus the needle which was 75 μl and we added this volume to the original volumes calculated that correspond to specific animal weight and all STZ dosages were adjusted afterwards. The last 2 columns are the ones we followed in our study.
## Appendix B: Glucose and weight measurements

Table B.1: Blood glucose and weight profiles for STZ-induced and non-induced control NSG mice.

### Non-STZ induced control NSG mice

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>day 0 (Induction)</th>
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<th>day 5 (Wounding)</th>
<th>day 12 (Sacrifice)</th>
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n=4

### Glucose change (mmol/L)

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n=4

### STZ-induced NSG mice

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n=11 day 0-5, n=9 day 12

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n=11 day 0-5, n=9 day 12
Appendix C: Wound digital images profile

Table C.1: Day 7 wound digital images of healthier and weaker hyperglycemic NSG mice

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### Weaker hyperglycemic mice

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Appendix D: Protocols used

D1. High-dose STZ induction protocol for mice

Based on the protocol provided by the: Animal Models of Diabetic Complications Consortium (AMDCC Protocols, version 1, edited by: The University of Michigan Medical Center, Frank Brosius, 04/03/09) with slight modifications.

Materials needed:
1. Streptozotocin (Sigma-Aldrich, S0130-1G)
2. Sodium citrate (Fisher, S273-3)
3. dd H₂O
4. Sterile 15 ml tubes
5. Eppendorf tubes (for STZ aliquot)
7. Syringes (1cc)
8. Ice bucket

Preparations needed:

• Streptozotocin powder (MWT: 265.22)
Weigh the appropriate amount of STZ to give a final concentration in the sodium citrate buffer of 22.5 mg/ml (see dosing chart in Appendix A); place in eppendorf tube and protect from light (foil can be used otherwise place immediately in ice bucket and cover the box). Label appropriately. If not used immediately, place at -20 °C.

• Sodium citrate buffer, 0.1 M, pH 4.6

Stock solutions:
1. Citric acid (0.1 M)
   Dissolve 0.105 g of citric acid monohydrate (Sigma C0706, MWT 210.1) into 5 ml dd H₂O, store at RT. Adjust volume as required.

2. Sodium citrate (0.1 M)
   Dissolve 0.147 g of sodium citrate (Fisher, S279-3, 28771, MWT=294.1) in 5 ml dd H₂O, store at RT. Adjust volume as required.

Working buffer solution:
Mix (25.5 ml) of 0.1 M citric acid and (24.5 ml) of 0.1 M sodium citrate, complete the volume to 100 ml with dd H₂O. Check pH 4.6 and then filter using (0.45 μm) syringe filter in sterile tubes and keep on ice. Must be prepared fresh for every injection. Adjust volume as required.
**Induction procedure:**

1. Measure blood glucose before fasting the animals.
2. Fast mice (4-6 hr) prior to injection.
3. Inside a biosafety cabinet, pipette the required volume of sodium citrate buffer into eppendorf tubes containing specific mass of STZ (see Appendix A). Mix thoroughly. Usually STZ dissolves within 1-2 minutes.
4. Using a 1 cc syringe, draw dissolved STZ solution and inject it IP as shown in the image below.
5. Repeat the same procedure for each animal
6. For the control group: inject only sodium citrate buffer, same volume adjusted for their weight.
D.2 Ki-67 staining protocol (ICC-IF)

**Ki-67**: is a nuclear antigen which is expressed in proliferating cells, normal and tumor tissues, but absent in resting cells (i.e. present in G1, S, G2, M phases, but not in G0).

**Materials needed:**
1. Mouse monoclonal Ki-67 antibody IgG (ab8191) (human only), 0.15 mg/ml, dilution: 1:300.
2. Secondary Ab used: Alexa Fluor® 488 goat anti-mouse IgG (H+L), invitrogen, A-11001, 2 mg/ml, dilution: 1:500.
3. 1% BSA in PBS
4. 0.05% Triton-X 100 in PBS

**Preparations needed:**

**BSA in PBS, 1% (w/v):**
- 1 g BSA + 100 ml 1X PBS. Adjust volume as needed.
- Put on shaker until it dissolves then filter the solution with a (0.45 μm) syringe filter.
- Keep at 4 °C.

**Triton-X 100, 0.05% (v/v):**
- This material is viscous. Pipette very slowly.
- Add PBS first to the tube then once you add the Triton X-100, start inverting the tube gently without shaking to prevent producing bubbles. Don’t stop until all clumps are dissolved.
- Store in the dark to prevent photo oxidation.

**Protocol:**
1. Wash cells cultured on glass coverslips inside 24 well plate with PBS (+/ +), 2 X, 3 min each.
2. **Fixation**: 10% NBF, 10 min, RT. Don’t let cells dry out, add fixative immediately.
3. Wash with PBS (+/ +), 3X, 3 min each.
4. **Permeabilization**: 0.05% Triton X in PBS, 10 min, RT, on shaker.
5. Wash with PBS (+/ +), 3X, 3 min each.
6. **Blocking**: 1% BSA in PBS, 1 hr, RT.
7. Wash with PBS (+/ +), 3X, 3 min each.
8. **Primary Ab (diluted in the blocking buffer)**: Place 50 μl aliquots on a parafilm paper inside a humidity chamber and mount coverslips (cell layer facing down) on the droplets. Incubate the chamber at 4 °C, overnight.
9. Next day: carefully lift coverslips and return them to the 24-well plate. Wash with PBS (+/ +), 3X, 3 min each.
10. **Secondary Ab (diluted in blocking buffer)**: Place 50 μl aliquots on a piece of parafilm as mentioned in step (8). Incubate for 1 hr, dark, RT.
11. Return coverslips back to the 24-well plate. Wash with PBS, 2X, 2 min each.
12. **Counterstaining**: Hoechst 33342, diluted in PBS (1:5000). Also place 50 μl droplets on a Parafilm and mount coverslips on top of them. Incubate for 5 min, dark, RT.
13. Rinse 2X with PBS (-/ -).
14. Store in the dark, in the fridge for short-term storage or at -20 °C for long-term storage.
15. Positive control: HeLa cells.
16. Negative control: primary Ab was deleted and replaced with a blocking buffer.

**Result calculation:**
1. Filters used: GFP filter for Ki-67 and DAPI filter Hoechst.
2. Take fluorescent images at 10x, 7-10 random fields of view. Nuclei will stain blue (Hoechst), and green for proliferating cells.
3. Count Ki-67 positive cells (green) in each image.
4. Count Hoechst positive cells (total blue nuclei) in each image.
5. Calculate proliferation index by measuring the number of proliferating cells as a percentage of the total cell count. The formula below can be used:

\[
\text{Ki-67 proliferation index} = \frac{\text{Total no. of Ki-67 positive nuclei (green)}}{\text{Total no. of nuclei (blue)}} \times 100
\]
D.3 Skin processing protocol for histological analysis

Materials used:
1. 10% neutral buffered formalin (Sigma)
2. Ethanol (EtOH)
3. Methyl benzoate (Sigma, # M29908)
4. Xylene (Caledon Laboratories Ltd.)
5. Distilled water DW
6. Harris hematoxylin (Leica Microsystems, # 3801601)
7. Eosin (Leica Microsystems, # 3801561)
8. Acid alcohol (0.7% in 70% EtOH)
9. Ammonia water 0.25%
10. Tissue softener (Molifex, EMD, Gibbstown, NJ, USA)
11. Paraffin (POLYFIN™ Embedding and Infiltration Paraffin, Triangle Biomedical Sciences®, Durham, NC, USA)
12. Mounting media (Krystalon, EMD, # 64969-71)

Equipment used:
1. Embedding station (Leica EG 1160 Microsystems embedding station, Nussloch, Germany)
2. Leica microtome (American Optical 820 Rotary Microtome, Richmond, Illinois, USA)
3. Dissecting microscope (Heerbrugg Switzerland Wild M3Z, Type B, Germany)
4. Slide warmer (Fisher Scientific, model 77, 24”x7”, Cat# 12-594)
5. Microscope slides (VWR, frosted Goldline and superfrost®Plus)
6. Metal molds and embedding rings (Tissue-Tek®)
7. Vacuum oven (Fisher Scientific, model 280)
8. Water bath (Tissue-Tek® II, model 4674)
9. Slide coverslips (VWR, size: 24x60 mm)
10. Glass trays and slides rack

Fixation and processing:

Day 0
After sacrifice, the whole animal was fixed in 10% neutral buffered formalin (NBF) overnight at 4 °C. Small pins were used to slightly stretch the skin around the wounds to reduce skin folding while in the fixative.

Day 1
The wounded dorsal area was cut as a big square with the spine, ribs, and dorsal musculature. It was then pinned to a flat piece of cork, placed inverted (tissue facing down) in NBF container, and left for another 24 hr at 4 °C, Fig. 2.5.

Day 2
Under the dissecting microscope, the excised tissue was cut in half along the spine and carefully freed from the spine and ribs, using a small surgical scissor and a blade. It was then pinned back to the piece of cork and placed inverted in NBF container, and left for 24 hr at 4 °C, Fig. 2.5.
**Day 3**

1. Samples were imaged under the dissecting microscope for documentation.

2. **Washing:** the fixative was discarded and samples were rinsed with PBS, 1 hr, RT, on shaker.

3. **Dehydration:** PBS was discarded and samples were washed with increasing grades of alcohol (70, 95, and 100%), 30 min-1 hr each, RT, on shaker. Then, samples were incubated with Methyl benzoate for 24 hr, 4 °C.

4. **Clearing:** samples were placed in xylene (to remove alcohol), 1 hr, RT, on shaker.

5. **Infiltration with the embedding material (paraffin):** xylene was discarded and melted paraffin was added. Vials were then placed in the vacuum oven at 60 °C for 1hr. The process was repeated 2 more times and the last step was done under vacuum pressure 20"Hg.

6. **Embedding:** Using the hot side of the embedding station, an off-center cut (across the widest wound diameter far from the centre) was made for each wound (Fig. 2.5) and the largest piece was vertically placed in the metal molds (cut side facing down) containing little melted paraffin. The mold was then moved to the cold side of the station for few seconds to stabilize the positioning of the sample. A ring was placed on top of the mold and embedding was completed with melted paraffin. Blocks were then left on the cold side of the embedding station to harden. Blocks should be cooled slowly to avoid cracking. Blocks were stored at 4 °C.

**Sectioning paraffin blocks:**

1. Before actual sectioning began, samples were trimmed first until reached the surface of the tissue and were then placed (trimmed surface facing down) in a tray containing a piece of gauze soaked with tissue softener for several days.

2. Using a microtome, serial sections (6-7 µm in thickness) were produced from each block.

3. The temperature of the water bath (filled with DW) was adjusted to ~40 °C.

4. From every 10 or 20 sections produced from each block, (3-4) attached sections were carefully placed on the surface of the water until no wrinkles could be seen. The quality of the tissue can be verified under a light microscope.

5. Sections were then picked up using a glass slides (coated ones are preferred), air dried for few minutes and then placed on a slide warmer, 37 °C, for 24 hr.

6. The following day, slides were processed for staining or stored in a box at RT for later use.
Staining paraffin-embedded sections with H&E:
1. Put slides in a slide rack and then place it inside an oven (~50 °C) for 5-10 min to slightly melt the paraffin.
2. Dip the rack into xylene to remove wax and then into EtOH to remove the xylene as follows:
   - Xylene: 3X, 3 min each.
   - 100% EtOH, 3X, 2 min each.
   - 95% EtOH, 2X, 2 min each.
   - 70% EtOH, 2X, 2 min each.
   - 50% EtOH, 2X, 2 min each.
3. Rinse with DW to remove EtOH, 2X, 2 min each.
4. Stain with hematoxylin, 10 min.
5. Rinse with tap water, 2 min.
6. Place in acid alcohol to remove background stain, 1-2 dips.
7. Rinse with tap water, 1 min.
8. Place in ammonia water, 1 min.
9. Rinse with DW, 2X, 2 min each.
10. Stain with eosin, 1 dip (30 – 60 sec, depending on section size, 45 sec is an average).
11. Dehydrate in graded EtOH as follows:
   - 95% EtOH, 2X, 15 dips each.
   - 100% EtOH, 2X, 15 dips each.
12. Place in xylene, 3X, 2 min each.

Mounting:
- Work is done inside a fume hood.
- Keep slides in xylene while mounting and never let them dry.
- Add 2-3 drops of the mounting medium (xylene based) around the sections and cover with a rectangular coverslip.
- Leave inside the hood on a clean flat surface until they dry.