ผลของเซลล์เอนโดทีเลียมโปรเจนนิเตอร์ร่วมกับเซลล์ต้นกำเนิดมีเซนไคมอลต่อการเกิดหลอดเลือดใหม่และการหายของแผลในหนูไมซ์ที่เป็นเบาหวาน

นางสาวศุภกานดา สุขแพทย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2554

EFFECTS OF COMBINED ENDOTHELIAL PROGENITOR CELLS (EPCs) AND MESENCHYMAL STEM CELLS (MSCs) ON ANGIOGENESIS AND WOUND HEALING IN DIABETIC MICE

Miss Supakanda Sukpat

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Thesis Title	EFFECTS OF COMBINED ENDOTHELIAL PROGENITOR CELLS (EPCs) AND MESENCHYMAL STEM CELLS (MSCs) ON ANGIOGENESIS AND WOUND HEALING IN DIABETIC MICE
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ศุภกานดา สุขแพทย์ : ผลของเซลล์เอนโดทีเลียมโปรเจนนิเตอร์ร่วมกับเซลล์ต้นกำเนิดมีเซนไคมอลต่อการเกิดหลอดเลือดใหม่และการหายของแผลในหนูไมซ์ที่เป็นเบาหวาน (EFFECTS OF COMBINED ENDOTHELIAL PROGENITOR CELLS (EPCs) AND MESENCHYMAL STEM CELLS (MSCs) ON ANGIOGENESIS AND WOUND HEALING IN DIABETIC MICE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. สุทธิลักษณ์ ปทุมราช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.นพ.ดร.นิพัญจน์ อิศรเสนา ณ อยุธยา, 83 หน้า.

ศึกษาผลของเซลล์เอนโดทีเลียมโปรเจนนิเตอร์ร่วมกับเซลล์ต้นกำเนิดมีเซนไคมอลต่อการเกิดหลอดเลือดใหม่และการหายของแผลในหนูไมซ์ที่เป็นเบาหวาน โดยนำหนูนู๊ดไมซ์มาแบ่งออกเป็น 5 กลุ่ม กลุ่มละ 6 ตัว กลุ่มที่ 1 เป็นกลุ่มที่ควบคุม กลุ่มที่ 2 คือ กลุ่มที่เป็นเบาหวานกลุ่มที่ 3 คือ กลุ่มที่เป็นเบาหวานซึ่งได้รับเซลล์ต้นกำเนิดมีเซนไคมอล (1×10^6) กลุ่มที่ 4 คือ กลุ่มที่เป็นเบาหวานซึ่งได้รับเซลล์เอนโดทีเลียมโปรเจนนิเตอร์(1×10^6) กลุ่มที่ 5 คือ กลุ่มที่เป็นเบาหวานซึ่งได้รับเซลล์เอนโดทีเลียมโปรเจนนิเตอร์ (0.5×10^6) ร่วมกับเซลล์ต้นกำเนิดมีเซนไคมอล (0.5×10^6) ซึ่งหนูกลุ่มเบาหวานจะถูกเหนี่ยวนำให้เป็นเบาหวานด้วย streptozotocin 45มิลลิกรัมต่อกิโลกรัมน้ำหนักตัวทางช่องท้อง วันละครั้งติดต่อกัน 5 วัน เมื่อครบ 6-7 สัปดาห์ จึงทำให้เกิดบาดแผลชนิด bilateral full thickness excisional wound ขนาด 0.6 x 0.6 ตารางเซนติเมตร จากนั้นทำการใส่ fibrin gel หรือเซลล์ต้นกำเนิดตามการแบ่งกลุ่มการเบื้องต้น ในวันที่7 และ 14 วันหลังการเกิดแผลจะทำการวัดพื้นที่แผลด้วยโปรแกรม Image Pro-Plus จากนั้นทำการตรวจหาการเกิดหลอดเลือดใหม่ด้วย intravital fluorescence microscopy และนำชิ้นเนื้อตัวอย่างไปย้อม H&E ดูการเกิดของ re-epithelialization และดูการบุกรุกของนิวโทรฟิวล์ สุดท้ายจะทำการตรวจหาปริมาณของVEGF โดยการทำ ELISA

ผลการทดลองพบว่า 1) ค่าระดับน้ำตาลในเลือดของหนูที่อยู่ในกลุ่มที่เป็นเบาหวานทุกกลุ่มมีค่าสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติทั้งในวันที่ 7 และ 14 หลังการเกิดแผลส่วนค่าน้ำหนักตัวนั้นพบว่าทุกกลุ่มมีค่าไม่แตกต่างกันอย่างมีนัยสถิติเมื่อเทียบกับกลุ่มควบคุม 2) การปิดของแผลในทุกกลุ่มมีค่าเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่เป็นเบาหวานทั้งในวันที่ 7 และ 14 หลังการเกิดแผล 3) ในวันที่ 7 พบว่าในทุกกลุ่มมีการเกิดหลอดเลือดใหม่เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่เป็นเบาหวาน โดยเฉพาะยิ่งในกลุ่มที่เป็นเบาหวานซึ่งได้รับเซลล์เอนโดทีเลียมโปรเจนนิเตอร์ร่วมกับเซลล์ต้นกำเนิดมีเซนไคมอล พบว่ามีการเกิดหลอดเลือดใหม่เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่เป็นเบาหวานซึ่งได้รับเซลล์ต้นกำเนิดมีเซนไคมอลและกลุ่มที่เป็นเบาหวานซึ่งได้รับเซลล์เอนโดทีเลียมโปรเจนนิเตอร์ ส่วนในวันที่ 14 พบว่าในกลุ่มที่เป็นเบาหวานซึ่งได้รับเซลล์เอนโดทีเลียมโปรเจนนิเตอร์ร่วมกับเซลล์ต้นกำเนิดมีเซนไคมอลมีการเกิดหลอดเลือดใหม่เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่เป็นเบาหวาน 4) การเกิดre-epithelialization ทั้งในวันที่ 7 และ 14 นั้นพบว่าทุกกลุ่มมีค่าไม่แตกต่างกันอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่เป็นเบาหวาน 5) ในวันที่ 7หนูในกลุ่มที่เป็นเบาหวานมีการบุกรุกของนิวโทรฟิวล์ที่มากกว่าอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่ควบคุม ส่วนในกลุ่มที่เป็นเบาหวานซึ่งได้รับเซลล์ต้นกำเนิดมีเซนไคมอล กลุ่มที่เป็นเบาหวานซึ่งได้รับเซลล์เอนโดทีเลียมโปรเจนนิเตอร์ และในกลุ่มที่เป็นเบาหวานซึ่งได้รับเซลล์เอนโดทีเลียมโปรเจนนิเตอร์ร่วมกับเซลล์ต้นกำเนิดมีเซนไคมอลพบว่ามีการบุกรุกของนิวโทรฟิวล์มีค่าไม่แตกต่างอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่เป็นเบาหวาน ในวันที่ 14 พบว่าในทุกกลุ่มจะมีปริมาณนิวโทรฟิวล์ที่ลดลงอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่เป็นเบาหวาน 6) ในวันที่ 7 การวัดปริมาณของ VEGF พบว่าทุกกลุ่มมีค่าเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่เป็นเบาหวาน

โดยสรุปการศึกษาครั้งนี้แสดงให้เห็นว่าการใช้เซลล์เอนโดทีเลียมโปรเจนนิเตอร์ร่วมกับเซลล์ต้นกำเนิดมีเซนไคมอลทำให้เพิ่มการหลั่งปริมาณของ VEGF ทำให้การเกิดหลอดเลือดใหม่ ส่งผลทำให้ปริมาณนิวโทรฟิวล์ที่ลดลงการหายของแผลในหนูไมซ์ที่เป็นเบาหวานเกิดได้ดีขึ้น

สาขาวิชา สรีรวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5187290520 : MAJOR PHYSIOLOGY
KEYWORDS: ENDOTHELIAL PROGENITOR CELLS (EPCS)/ MESENCHYMAL STEM CELLS (MSCS)/ ANGIOGENESIS/ WOUND HEALING/ DIABETES MELLITUS

SUPAKANDA SUKPAT: EFFECTS OF COMBINED ENDOTHELIAL PROGENITOR CELLS (EPCs) AND MESENCHYMAL STEM CELLS (MSCs) ON ANGIOGENESIS AND WOUND HEALING IN DIABETIC MICE.ADVISOR: ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D., CO-ADVISOR: ASST. PROF. NIPAN ISRASENA, M.D., Ph.D., 83 pp.

The effects of combined endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) on angiogenesis and wound healing in diabetic mice were studied by using Balb/c nude mice. The animals were divided into five groups. Group 1 is control group with implanted fibrin gel (Control; (n=6)). Group 2 is diabetic group with implanted fibrin gel (DM; (n=6)). Group 3 is diabetic group with implanted fibrin gel and $1X10^6$ cells MSCs (DM+MSCs; (n=6)). Group 4 is diabetic wounded group with implanted fibrin gel and $1X10^6$ cells EPCs (DM+EPCs; (n=6)). Group 5 is diabetic wounded group with implanted fibrin gel and combined $0.5X10^6$ cells MSCs and $0.5X10^6$ cells EPCs (DM+MSCs+EPCs; (n=6)). Diabetic groups were induced by injection of streptozotocin ((STZ) 45 mg/kg BW ip. daily for 5 days). After 6-7 weeks, all mice were anesthetized and created bilateral full-thickness excisional skin wounds on the dorso-rostral back (0.6x0.6 cm2). Each mouse received fibrin gel or stem cells injection into wound bed. On days 7 and 14 post- wound, the percentage of wound closure (%WC) was accessed by using digital image analysis(Image Pro-Plus). The percentage of capillary density (%CV) was determined by using intravital fluorescence microscopy The re-epithelialization and the number of neutrophil infiltration were analyzed from wound tissue samples using H&E staining. Tissue vascular endothelial growth factor (VEGF) level was detected by ELISA.

The results showed that: 1) Blood sugar level in all diabetic group was increased significantly as compared to Control on day 7 and 14 post-wound. However, body weights of all groups were not significant difference as compared to DM. 2) On day 7 and 14, %WC of all groups increased significantly as compared to DM. 3) On day 7, the % CV of all group increased significantly as compared to DM. The %CV of DM+EPCs+MSCs group was significantly higher than DM+MSCs and DM+EPCs groups ($P<0.000$). On day 14, the %CV of DM+EPCs+MSCs group was increased significantly as compared to Control. 4) %Re-epithelialization in all groups was not significant difference as compared to DM. 5) On day 7, number of neutrophil infiltration in DM was increased significantly as compared to Control. DM+MSCs, DM+EPCs and DM+EPCs+MSCswere no significant difference when compared to DM group. On day 14, the number of neutrophil infiltration of all groups increased significantly as compared to DM. 6) On day 7, VEGF levels of all groups increased significantly as compared to DM.

In conclusion, the present study has demonstrated that the combined EPCs and MSCs can increase VEGF level and increased angiogenesis which lead to reduce number of neutrophil infiltration and enhanced wound healing in diabetic mice model.

Field of Study : Physiology Student's Signature .....

Academic Year : 2011 Advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

AGE's	=	Advanced glycation end products
bFGF	=	Basic fibroblast growth factor
BM	=	Bone marrow
CD	=	cluster of differentiation
CXCR-4	=	C-X-C chemokine receptor type 4
DM	=	Diabetes mellitus
DNA	=	Deoxyribonucleic acid
ECM	=	extracellular matrix
EGF	=	Epidermal growth factor
eNOS	=	Endothelial nitric oxide synthase
EPCs	=	Endothelial progenitor cells
G-CSF	=	Granulocytes colony-stimulating factor
HIF-1	=	Hypoxia-inducible transcription factor-1
IDF	=	International Diabetes Federation
IGF-1	=	Insulin-like growth factor 1

IGT	=	Impaired glucose tolerance
IL-8	=	Interleukin-8
KGF	=	Keratinocyte growth factor
LTs	=	leukotrienes
MMP-9	=	Matrix metalloproteinase -9
MSCs	=	Mesenchymal stem cells
NO	=	Nitrous oxide
$O2^{\circ}$	=	Superoxide anions
PBMCs	=	Bone marrow-derived Peripheral blood mononuclear cells
PDGF	=	platelet-derived growth factor
PF4	=	Platelet factor 4
PGE_2	=	prostaglandin E_2
ROS	=	Reactive oxygen species
SDF-1	=	Stromal-derived factor-1
STZ	=	Streptozotocin
TGF–α	=	Transforming growth factor α

TGF-β	=	Transforming growth factor β
TNF-α	=	Tumor necrosis factor-α
TSG-6	=	Tumor necrosis factor-α stimulated gene/protein 6
T1D	=	Type 1 diabetes mellitus
T2D	=	Type 2 diabetes mellitus
VCAM-1	=	Vascular cell adhesion molecule 1
VE-cadherin	=	Vascular endothelial cadherin
VEGF	=	Vascular endothelial growth factor
VEGFR-1	=	Vascular endothelial growth factor receptor-1
VEGFR-2	=	vascular endothelial growth factor receptor-2
vWF	=	Von Willebrand factor

CHAPTER I

INTRODUCTION

Diabetes mellitus (DM) is the most common severe chronic illnesses, in 2009, affecting over 230 million people worldwide with an estimated global prevalence of 5.1% (Reimann et al., 2009: 317-331). DM is a syndrome of disordered metabolism, resulting in hyperglycemia (Reimann et al., 2009: 317-331). Hyperglycemia induced glycolysis and mitochondrial reactions which lead to increase superoxide anions ($O_2^{\cdot}$) and reactive oxygen species (ROS) are commonly referred as underlining causes of diabetic complications (Maechler, Jornot, and Wollheim, 1999: 27905-27913). The development and progression of diabetic complications including nephropathy, retinopathy, neuropathy, diabetic ulcer, macro- and micro-vascular damages were significantly associated with hyperglycemia-induced oxidative stress. Particularly, one of diabetic complications which is characterized by poor circulation and impaired angiogenesis, appears to contribute to the frequent skin lesions and poor wound healing. Non-healing ulcers are the most common with high risk cause of amputation. In 2000, the risk of patients with diabetic ulcers was leading up to 85% for amputations (Apelqvist and Larsson, 2000: S75-83; Mayfield et al., 2000: 499-504; Pecoraro, Reiber, and Burgess, 1990: 513-521).

Not only poor blood supply, diabetic wounds are also shown the reduced chemotactic ability to recruit inflammatory cells into the injury tissue as which lead to prolong inflammation (Medina et al., 2005: 306-319). In addition, it has been reported that the production of growth factor and angiogenesis which caused by dysfunction of endothelial progenitor cells

(EPCs) and dysfunction of endothelial cells in diabetic wounds are significantly reduced (Fadini, Agostini, and Avogaro, 2005: 41-58; Imanishi, Tsujioka, and Akasaka, 2008: 275-286; Loomans et al., 2004: 195-199).

Moreover, the abnormal granulation tissue and collagen formation are identified in diabetic wounds as well. It is suggested that the lesion of diabetic tissue formation is caused by failure in the fibroblast and collagen synthesis (Spanheimer, Umpierrez, and Stumpf, 1988: 371-376). Therefore, under these multiple factors contributing to poor and prolong wound healing in diabetic patients, there are many studies that attempted to overcome these problems with multiple approaches, including tissue engineering and stem cells (Keswani et al., 2004: 497-504; Kwon et al., 2008: 453-463).

Circulating EPCs are thought to be a subset of bone marrow (BM)-derived peripheral blood mononuclear cells (PBMCs). Up to now, with advancing technology, EPCs can be isolated from peripheral and umbilical cord blood, and bone marrow. The origin of EPCs may not be limited to BM, tissue specific stem/progenitor cells possibly provide 'in situ EPCs' as other sources of EPCs than BM (Zammaretti and Zisch, 2005: 493-503). The recent studies reported that EPCs cultured from type 1 diabetic patients were reduced both in number and function. As a consequence, EPCs dysfunction could cause the reduction of vascular regenerative potential in diabetic patients, thereby, contribute to the pathogenesis of vascular complications, in particular diabetic ulcers (Loomans et al., 2004: 195-199). Numerous researches indicated that EPCs can increase neovascularization and improve wound healing in diabetic and non-diabetic wound (Sivan-Loukianova et al., 2003: 368-377; Suh et al., 2005: 1571-1578).

Mesenchymal stem cells (MSCs) are non-haematopoietic stromal cells that are able to isolate from the bone marrow. They are multipotent cells that differentiate into a variety of cells i.e. adipocytes, osteoblasts, and chondrocytes (Pountos et al., 2007: S23-S33). Several recent studies suggested that MSCs can promote wound healing, angiogenesis and release of pro-angiogenic factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and stromal-derived factor-1 (SDF-1) (Debin et al., 2008: 106-115; Iwase et al., 2005: 543-551; Wu et al., 2007: 2648-2659).

Recently, some studies have shown that combined MSCs and EPCs can increase neovascularization, release of pro-angiogenic factors, producing high density and stable vascular networks (Melero-Martin et al., 2008: 194-202). However, there is no evidence in diabetic wound model. Therefore, our objective is to study the effects of combined EPCs and MSCs on angiogenesis and wound healing in diabetic mice model.

Research questions

Whether the combination of EPCs and MSCs could increase angiogenesis and wound healing in diabetic mice?

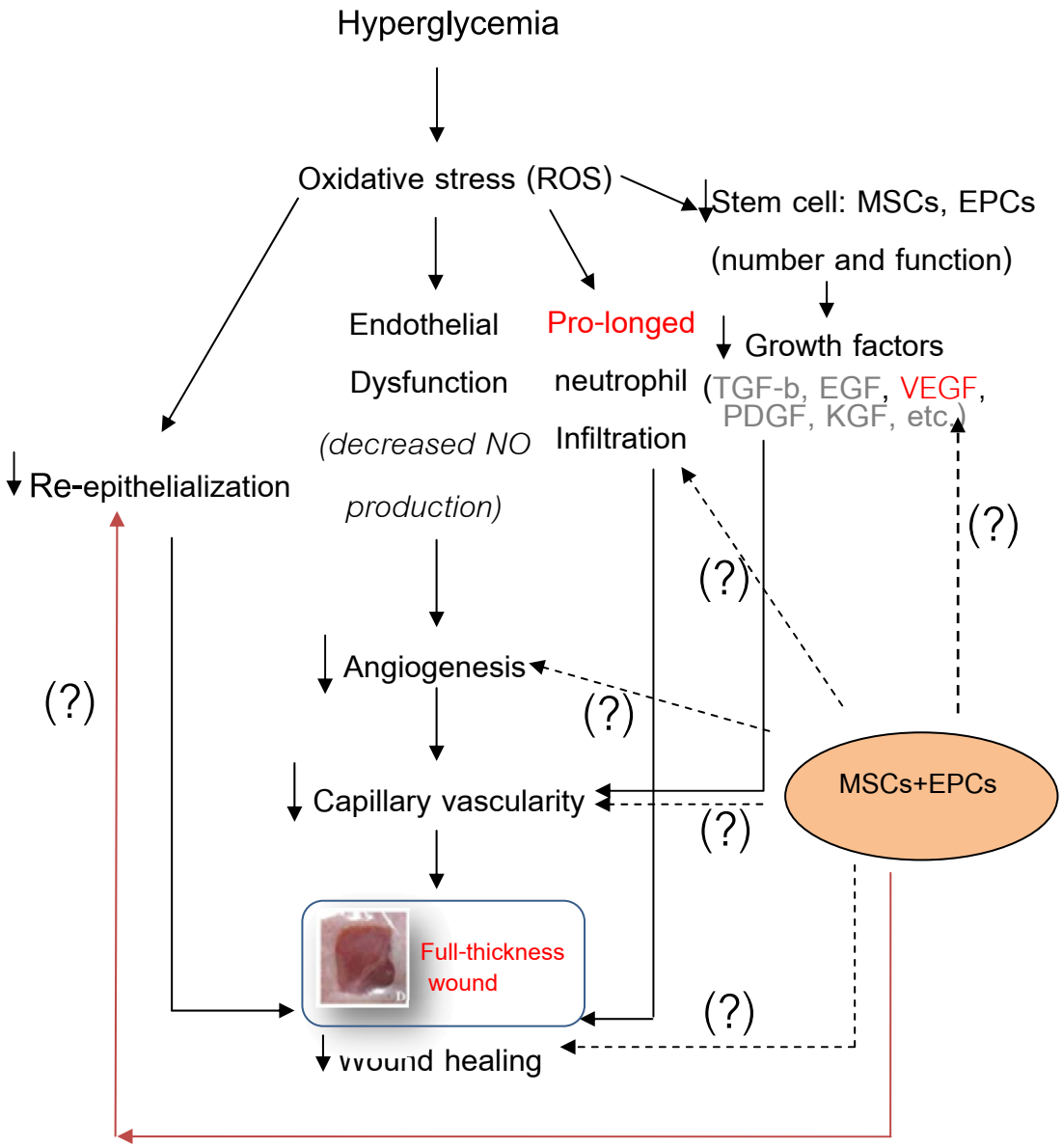
Hypothesis

The combination of EPCs and MSCs can increase angiogenesis and wound healing in diabetic mice.

Research Objective

To study the effects of combined EPCs and MSCs on angiogenesis and wound healing in diabetic mice.

Conceptual framework



CHAPTER II

LITERATURE REVIEWS

The skin

The skin is the largest organ of body. The average adult skin accounts for about 15% of the total body weight and has a surface area of about 1.7-2 m^2(Celleno and Tamburi, 2009: 3-45; Lai-Cheong and McGrath, 2009: 223-226).

The structure of the skin

Skin is composed three layers. The most external layer is epidermis, and the below the epidermis it is dermis, hypodermis or subcutis is inner layer. (Figure 2.1)

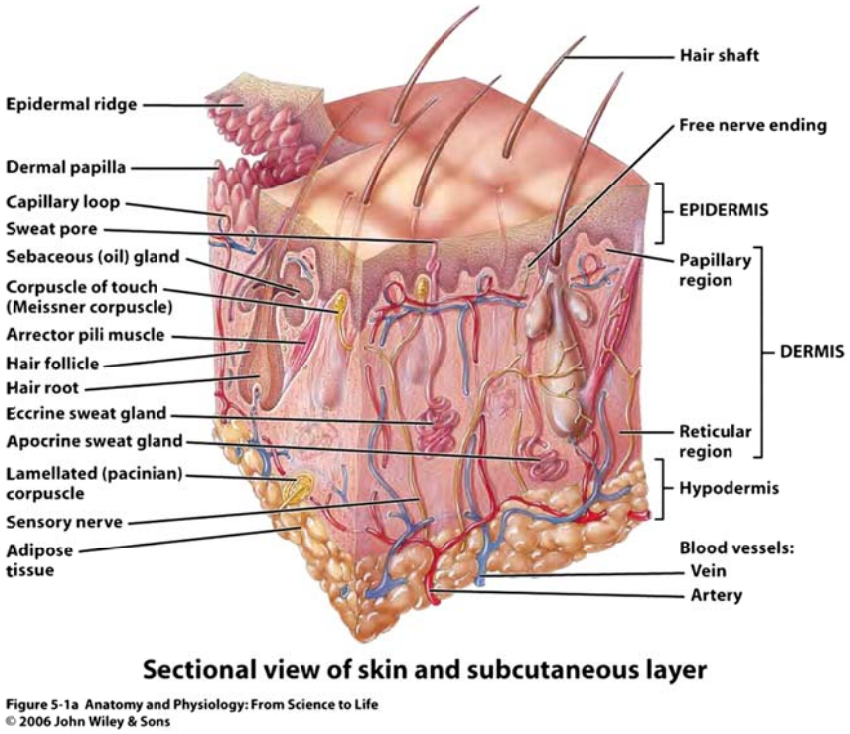


Figure 2.1 Structure of the skin. (Jenkins, Tortora, and Kemniitz, 2006; 148–171)

1. Epidermis

Keratinocytes make up 95% of the epidermis. The remaining 5% are Melanocytes, Langerhans cells and Merkel cells. The epidermis is divided into five main layers depending on the state of keratinocyte differentiation. The first layer is stratum basale which is consists of a single layer of keratinocytes. These cells can proliferate and differentiate into stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum are show in Figure 2.2. (Celleno and Tamburi, 2009: 3-45)

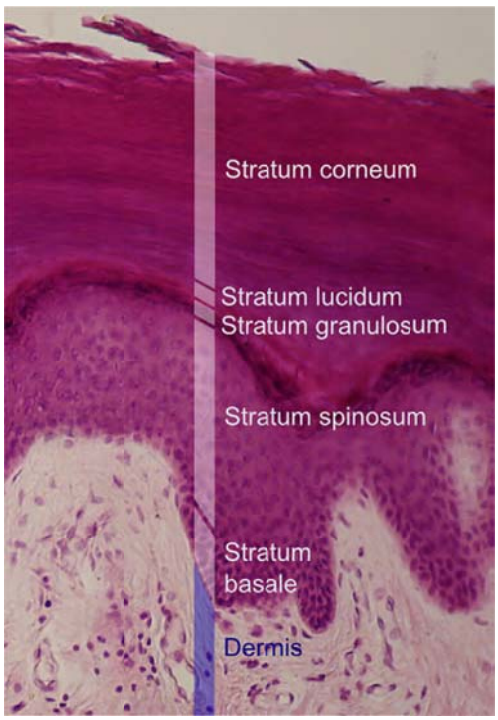


Figure 2.2 Structure of the epidermis (Gantwerker and Hom, 2012: 85-97).

2. Dermis

The dermis is 0.55 mm thick, which is below the epidermis and is composed of collagen fibres, elastic tissue, ground substance and cellular (fibroblast, mast cell, plasma cell, dermal dendritic cell and histiocyte)

components, blood vessels (superficial and deep plexi), lymphatic vessels and sensory nerves. The dermis is subdivided into two main layers:

• papillary dermis, which is in contact with the epidermis and is richly supplied by blood vessels and sensory nerve endings

• reticular dermis, which is the principal part of the dermis and is contacted with the subcutis. It is composed collagen fibers, cells, ground substance, vessels, especially arterioles and venules (Celleno and Tamburi, 2009: 3-45; Lai-Cheong and McGrath, 2009: 223-226).

3. Hypodermis or subcutis

The hypodermis or subcutis is the innermost layer of the skin and is composed of lipocytes (Lai-Cheong and McGrath, 2009: 223-226).

Wound

Types of deep Wounds

Wound is classicized in three types: Superficial wounds, deep dermal wounds, full thickness wounds

1. Superficial wounds

Superficial wounds are defined as those involving only the epidermis and the papillary dermis. wound healing by epithelialization, that is no significant scar formation and no wound contraction.

2. Deep dermal wounds

Deep dermal wounds or Partial thickness is wound which loss of epidermis and dermis. Wound presenting as a shallow open ulcer with a red

pink wound bed, without slough. These wounds normally heal within 10–21 days.

3. Full thickness wounds

These wounds are completed removal of the epidermis and dermis. Subcutaneous fat, bone, tendon or muscle may be damage. (Percival, 2002: 114-117).

Wound healing process

The wound healing process is a complex process that can be divided into 4 overlapping phases of hemostasis, inflammation, proliferation and maturation/remodeling show in Figure 2.3 (Gantwerker and Hom, 2012: 85-97).

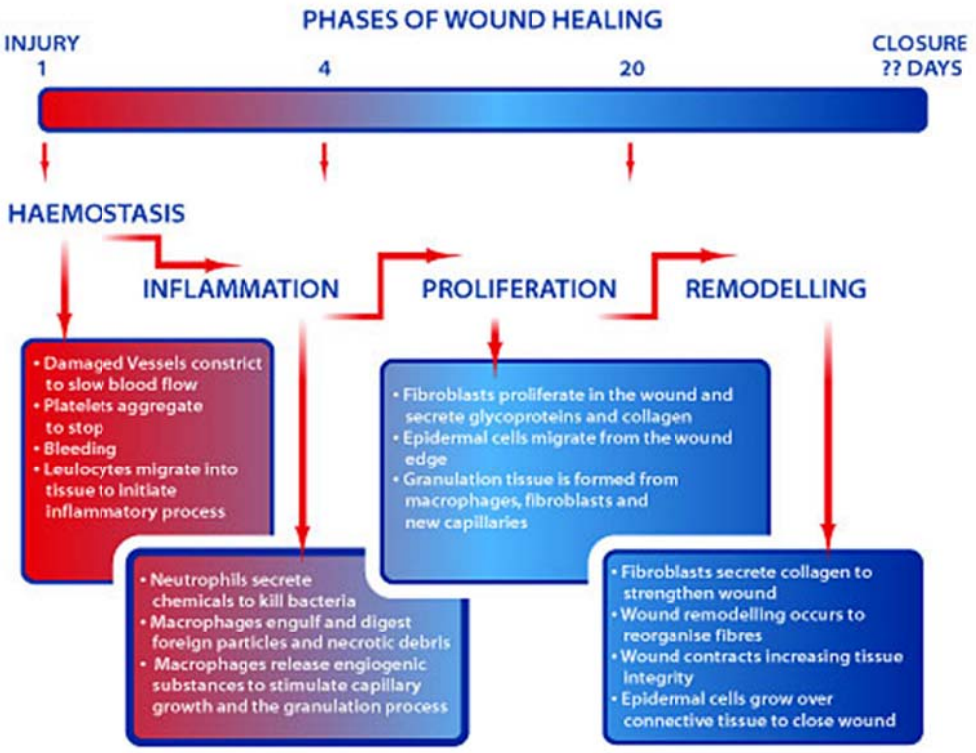


Figure 2.3 The 4 phases of wound healing and the many processes that occur at each phase of healing [Copied from the website: (http://www.advancedproteinsystems.co.uk/active2.html)].

1. Hemostasis

Initial phase occurs when the body injury in seconds to minutes that is hemostasis. Platelets serve to provide initial hemostasis and release multiple cytokines, hormones, and chemokine including transforming growth factor (TGF)–α, TGF-β and platelet-derived growth factor (PDGF) to set off the remaining phases of healing. The action of vasoactive substances such as catecholamine and serotonin act via specialized receptors on the endothelium to cause vasoconstriction of the surrounding blood vessels. Smaller vessels are vasodilation to cause influx of leukocytes, red blood cells and plasma proteins to tissue injury (Gantwerker and Hom, 2012: 85-97; Li, Chen, and Kirsner, 2007: 9-18).

2. Inflammation

The inflammatory phase is the influx of neutrophils, macrophages, and lymphocytes to the area of injury. In the early inflammatory state, neutrophils are the first leukocytes on site within the first 24 hours by chemotactic factors released during hemostasis and by mast cells such as kallikrein, fibrinopeptides, tumor necrosis factor, histamine, proteases, leukotrienes (LTs), and cytokines (interleukins) (Noli and Miolo, 2001: 303-313). Neutrophils are killed and phagocyte bacteria and damaged matrix proteins within the wound. Neutrophil infiltration normally declines for only a few days. However, in infection wound is prolonged the presence of neutrophils within the wound and may delay healing (Simpson and Ross, 1972: 2009-2023).

And then, macrophages and other lymphocytes appear to begin to clear debris and bacteria in the wound. Macrophages infiltration begin at approximately 48 hours post injury and stay until the finish of the inflammatory

phase. Macrophages release chemotactic factors (e.g., fibronectin) which attract fibroblasts to the wound site. In addition, macrophages can be produce PDGF, fibroblast growth factor, VEGF, TGF-β, and TGF-α. They are important cytokines in inducing cell migration, proliferation, and matrix production are showed in Figure 2.4 (Gantwerker and Hom, 2012: 85-97; Li, Chen, and Kirsner, 2007: 9-18).

3. Proliferation

The proliferative phase has the major events during this phase which are the creation of a permeability barrier (ie, re-epithelialization), the establishment of proper blood supply (ie, angiogenesis), and reinforcement of the injured dermal tissue.

The epithelialization is marked by the proliferation and influx of keratinocytes of the wound. In an early event in wound re-epithelialization keratinocyte initially migrate from the edges of the wound within 24 hours. The beginning of re-epithelialization is epidermal stem cells from the hair follicle and apocrine glands begin to differentiate into keratinocytes and lie in the stratum basale. The mesenchyme of the extracellular matrix (ECM) which attach near the inner wound edge and lay down a new basement membrane. Another row of keratinocytes migrates over the newly laid epithelial cells to fill in the defect. These cells stop migration, signaled by contact inhibition from neighboring keratinocytes (Gantwerker and Hom, 2012: 85-97; Li, Chen, and Kirsner, 2007: 9-18; Pilcher et al., 1999: 12-24).

Angiogenesis is the formation of new blood vessels that necessary to wound process. The induction of angiogenesis is initially attributed by many other angiogenic activity molecules such as fibroblast growth factor, VEGF,

TGF-β, angiogenin, angiotropin, angiopoietin 1 and thrombospondin. On the first three days after injury, bFGF may set the stage for angiogenesis of wound healing (Singer and Clark, 1999: 738-746). Nevertheless, VEGF is critical for angiogenesis during the formation of granulation tissue within 4 to 7 days (Nissen et al., 1998: 1445-1452).

Granulation tissue consists of fibroblasts, new budding vessels, and immature collagen (collagen type III). Some fibroblasts will begin to differentiate in this phase into myofibroblasts which have contractile function of edges wound (Figure 2.4) (Gantwerker and Hom, 2012: 85-97; Li, Chen, and Kirsner, 2007: 9-18; Robson, 2003: 557-569).

4. Maturation/remodeling

This phase is phase begins as the ECM and type III collagen is replaced with type I collagen (Welch, Odland, and Clark, 1990: 133-145). The remaining cell types of the previous phases are apoptosis. The tensile strength of the wound will increase due to the laying down of type I collagen of wound. Granulation tissue begins to involute and excess blood vessels retract. This phase lasts the longest and results in the final appearance of the wound following healing (Figure 2.4) (Gantwerker and Hom, 2012: 85-97; Li, Chen, and Kirsner, 2007: 9-18).

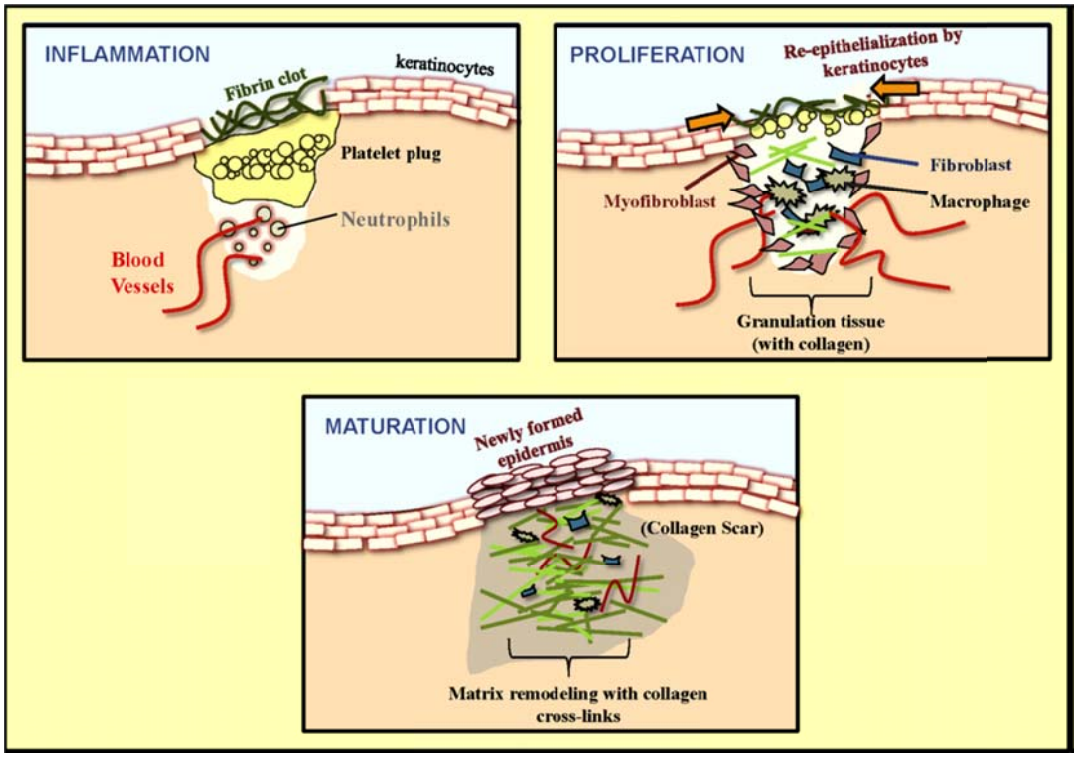


Figure 2.4 The event of wound healing in process of the inflammation, proliferation and maturation (Nauta et al., 2011: 103-127).

Angiogenesis

Angiogenesis is a process of new blood vessel formation from pre-existing ones (Carmeliet, 2000: 389-395). In response of tissue injury, an angiogenic process is initiated by activation of endothelial cells and local degradation of their basement membrane. Capillary buds and sprouts is occurred into process of wounded clot (Li, Chen, and Kirsner, 2007: 9-18). In wound repair, the soluble factors can stimulate angiogenesis such as bFGF, VEGF, TGF-β, TGF-α, tumor necrosis factor α (TNF-α), platelet factor 4 (PF4), angiogenin, angiotropin, angiopoietin, IL-8, PDGF, thrombospondin, and nitric oxide (NO) (Montrucchio et al., 1997: 557-563; Risau, 1997: 671-674; Singer and Clark, 1999: 738-746). This cytokines stimulate EPCs migration, proliferation and maturation into endothelial cells, resulting in

capillary structure formation and extension. Capillary sprouts latterly branch and join to form capillary arcades. New sprouts then extend from these loops to form capillary networks, apparently under the influence of VEGF, FGF and degrade new capillary in remodeling phase (Hristov and Weber, 2004: 498-508; Tonnesen, Feng, and Clark, 2000: 40-46).

Diabetes mellitus (DM)

Diabetes mellitus is one of the most rapidly growing diseases worldwide. The International Diabetes Federation (IDF) reported that the prevalence of diabetes mellitus in 2010 indicate that 285 million adults have diabetes. Which increased of 39 million from 2007 and an expected continued increase to 439 million in 2030 (Egede and Ellis, 2010: 302-312). DM can be classified in 2 types.

Type 1 diabetes mellitus (T1D) has currently reported incidents for 10% of all diabetes cases. T1D is due to the autoimmune destruction of insulin-producing pancreatic beta-cells via auto-aggressive T-cells and pancreatic macrophage infiltration.

Type 2 diabetes mellitus (T2D) is the most common endocrine disorder worldwide, covering 90–95% of all diabetes cases. The T2D has abnormalities in glucose and lipid metabolism, including insufficient insulin secretion from pancreatic beta-cells and resistance of insulin activity.

In T1D and T2D, beta-cell mass loss more than 50% which is lead to hyperglycemia. Insulin resistance and impaired glucose tolerance (IGT) the

both conditions preceding the development of T2D are closely related to obesity (Reimann et al., 2009: 317-331).

Diabetic and wound

Hyperglycemia is caused of formation of advanced glycation end products (AGE's) which bind cell membrane and extracellular matrix proteins and impedes their function (Ahmed, 2005: 3-21). In recent study of the diabetic wound microenvironment abnormalities a number of local cytokine and cellular in wound and the growth factors such as PDGF, TGF-β, and VEGF have all been found to be deficient in the diabetic wound whereas levels of matrix metalloproteinases and superoxide are elevated in diabetic wound fluid to contribute to the delay in wound healing (Blakytny and Jude, 2006: 594-608; Lobmann et al., 2002: 1011-1016).

In addition, the reports indicated that in animal models of diabetes and patients with type 1 and type 2 diabetes have endothelial dysfunction, reduction in wound NO expression, reduced collagen accumulation and wound breaking strengths which correlate to impaired healing (Schaffer et al., 1997: 513-519). Furthermore, Macrophage in diabetic wounds had also demonstrably abnormal function and fail to progress in a timely fashion through the inflammatory phase of healing (Wetzler et al., 2000: 245-253). The reduction in cutaneous blood flow, reduced skin oxygen tension and abnormal angiogenesis support the theory of impaired endothelial function impeding wound repair in diabetes (Laing et al., 2007: 1029-1031; Martin, Komada, and Sane, 2003: 117-145)

Endothelial Progenitor Cells (EPCs)

EPCs are a small subpopulation (<0.05%) of the leukocyte fraction of bone marrow-derived mononuclear hematopoietic cells in the adult. Typically, mononuclear cells are isolated from bone marrow, peripheral blood, or cord blood by using density gradient centrifugation and/or separation by magnetic beads or flow cytometry with antibodies against surface antigens such as cluster of differentiation (CD) 34 (Zammaretti and Zisch, 2005: 493-503).

EPCs phenotype

EPCs are identified cell surface marker expressing CD34, CD133, and the VEGFR-2 (KDR) in early EPCs and in late EPCs lost CD133 and express endothelial lineage cell markers, including von Willebrand factor (vWF), endothelial nitric oxide synthase (eNOS) and Ve-cadherin(Hristov, Erl, and Weber, 2003: 1185-1189; Urbich and Dimmeler, 2004: 343-353; Zammaretti and Zisch, 2005: 493-503).

EPCs mobilization

EPCs mobilization is regulated by hypoxia, limb ischemia, vessel wall damage, atherogenic plaque, coronary thrombosis or bypass surgery, which can recruit EPCs and rapidly increase their number.

The injury site releases VEGF and other cytokines such as granulocytes colony-stimulating factor (G-CSF), hypoxia-inducible transcription factor (HIF-1) promoting the recruitment of EPCs (Figure 2.5) which is activation of matrix metalloproteinase-9 (MMP-9) transforming the membrane-bound Kit ligand to a soluble Kit and could move cKit-positive stem and EPCs to the vascular zone of BM. The study found that the recruitment of stem cells and

EPCs is also influenced by eNOS through the increase of pro-MMP-9 expression. VEGF induces EPCs to migrate by binding vascular endothelial growth factor receptor-1 (VEGFR-1, flt-1) and vascular endothelial growth factor receptor-2 (VEGFR-2, flk-1). SDF-1 binds to C-X-C chemokine receptor type 4 (CXCR-4) on the surface of EPCs enhancing their mobilization and homing to the site of injury. EPCs are also involved into angiogenesis, the developmental process involving the growth of new blood vessels from pre-existing ones. They take part into formation of vessel walls by using adhesion molecules (i.e. vascular endothelial (VE)-cadherin, which are actively present at the site of angiogenesis) (Napoli et al., 2011: 9-22).

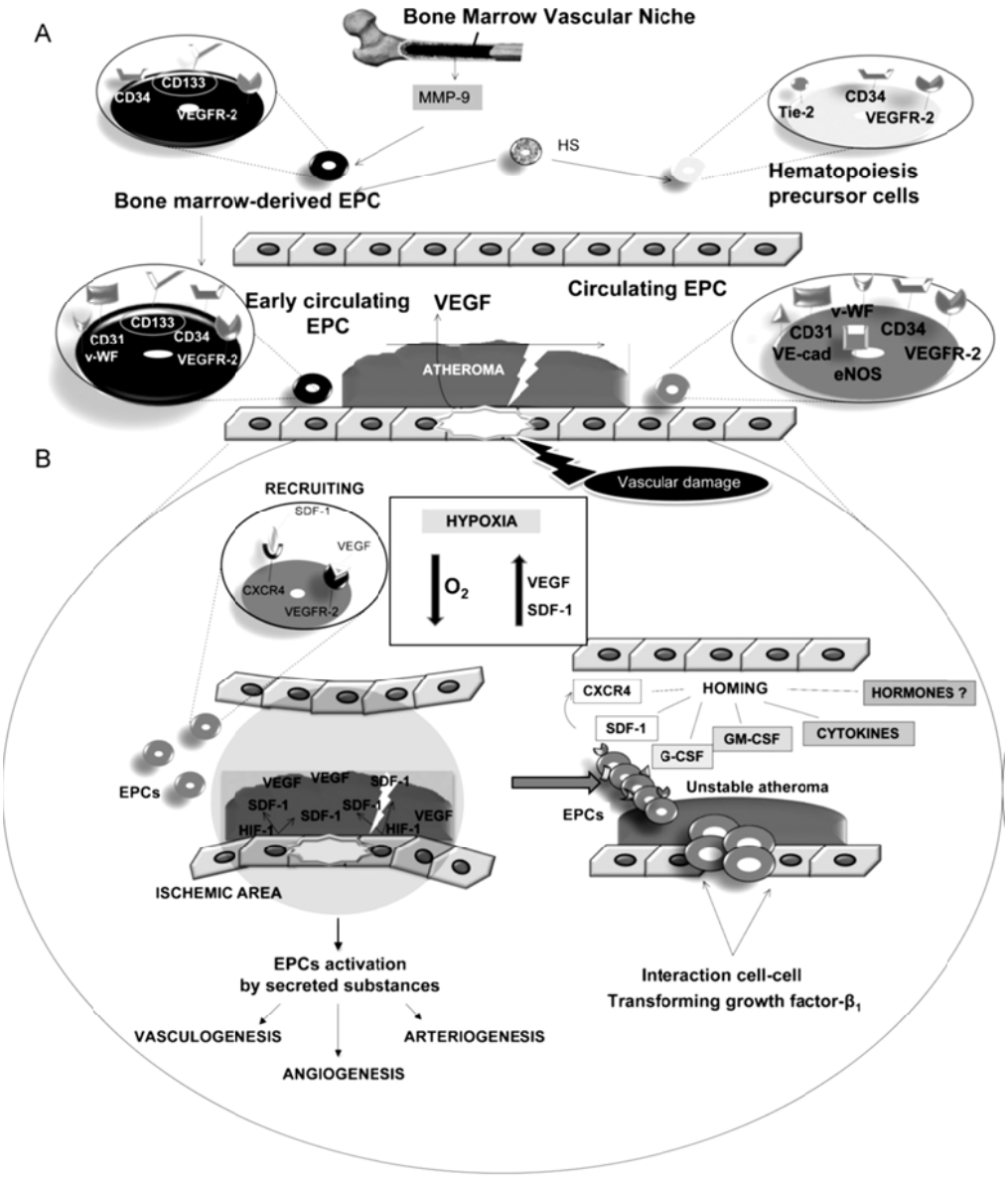


Figure 2.5 From bone marrow to blood circulation: differentiation and key role of endothelial progenitor cells (Napoli et al., 2011: 9-22).

Mesenchymal stem cells (MSCs)

1. Phenotype of MSCs

The recent study, MSCs is not phenotypic criteria ensure of MSCs. Therefore, many reports used the phenotypic markers are identified to be expressed on MSCs, there are still no unique specific markers that could be

used to ensure homogeneity of MSCs which expression of CD73, CD90, and CD105 and lack the expression of CD14, CD19, CD31, CD34, CD45, and HLA-DR surface molecules. The MSCs can differentiate into osteoblasts, chondrocytes, adipocytes and connective stromal cell. Moreover, it also has been suggested that MSCs might also differentiate into not only ectodermal lineage as neuron, epithelial cells, but also endodermal lineage as muscle and hepatocyte (Si et al., 2011: 93-103).

2. Migration and homing potential of MSCs

MSCs could migrate to injured, inflamed tissues. The detailed mechanism and efficiency of MSCs migration might involve: Specific receptors or ligands upregulated by injury tissues not only facilitate trafficking, adhesion, and infiltration of MSCs, but also provide MSCs with a specialized microenvironment or niche to support their self-renewal and maintain their multi-potentiality. Integrins, selectins, and chemokine receptors expressed on MSCs are involved in migration of MSCs across the endothelium. MSCs are passively arrested in capillaries or microvessels including arterioles and post-capillary venules, and then directly interact with accessory cells and the release a wide array of soluble growth factors and trophic cytokines (Si et al., 2011: 93-103).

EPCs and MSCs treatment of diabetic wounds

Prolonged and incomplete wound healing, caused by reduced production of growth factors, impaired angiogenesis, and compromised formation of collagen matrixes, is present as a complication of DM. The

characteristics of diabetic wounds are poor neovascularization, presence of abundant inflammatory infiltrates contain polymorphonuclear cells and neutrophils of necrotic tissue. In addition, disturbances in collagen metabolism and compromised production and decrease of growth factors such as TGF-β, epidermal growth factor (EGF), VEGF, PDGF, and keratinocyte growth factor (KGF). There are main factors responsible for the pathogenesis of poor wound healing (Volarevic et al., 2011: 5-10).

Systemic and local administration of bone marrow-derived MSCs improves healing of diabetic wounds in rats and mice. After administration of MSCs, diabetic wounds showed increased collagen levels, increase moderate (TGF-β, KGF) or significant (EGF, PDGF, and VEGF) in the production of growth factors, These factors stimulated cell adhesion at the site of injury and induced cells to secrete more chemokines resulting in neovascularization and formation of inflammation infiltrate, containing predominantly mononuclear cells, without tissue necrosis. MSCs can help to improve diabetic wounds (Volarevic et al., 2011: 5-10).

Although MSCs were not found in the vascular structures of diabetic wounds, it was documented that, after MSCs treatment, there was enhanced capillary density in those, suggesting that MSCs promoted angiogenesis that was very important for successful healing. In diabetic wounds, MSCs settled predominantly in the newly formed dermis, and to a lesser extent in the epidermis, however, none were detected in the undamaged skin. MSCs have already shown efficacy in the treatment of foot ulcerations in diabetic patients (Volarevic et al., 2011: 5-10).

The contribution of BM EPCs to neovascularization in wounds results from a multistep process. Circulating EPCs of diabetes into wounds may be decreased number and function. Therefore, a full understanding of impaired homing mechanisms in diabetic wounds is crucial for enhancing EPCs recruitment and engraftment. The recently study showed that the local concentration of SDF-1α is significantly decreased in a diabetic wound. epithelial cells and myofibroblasts appeared to be responsible for the down regulation of SDF-1α in diabetic wounds (Liu and Velazquez, 2008: 1869-1882).

CHAPTER III

MATERIALS AND METHODS

Chemical substances

chemical substances were used in following lists:

- Streptozotocin (Sigma Chemical Co, USA.)
- Citrate buffer pH 4.5 (Sigma Chemical Co, USA.)
- Fibrin gel (Shanghai RAAS blood products co. ltd, China)
- Fluorescein isothiocyanate (FITC)-labeled dextran, MW.250,000 (Sigma Chemical Co, USA)
- Thiopental (pentobarbital) (Sigma Chemical Co, USA)
- RIPA lysis buffer (Cell Signaling Technology, Inc., USA)
- Phosphatase inhibitor cocktails (Sigma Chemical Co, USA)
- Protease inhibitor cocktails (Sigma Chemical Co, USA)

Animal preparation

Male Balb/c nude mice (7-8 week-old, weight 20–25 g.) were purchased from the National Laboratory Animal Center, Salaya Campus, Bangkok. The experiment procedures were conducted according to the guideline of experimental animals by The National Research Council of Thailand and approved by the ethics committee, Faculty of Medicine, Chulalongkorn University. Mouse were kept in temperature room was maintained at 25±3°C. The one animal was housed per cage and fed standard chow and sterilized water.

All mice are divided into five groups.

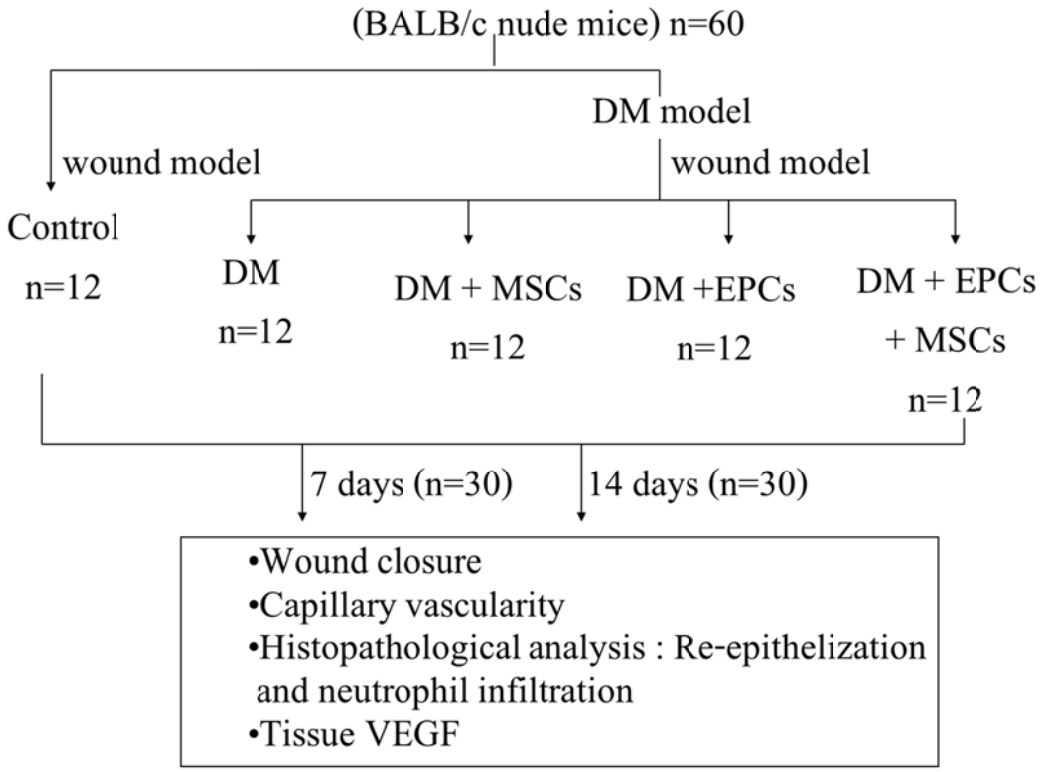
Group 1 is wounded control group with implanted fibrin gel (Control).

Group 2 is diabetic wounded group with implanted fibrin gel (DM)

Group 3 is diabetic wounded group with implanted fibrin gel and MSCs (DM+MSCs).

Group 4 is diabetic wounded group with implanted fibrin gel and EPCs (DM+EPCs).

Group 5 is diabetic wounded group with implanted fibrin gel and combined EPCs and MSCs (DM+EPCs+MSCs).



Diabetic model

The animals in diabetic groups were induced by injection of streptozotocin (Sigma Chemical Co, USA.) in Citrate buffer pH 4.5 (Sigma Chemical Co, USA.) dose 45 mg/kg intraperitoneal injection (i.p.) daily for 5 days. Two weeks later, glucose level was measured from tail-vein blood by used glucometer (Advance Glucometer, Bochringer Mannheim, Germany), and mice with glycemia over 200 mg/dl were selected for the study.

Preparation of mouseMSCs

mouseMSCs cultured in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C 5%CO_2 (supported by Stem cell and Cell Therapy Reach Unit). Each day from day 3 to day 7 of culture, medium is refreshed by removing and adding 4 ml DMEM to the plate. (Figure 3.1A). MSCs were confirmed by using flow cytometry to detect CD 105.

Preparation of humanEPCs

humanEPCs (Lonza Group Ltd.) cultured in endothelial cell basal medium 2 (EBM-2) at 37°C 5%CO_2 (supported by Stem cell and Cell Therapy Reach Unit). Each day from day 3 to day 7 of culture, medium is refreshed by removing the medium from the flask and adding 4 ml EGM-2 to the plate which coated with 10 µl fibronectin and 1 ml phosphate-buffered saline (PBS). (Figure 3.1B)

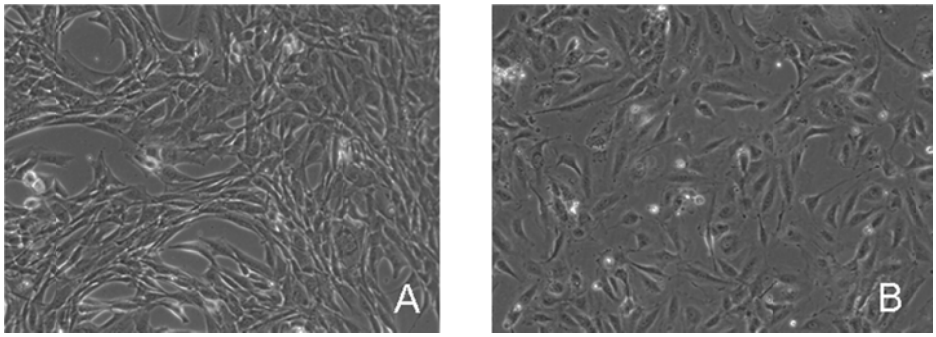


Figure 3.1 Morphology of mouseMSCs (A) and humanEPCs (B). Magnified at 100 power

Preparation of stem cells and fibrin gel

Washing plate by sterile PBS and cells were incubated with 2 ml 0.25% trypsin for 1 minute at 37°C. After, 10 ml culture medium were added to plate for stop trypsin activity. Cell suspension was transferred in tube and counted by microscope with 100X magnification. And then, Centrifuge the required number of cell suspension for 5 min at 1000 rpm in room temperature. The supernatant was removed and cell pellet was added 30 µl fibrinogen per wound (fibrin gel is contain with fibrinogen and thrombin, Shanghai RAAS blood products co. ltd, China). Mix by pipet and keep cells in 4°C before wounding.

Wound healing model

6 - 7 weeks after induced streptozotocin. Anesthetized mice with 55 mg/kg sodium pentobarbital i.p. and swab with alcohol on dorsal-rostral back. Bilateral full-thickness excisional skin wounds (0.6x0.6 cm^2) were created on each side of the midline. Each mouse received 30 µl fibrinogen and 30 µl thrombin (Control and DM), $1x10^6$ cells MSCs in 30 µl fibrinogen and add 30 µl thrombin (DM+MSCs), $1x10^6$ cells EPCs in 30 µl fibrinogen and add

30 µl thrombin (DM+EPCs) and the combined $0.5x10^6$ cells MSCs and $0.5x10^6$ cells EPCs in 30 µl fibrinogen and add 30 µl thrombin (DM+EPCs+MSCs). Then the wounds covered by Tegaderm (3M, USA.) as shown in Figure 3.2. EPCs and MSCs were supported by the Stem cell and Cell Therapy Research Unit, Chulalongkorn University. After 7 and 14 day wounded, mouse was anesthetized with 50 mg/kg sodium pentobarbital i.p. and measured body weight. And the end experiment, blood samples were collected by cardiac puncture for determined using enzymatic reference method (BRiA LAB CO., Thailand).

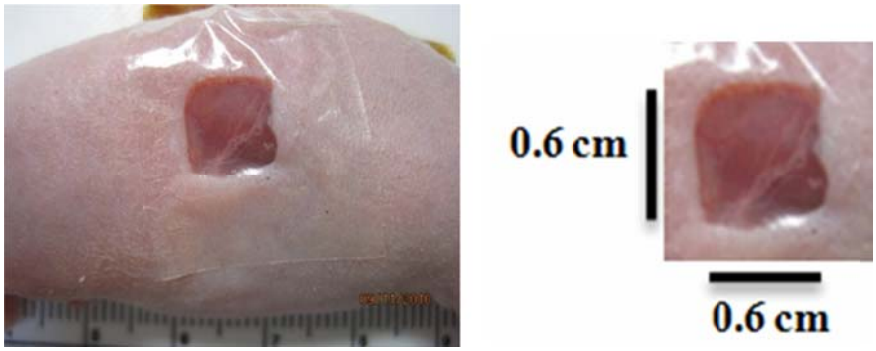


Figure 3.2 Bilateral full-thickness excisional skin wounds (0.6x0.6 cm^2) were created on each side of the midline.

Wound analysis

Digital photographs of wounds were taken at days 0, 7 and 14 by canon digital camera IXUS 9515 (10.0 MEGA PIXES). Wound area was analyzed by digital image software analysis (Image Pro Plus 6.1) as shown in Figure 3.3. The percentage of wound closure (%WC) was calculated by formula below:

$$\%\ WC = \frac{(\text{Area of original wound} - \text{Area of actual wound}) \times 100}{\text{Area of original Wound}}$$

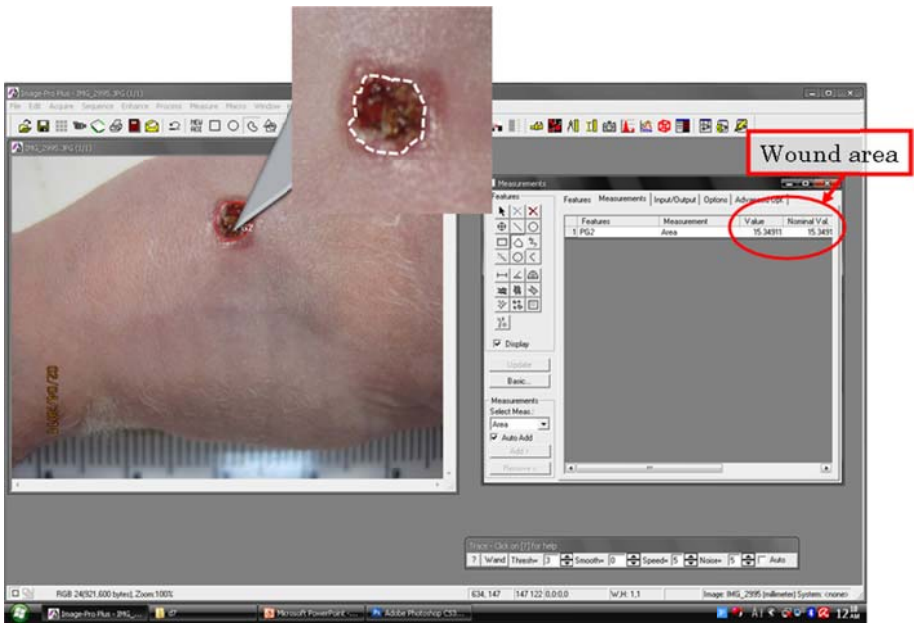


Figure 3.3 Method of the wound area (mm^2) measured by using Image-Pro Plus 6.1 software.

Measurement of capillary vascularity

At day 7 and 14 post wounding. Anesthetized mice with 50 mg/kg sodium pentobarbital i.p. The right jugular vein was canulated by inserting a polyethylene catheter (PE 10, inner diameter of 0.28 mm.) for injection of 0.2 ml of 5% fluorescein isothiocyanate (FITC)-labeled dextran (MW.250,000, Sigma Chemical Co, USA). The dorsal skin around the wound was cut open using scissors. (Figure 3.4)

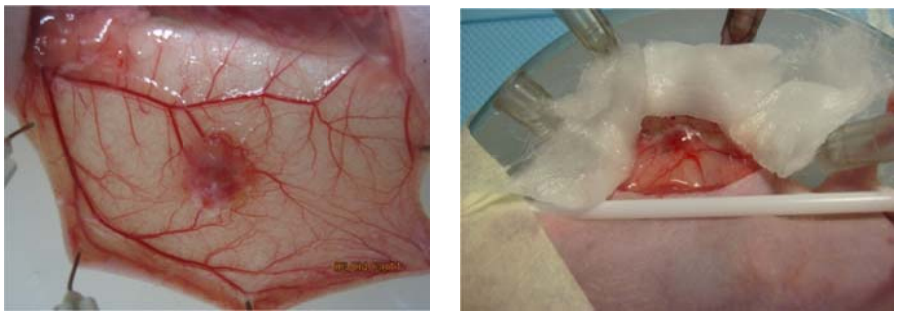


Figure 3.4 The microcirculation in wound skin when dorsal skin opened.

The mouse was plated to stage under intravital fluorescence microscope and was injected into right jugular vein. The number of capillary vascularity (CV) were examined with intravital fluorescence video microscopy technique at 10x objective lens. the fluorescence video microscopic system was consisted of a video microscope (Nikon E50i, Japan) with a 10x objective and eyepieces lens, camera controller (Hamamatsu C2400, Japan), video timer (For. A company VTG-33, Japan) and video recorder (Panasonic NV-HD620, Japan). The real time video image of microcirculation in wound area was observed on monitor. (Sony PVM-145E, Korea) (Figure 3.5.)

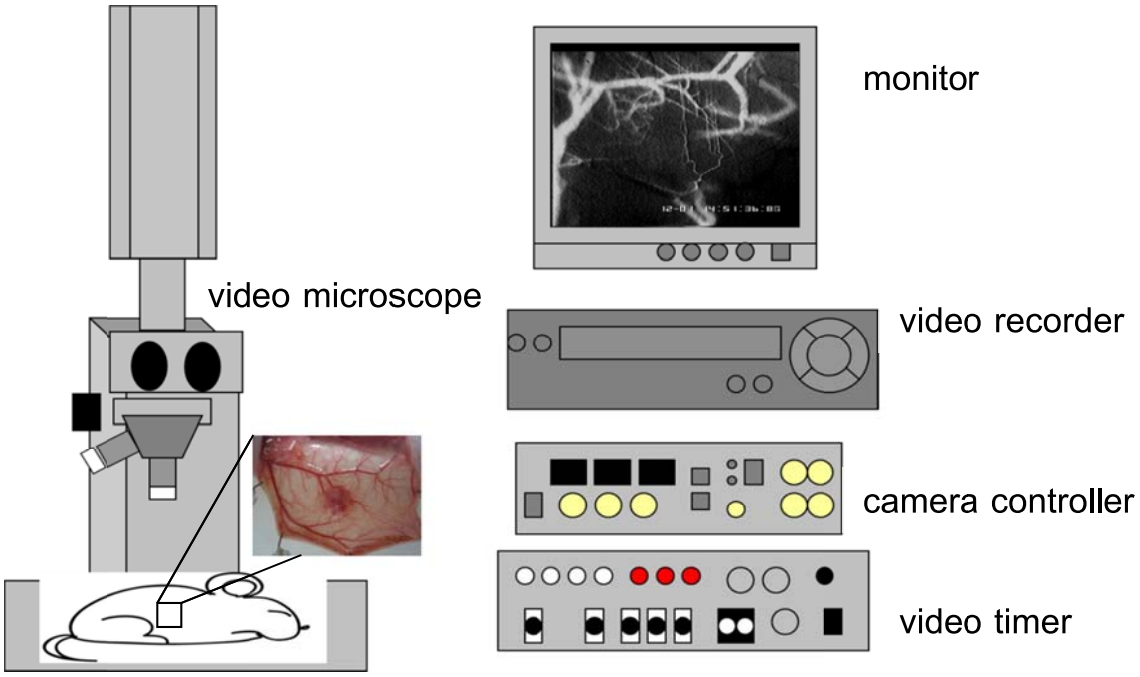


Figure 3.5 The fluorescence video microscopic system.

The video image of each animal was used to analyze changes in capillary vascularity on day 7 and 14 using the software Image Pro-plus (Figure 3.6.) and calculated %CV by formula below. (capillaries whose diameter was less than 15 μm.)

$$\% \text{ CV} = \frac{\text{(Number of pixels within the capillaries) x 100}}{\text{(Total numbers of pixels within the 100X100 window frame area)}}$$

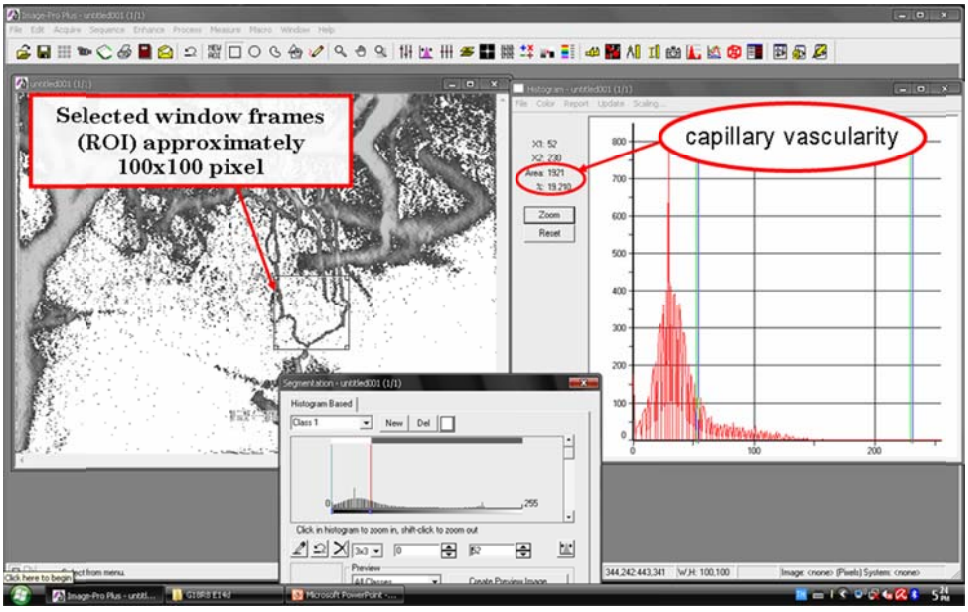


Figure 3.6 Method for measurement the capillary vascularity by using Image-Pro Plus 6.1 software.

Measurement of re-epithelialization

Mice were sacrificed at 7 and 14 days, wound samples were harvested in size 0.6x0.6 cm^2. Tissue specimens were fixed in 10% formaldehyde for 24 hours. The center of wound samples were cut and embedded in paraffin. And then 4 µm thick sections were stained with hematoxylin-eosin (H&E). The re-epithelialization was measured by Stero type microscopy (Nikon SM2800, Japan) under 20 magnification. Wound image was show in digital sight for microscope (Nicon DS-L2, Japan). The re-epithelialization was analysis by image Pro Plus 6.1 software (Figure 3.7) and was calculated by formula below (Mori et al., 2004):

$$\% \text{ Re-epithelialization} = \frac{\text{distance covered by epithelium (r) x 100}}{\text{distance of the wound edges (R)}}$$

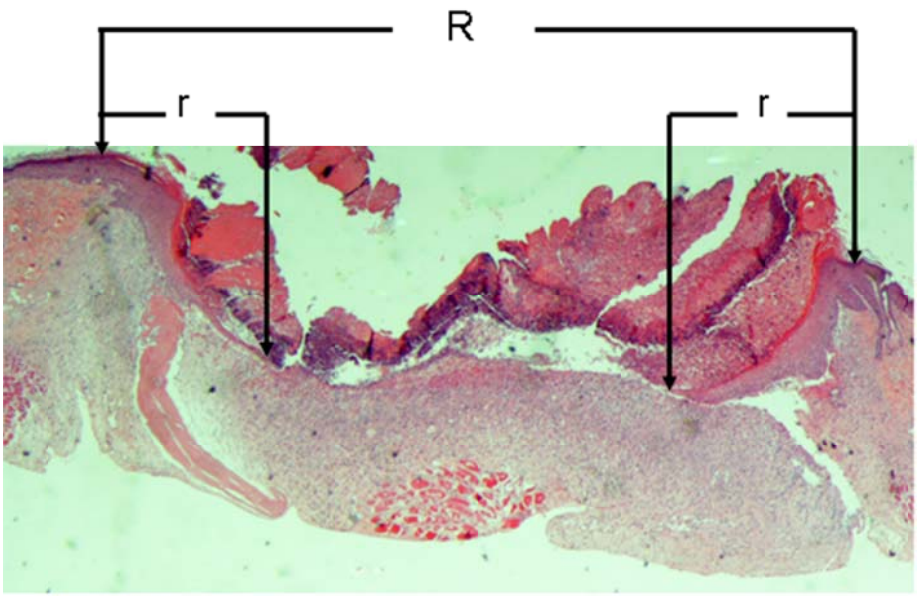


Figure 3.7 Histological section showing the re-epithelialization of skin wound. H&E-stained section was photographed at 20 power. R is the distance of the wound edges and r is the distance covered by epithelium.

Measurement of neutrophil infiltration

The neutrophil infiltration was measured by using microscope (Nikon E50i, Japan) at 400x (Figure 3.8). The specimens were evaluated for inflammatory wound area and analysis by image Pro Plus 6.1 software show in Figure 3.9.

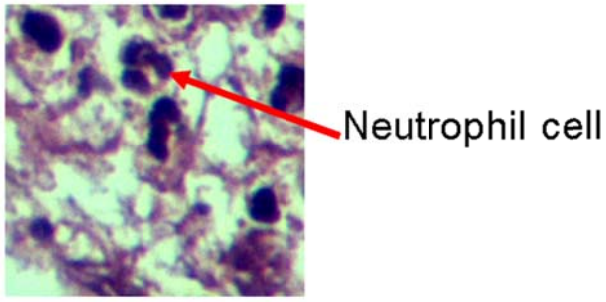


Figure 3.8 Morphology of neutrophil cell into wound area. H&E-stained section was photographed at 400 power.

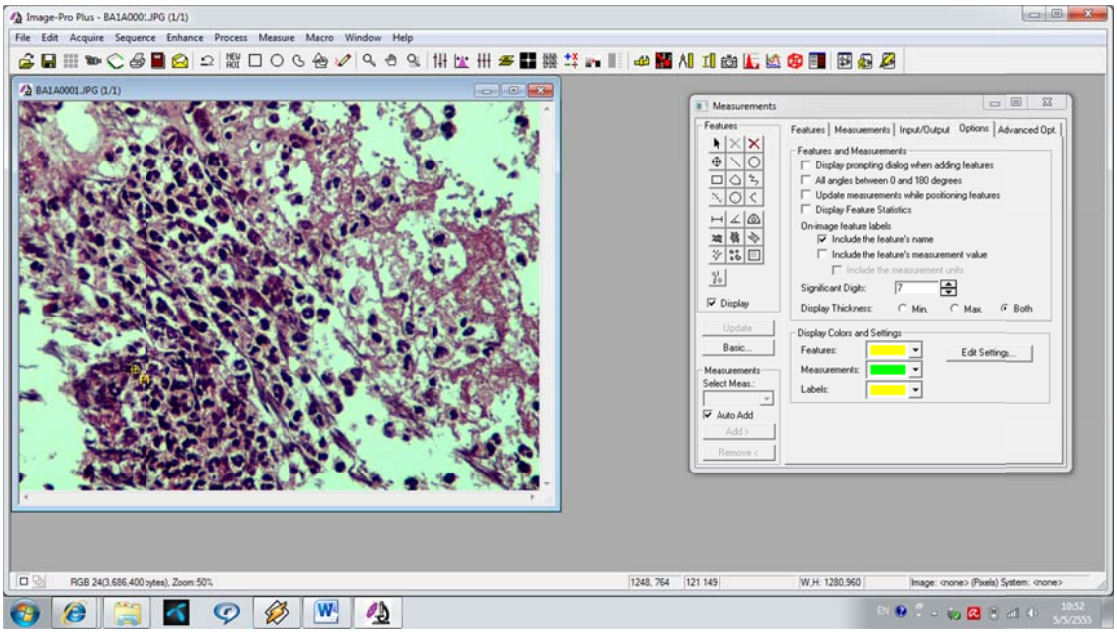


Figure 3.9 Method for measurement the number of neutrophil infiltration into wound area by using Image-Pro Plus 6.1 software.

Measurement of tissue VEGF level

Tissue sample was harvested from each mouse at 7 and 14 days post-wound and then frozen at -80 °C. 50 mg tissue sample was homogenized in 50 ml RIPA lysis buffer (Cell Signaling Technology, Inc.) with protease inhibitor cocktails (Sigma Chemical Co, USA.) and phosphatase inhibitor cocktails (Sigma Chemical Co, USA.), sonicated and centrifugated at 10,000 rpm. for 10 min. The supernatants of each tissue sample were used to analyze VEGF levels by ELISA (R&D Systems).

The experimental procedures were performing as following:

All reagent and sample in room temperature were prepared before do experiment. The each well were added 50 µl of assay diluent RD1N, standard and sample. Microplate was mixed by gently tapping for one minute and incubated for two hours at room temperature. Aspiration and wash the each well by 400 µl of wash buffer. This process was repeated for four times. 100 µl of Mouse VEGF Conjugate was added to each well and incubated at room temperature for two hours. The each well was aspirated and washed by 400 µl of wash buffer. This process was repeated for four times. 100 µl of Substrate Solution was added to each well. Incubation at room temperature for 30 minutes and protect from light. Then, the each well was added 100 µl of Stop Solution and mix by gently tapping. Optical density of each well was detected at 450 nm by using microplate reader, BIO-RAD Model 680.

Calculation of results

Calculate average the duplicate readings for standard optical density and sample optical density. And subtract average optical density of the standard mVEGF 0 pg/ml. Create a standard curve by Microsoft excel. The

calibration graph and the equation of best fitted linear line are show in Figure 3.10

mVGEF (pg/ml)	Optical Density			
	I	II	Average	Corrected
0	0.110	0.105	0.108	0.000
7.8	0.137	0.127	0.132	0.025
15.6	0.169	0.164	0.167	0.059
31.2	0.225	0.226	0.226	0.118
62.5	0.319	0.342	0.331	0.223
125	0.507	0.579	0.543	0.436
250	0.856	0.994	0.925	0.818
500	2.159	1.501	1.830	1.723

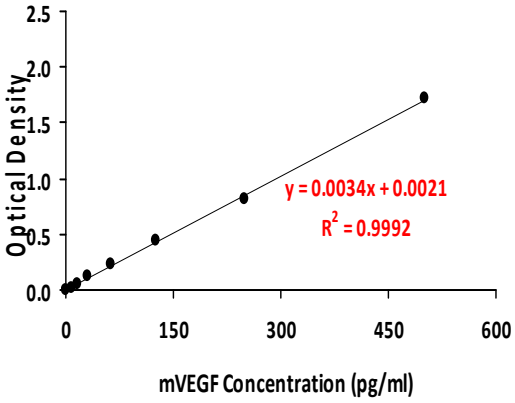


Figure 3.10 Calculation of optical density for VEGF analysis

To determine the total tissue protein

The supernatants of each tissue sample were used to analyze the total tissue protein by microplate BCA protein assay kit (Thermo scientific, USA)

The experimental procedures were performing as following:

The 9 µl of standard, 9 µl of sample and 4 µl of compatibility reagent solution was added to central microplate. Plate was covered and mixed by plate shaker in medium speed for 1 minute. And then plate was incubated for 15 minutes at 37°C and cooled at room temperature for 5 minutes. Optical density of each well detected at 570 nm by using microplate reader, BIO-RAD Model 680.

Calculation of results

Calculate average the duplicate readings for standard optical density and sample optical density. And subtract average optical density of the standard H (0 µg/ml). Create a standard curve by Microsoft excel. The calibration graph and the equation of best fitted linear line are show in Figure 3.11

Protein	Optical Density			
(µg/ml)	I	II	Average	Corrected
2000	0.862	0.795	0.829	0.707
1500	0.594	0.631	0.613	0.491
1000	0.477	0.484	0.481	0.359
750	0.392	0.401	0.397	0.275
500	0.320	0.326	0.323	0.201
250	0.231	0.242	0.237	0.115
125	0.186	0.184	0.185	0.063
0	0.122	0.122	0.122	0.000

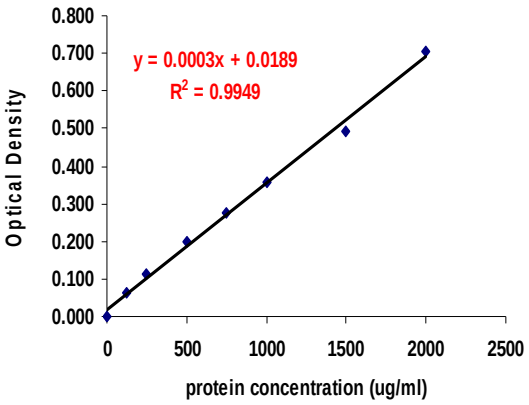


Figure 3.11 Calculation of optical density for total protein concentration analysis.

The results of VEGF were divided by the total protein concentration and the results of VEGF were show in pg/mg protein unit.

Data analysis

All data expressed as means ± standard error of the mean (SEM). The statistical differences between all groups were analyzed by using one-way analysis of variance (one-way ANOVA) and followed by Bonferroni's post-test for analysis of %WC and %CV and Least significant difference's post-test (LSD) for analysis of re-epithelialization, neutrophil infiltration, tissue VEGF

levels. The correlation analysis was analyzed by using two tails Pearson's correlation. The differences were considered statistically significant at p-value less than 0.05 ($P<0.05$).

CHAPTER IV

RESULTS

This chapter of results contented of five parts which were used to determine the effects of combined endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) on angiogenesis and wound healing in diabetic mice.

Physiological characteristics

The results of body weight showed no significant difference between the five groups on day 7 and 14. (Table 4.1and Figure 4.1),

On day 7 and 14 post wounding, the blood sugar in DM group, DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group were significantly higher than Control group ($p=0.0003$, $p=0.0001$, $p=0.0003$, $p=0.0013$ within day 7 and $p=0.0075$, $p=0.0030$, $p=0.0100$, $p=0.0101$, respectively). The results were showed in Table 4.2 and Figure 4.2

Table 4.1 Means ± SEM of the body weight on day 7 and 14 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group.

Group	Body weight (g)	
	Day 7	Day 14
Control	25.91 ± 1.22	26.06 ± 1.45
DM	23.86 ± 2.07	24.77 ± 0.72
DM+MSCs	21.81 ± 1.40	22.61 ± 2.32
DM+EPCs	24.76 ± 1.41	22.86 ± 1.30
DM+EPCs+MSCs	24.57 ± 1.07	23.60 ± 0.71

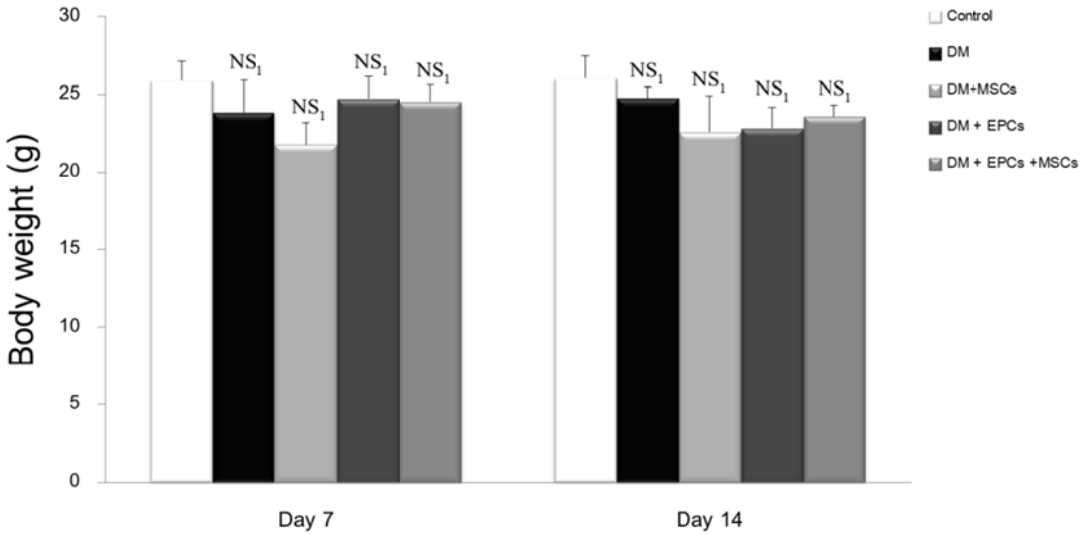


Figure 4.1 The body weight on day 7 and 14. The data is presented as means ± SEM.

NS_1 no significant difference as compared to Control group

Table 4.2 Means ± SEM of the blood glucose levels on day 7 and 14 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group.

Group	Blood glucose (mg/dl)	
	Day 7	Day 14
Control	115.17 ± 14.28	120.40 ± 11.52
DM	347.83 ± 49.41**	362.83 ± 57.56**
DM+MSCs	361.33 ± 18.63**	425.00 ± 104.74**
DM+EPCs	344.17 ± 61.21**	362.40 ± 35.56*
DM+EPCs+MSCs	312.33 ± 15.05**	376.5 ± 82.19*

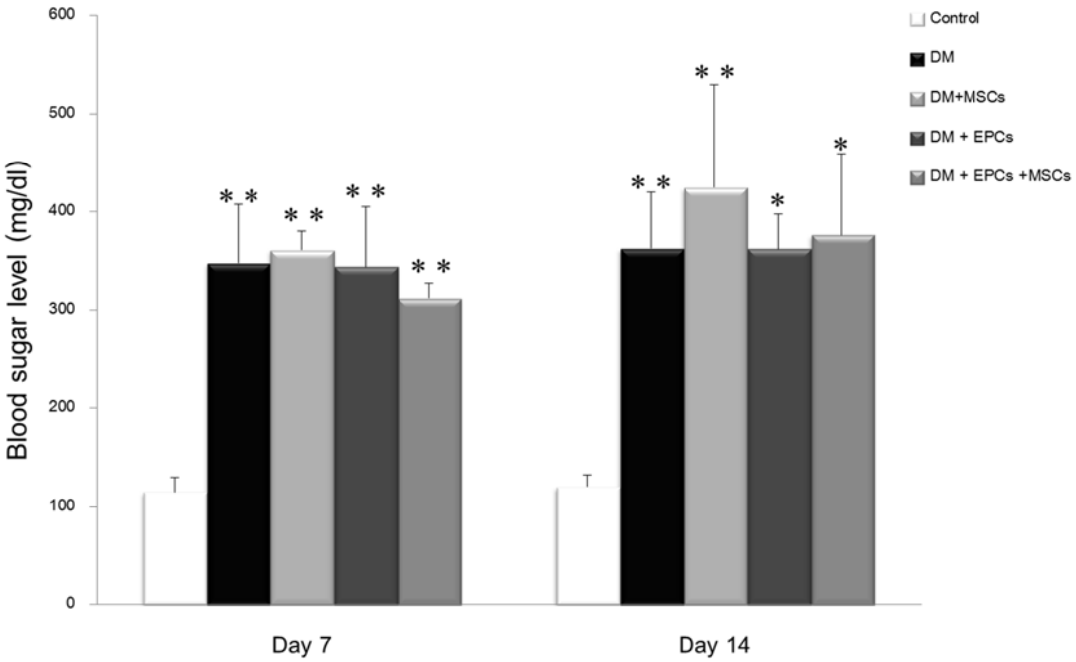


Figure 4.2 The blood sugar levels on day 7 and 14. The data is presented as means ± SEM.

* $P < 0.05$ significant difference as compared to Control group

** $P < 0.01$ significant difference as compared to Control group

Effects of combined EPCs and MSCs on the wound closure

We made bilateral full-thickness excisional skin wounds on the dorsal skin in diabetic mice and the wound healing process was monitored in Figure 4.3 showed the wound image on day 0, 7 and 14.

On day 7 post-operative wound, the percentage of wound closure in Control group was significantly increased than DM group (p=0.0157). The DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group were significantly higher the %WC than DM group (p=0.0012, p=0.0008, p=0.0005, respectively). However, there was no significant difference between groups of DM+MSCs, DM+EPCs and DM+EPCs+MSCs (Table 4.3 and Figure 4.4).

On 14 days after wounding, in Control group, the percentage of wound closure was significantly accelerated when compared to DM group (p=0.0066). The percentage of wound closure in DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group were significantly increased, compared to DM group (p=0.0358, p=0.0001, p=0.0344, respectively). However, there were not significant difference between groups of DM+MSCs, DM+EPCs and DM+EPCs+MSCs (Table 4.4 and Figure 4.5).

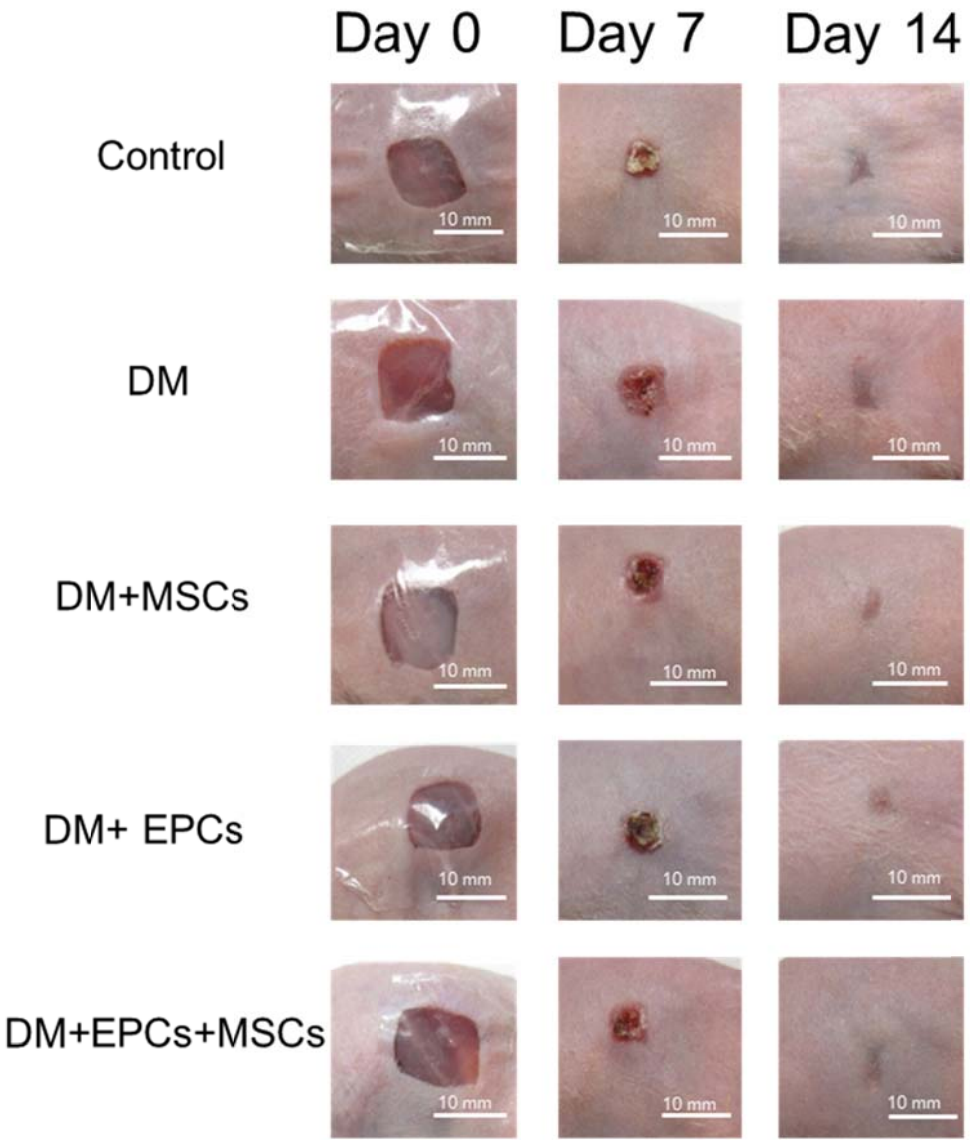


Figure 4.3 Wound image on day 0, 7 and 14 in wounded control group with implanted fibrin gel (Control), diabetic wounded group with implanted fibrin gel (DM), diabetic wounded group with implanted fibrin gel and MSCs (DM+MSCs), diabetic wounded group with implanted fibrin gel and EPCs (DM+EPCs) and diabetic wounded group with implanted fibrin gel and combined MSCs and EPCs (DM+EPCs+MSCs).

Table 4.3 Means ± SEM of percentage of wound closure on day 7 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+MSCs+EPCs group.

Group	%WC	p-value
	Day 7	
Control	72.47 ± 4.52	0.0157
	n=4	
DM	55.65± 3.92	0.0012
	n=6	
DM +MSCs	78.28 ± 4.16	0.0008
	n=4	
DM + EPCs	78.66 ± 2.29	0.0005
	n=5	
DM + EPCs +MSCs	72.04 ± 4.12	
	n=5	

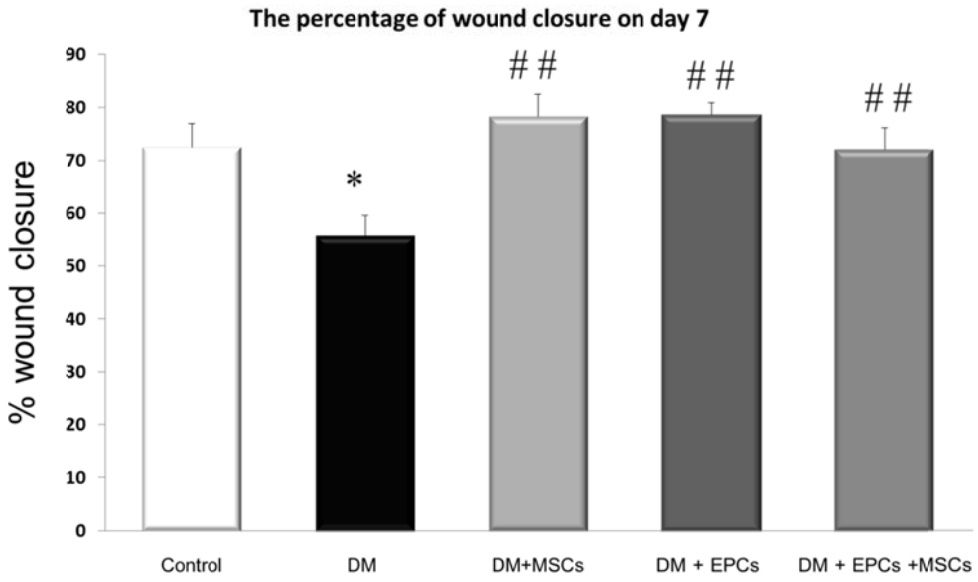


Figure 4.4 The percent of wound closure on day 7. The data is presented as means ± SEM.

* P < 0.05 significant difference as compared to Control group

P < 0.01 significant difference as compared to DM group

Table 4.4 Means ± SEM of percentage of wound closure on day 14 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+ EPCs+ MSCs group.

Group	%WC	p-value
	Day 14	
Control	86.36 ± 0.84	0.0066
	n=6	
DM	80.42 ± 1.37	0.0358
	n=5	
DM +MSCs	85.43 ± 1.58	0.0001
	n=5	
DM + EPCs	89.65 ± 1.07	0.0344
	n=5	
DM + EPCs +MSCs	85.42 ± 0.64	
	n=5	

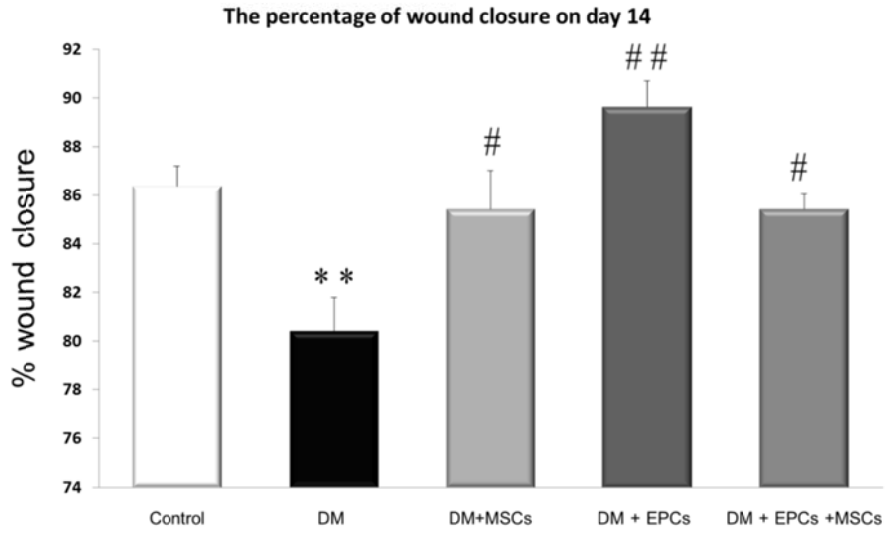


Figure 4.5 The percent of wound closure on day 14. The data is presented as means ± SEM.

* P < 0.05 significant difference as compared to Control group

\# P < 0.05 significant difference as compared to DM group

\## P < 0.01 significant difference as compared to DM group

Effects combined EPCs and MSCs on the angiogenesis in the wound area.

The during wound healing, angiogenesis was evaluated in the capillary vascularity increased within the 7 and 14 days. Figure 4.6 show angiogenesis of wound site in each group.

On day 7, the percentage of capillary vascularity (%CV) in Control group was significantly increased than DM group (p=0.0209). The DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group were significantly higher the %CV than DM group (p=0.0031, p=0.0002, p=0.0000, respectively). The %CV of DM+EPCs+MSCs was significantly increased than that in DM+MSCs group, DM+EPCs group (p=0.0004, p=0.0018 respectively). (Table 4.4 and Figure 4.7).

On 14 days, in Control group, the percentage of capillary vascularity was significantly higher than DM group (p=0.0493). The %CV in DM+EPCs+MSCs group was significantly increased compared to DM group (p=0.0020). However, there were not significant difference between groups of DM+MSCs, DM+EPCs and DM+EPCs+MSCs (Table 4.5 and Figure 4.8).

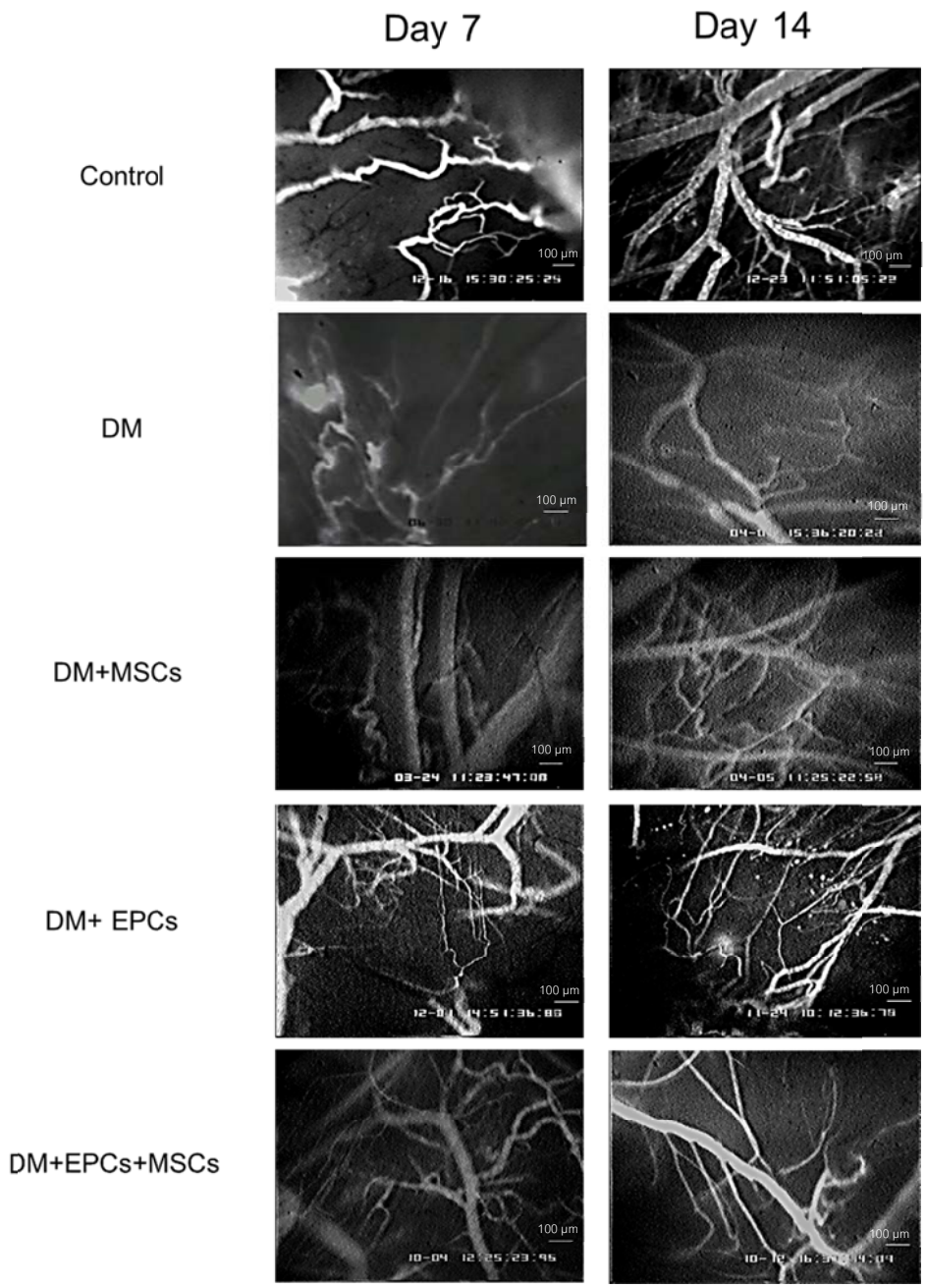


Figure 4.6 Showed angiogenesis of wound area on day 7 and 14 in wounded control group with implanted fibrin gel (Control), diabetic wounded group with implanted fibrin gel (DM), diabetic wounded group with implanted fibrin gel and MSCs (DM+MSCs), diabetic wounded group with implanted fibrin gel and EPCs (DM+EPCs) and diabetic wounded group with implanted fibrin gel and combined MSCs and EPCs (DM+MSCs+EPCs). (Scale bar: 100µm)

Table 4.5 Means ± SEM of percentage of capillary vascularity on day 7 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group.

Group	%CV	p-value
	Day 7	
Control	19.95 ± 0.82	
	n=6	0.0209
DM	14.72 ± 1.49	
	n=6	0.0031
DM +MSCs	21.42 ± 0.61	0.0002
	n=5	0.0000
DM + EPCs	22.86 ± 1.20	0.0018
	n=5	0.0004
DM + EPCs +MSCs	27.44 ± 1.07	
	n=5	

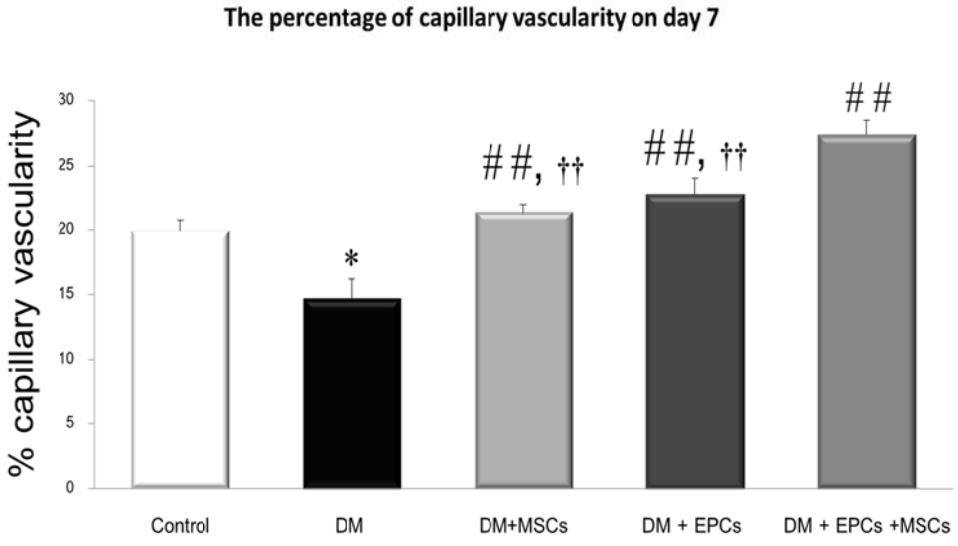


Figure 4.7 The percent of capillary vascularity on day 7. The data is presented as means ± SEM.

* P < 0.05 significant difference as compared to Control group

P < 0.01 significant difference as compared to DM group

†† P < 0.01 significant difference as compared to DM+EPCs+MSCs group

Table 4.6 Means ± SEM of percentage of capillary vascularity on day 14 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group.

Group	%CV	p-value
	Day 14	
Control	24.09 ± 2.33	0.0493
	n=4	
DM	16.72 ± 1.39	
	n=6	
DM +MSCs	20.24 ± 0.81	0.0020
	n=5	
DM + EPCs	21.50 ± 1.09	
	n=4	
DM + EPCs +MSCs	24.92 ± 1.81	
	n=5	

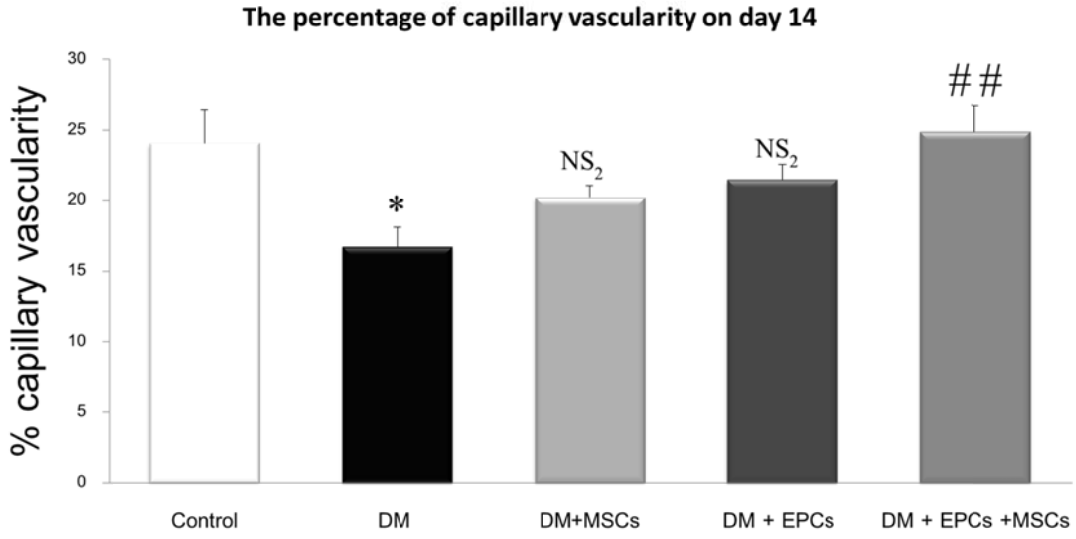


Figure 4.8 The percentage of capillary vascularity on day 14. The data is presented as means ± SEM.

* $P < 0.05$ significant difference as compared to Control group

$P < 0.01$ significant difference as compared to diabetic group

NS_1 no significant difference as compared to diabetic group

Effects combined EPCs and MSCs on re-epithelialization

The re-epithelialization was used to evaluate wound healing on day 7 and 14 after wounding. Figure 4.9 show histological of wound skin in the each group on day 7and 14.

On day 7 and 14, the results showed no significant difference in re-epithelialization between the five groups. (Table 4.7-4.8 and Figure 4.10-4.11)

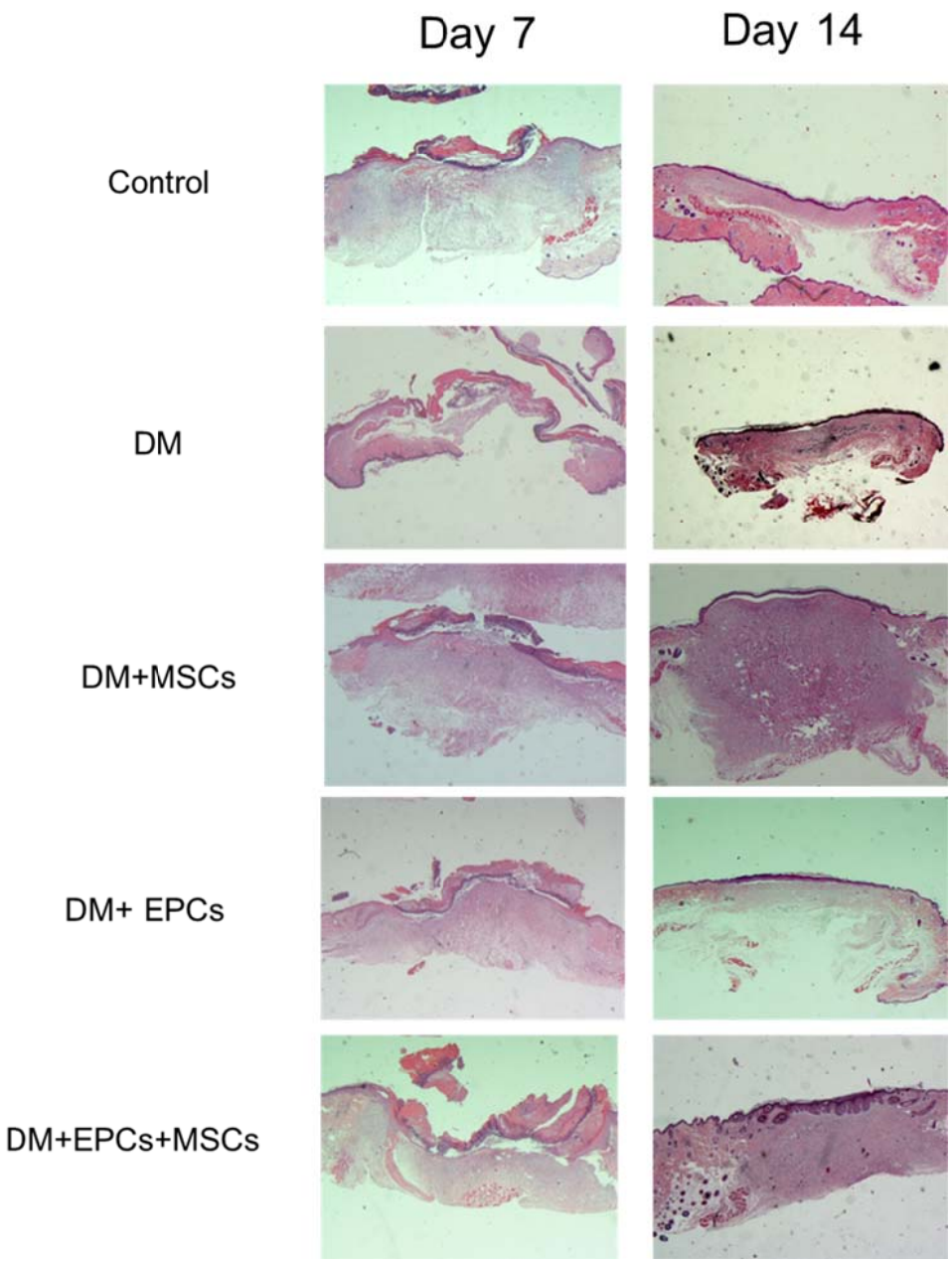


Figure 4.9 Re-epithelialization in wound area on day 7 and 14 in wounded control group with implanted fibrin gel (Control), diabetic wounded group with implanted fibrin gel (DM), diabetic wounded group with implanted fibrin gel and MSCs (DM+MSCs), diabetic wounded group with implanted fibrin gel and EPCs (DM+EPCs) and diabetic wounded group with implanted fibrin gel and combined MSCs and EPCs (DM+EPCs+MSCs). H&E-stained section was photographed at 20 power.

Table 4.7 Means ± SEM of percentage of re-epithelialization in wound area on day 7 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group.

Group	% re-epithelialization	p-value
	Day 7	
Control	69.03 ± 9.62	
	n=5	
DM	65.13 ± 12.23	
	n=4	
DM +MSCs	85.99 ± 7.12	
	n=5	
DM + EPCs	90.30 ± 5.99	
	n=5	
DM + EPCs +MSCs	72.77 ± 7.54	
	n=4	

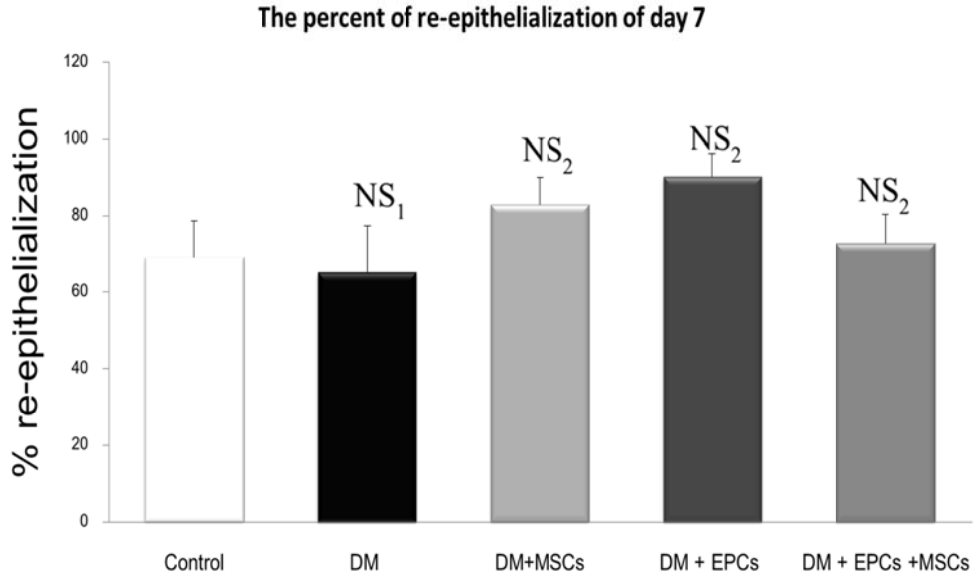


Figure 4.10 The percentage of re-epithelialization in wound area on day 7. The data is presented as means ± SEM.

NS_1 no significant difference as compared to Control group

NS_2 no significant difference as compared to diabetic group

Table 4.8 Means ± SEM of percentage of re-epithelialization in wound area on day 14 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group.

Group	% re-epithelialization	p-value
	Day 14	
Control	100.00 ± 0.00	
	n=6	
DM	99.76 ± 0.29	
	n=6	
DM +MSCs	100.00 ± 0.00	
	n=6	
DM + EPCs	100.00 ± 0.00	
	n=5	
DM + EPCs +MSCs	100.00 ± 0.00	
	n=5	

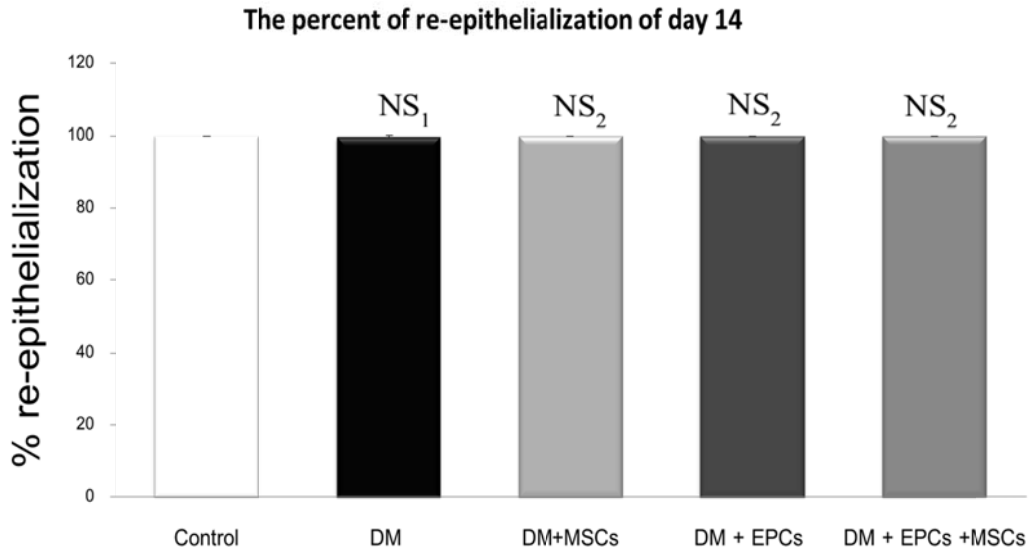


Figure 4.11 The percentage of re-epithelialization in wound area on day 14. The data is presented as means ± SEM.

NS_1 no significant difference as compared to Control group

NS_2 no significant difference as compared to diabetic group

Effects combined EPCs and MSCs on the neutrophil infiltration

Figure 4.12 show number of neutrophils infiltration in wound area between five groups.

On day 7, Number of neutrophils in DM group significantly increased more than that in Control group (p=0.0227). DM+MSCs, DM+EPCs and DM+EPCs+MSCs showed no significant difference when compared to DM group (Table 4.9 and Figure 4.13).

On day 14, Number of neutrophils in DM group significantly increased more than that in Control group (p=0.0115). DM+MSCs, DM+EPCs and DM+EPCs+MSCs were significant reduced number of neutrophil, compare to DM group (p=0.0044, p=0.0061, p=0.0153, respectively). However, there were not significant difference between groups of DM+MSCs, DM+EPCs and DM+EPCs+MSCs (Table 4.10 and Figure 4.14).

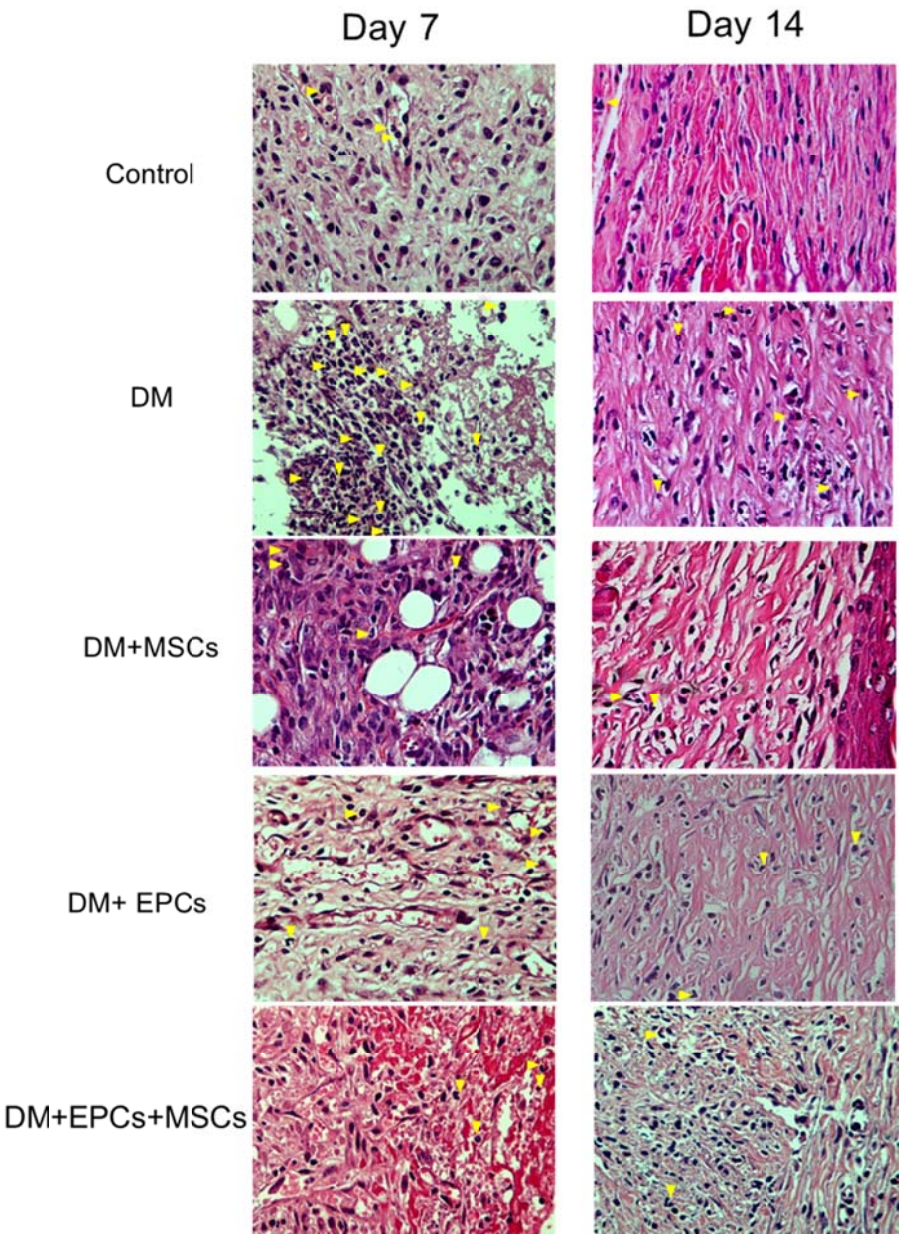


Figure 4.12 The number of neutrophil infiltration in wound area on day 7 and 14 in wounded control group with implanted fibrin gel (Control), diabetic wounded group with implanted fibrin gel (DM), diabetic wounded group with implanted fibrin gel and MSCs (DM+MSCs), diabetic wounded group with implanted fibrin gel and EPCs (DM+EPCs) and diabetic wounded group with implanted fibrin gel and combined EPCs and MSCs (DM+EPCs+MSCs). H&E-stained section was photographed at 400 power.

Table 4.9 Means ± SEM of number neutrophil infiltration in wound on day 7 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group.

Group	neutrophil infiltration (cells)	p-value
	Day 7	
Control	15.00 ± 3.33	0.0227
	n=5	
DM	30.25 ± 6.14	
	n=4	
DM +MSCs	21.80 ± 4.02	
	n=5	
DM + EPCs	20.00 ± 4.00	
	n=5	
DM + EPCs +MSCs	21.25 ± 2.66	
	n=4	

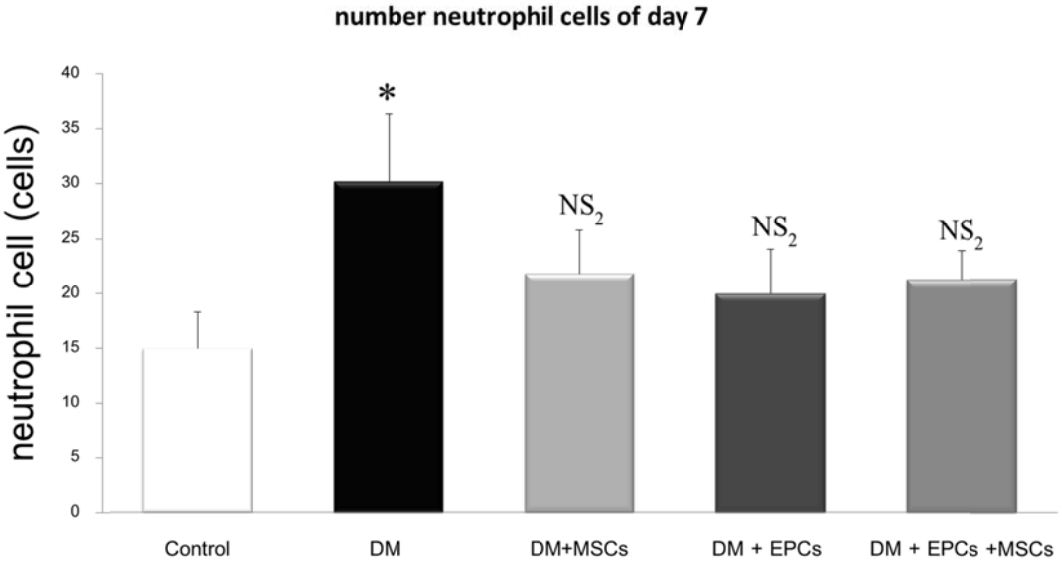


Figure 4.13 The number neutrophil infiltration on day 7. The data is presented as means ± SEM.

* $P < 0.05$ significant difference as compared to Control group

NS_2 no significant difference as compared to diabetic group

Table 4.10 Means ± SEM of number neutrophil infiltration in wound on day 14 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group.

Group	neutrophil infiltration (cells)	p-value
	Day 14	
Control	13.00 ± 2.16	0.0115
	n=4	
DM	34.25 ± 4.59	0.0044
	n=4	
DM +MSCs	11.60 ± 3.14	0.0061
	n=5	
DM + EPCs	12.40 ± 2.40	0.0153
	n=5	
DM + EPCs +MSCs	14.60 ± 1.63	
	n=5	

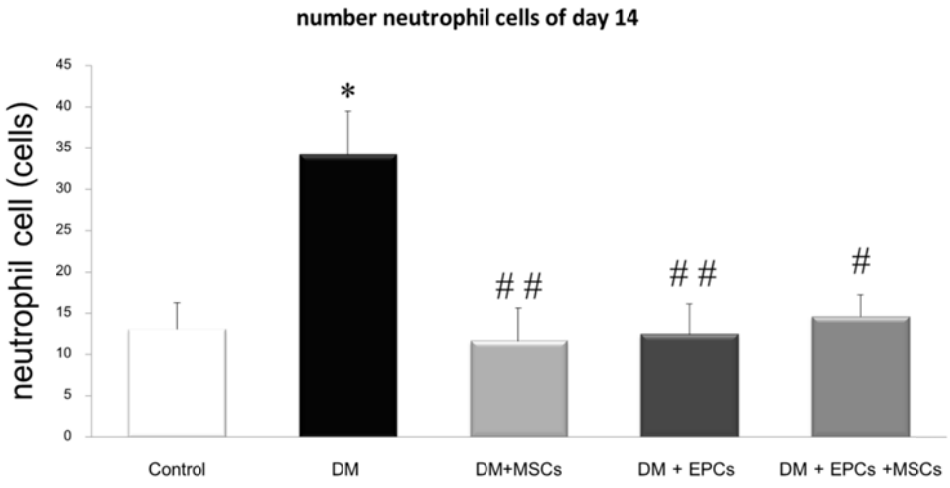


Figure 4.14 The number neutrophil infiltration on day 14. The data is presented as means ± SEM.

* $P < 0.05$ significant difference as compared to Control group

\# $P < 0.05$ significant difference as compared to DM group

\## $P < 0.01$ significant difference as compared to DM group

Effects combined EPCs and MSCs on the tissue VEGF level

On day 7, VEGF levels in Control group was significantly increased compared to DM group (p=0.0021). The DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group were significantly higher VEGF level than DM group (p=0.0036, p=0.0122, p=0.0039, respectively). However, there were not significant difference between groups of DM+MSCs, DM+EPCs and DM+EPCs+MSCs (Table 4.11 and Figure 4.15).

On day 14, the results showed no significant difference among all groups. (Table 4.12 and Figure 4.16)

Table 4.11 Means ± SEM of tissue VEGF levels on day 7 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group.

Group	Tissue VEGF level (pg/mg protein)	p-value
	Day 7	
Control	232.30 ± 44.09	0.0021
	n=4	
DM	44.06 ± 10.70	0.0036
	n=4	
DM +MSCs	218.84 ± 27.50	0.0122
	n=4	
DM + EPCs	188.43 ± 28.09	0.0039
	n=4	
DM + EPCs +MSCs	216.45 ± 53.01	
	n=4	

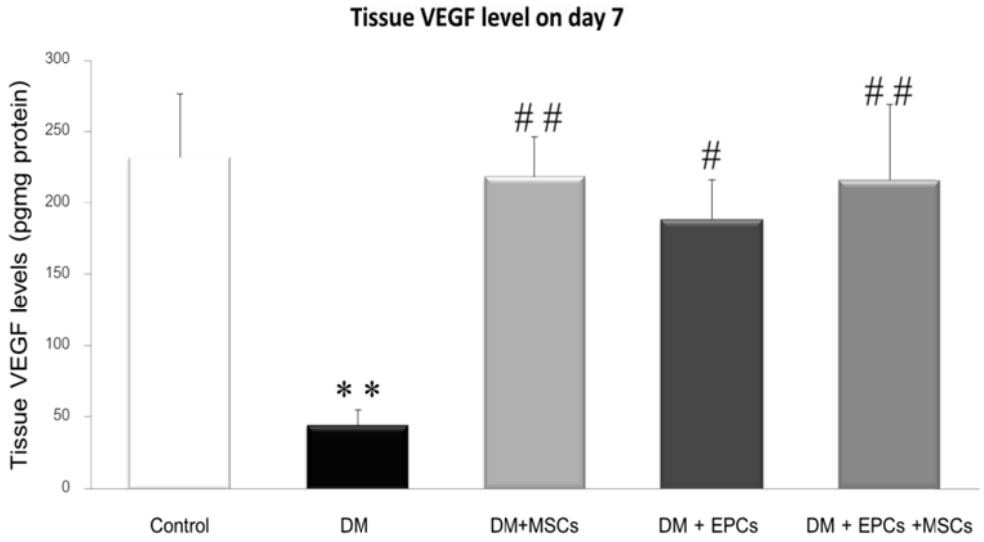


Figure 4.15 The tissue VEGF levels on day 7. The data is presented as means ± SEM.

** P < 0.01 significant difference as compared to Control group

# P < 0.05 significant difference as compared to DM group

P < 0.01 significant difference as compared to DM group

Table 4.12 Means ± SEM of tissue VEGF levels on day 14 in Control group, DM group, DM+MSCs group, DM+EPCs group and DM+EPCs+MSCs group.

Group	Tissue VEGF level (pg/mg protein)	p-value
	Day 14	
Control	45.30 ± 7.91	
	n=4	
DM	28.18 ± 2.19	
	n=4	
DM +MSCs	44.99 ± 9.58	
	n=4	
DM + EPCs	40.36 ± 14.41	
	n=4	
DM + EPCs +MSCs	50.73 ± 8.97	
	n=4	

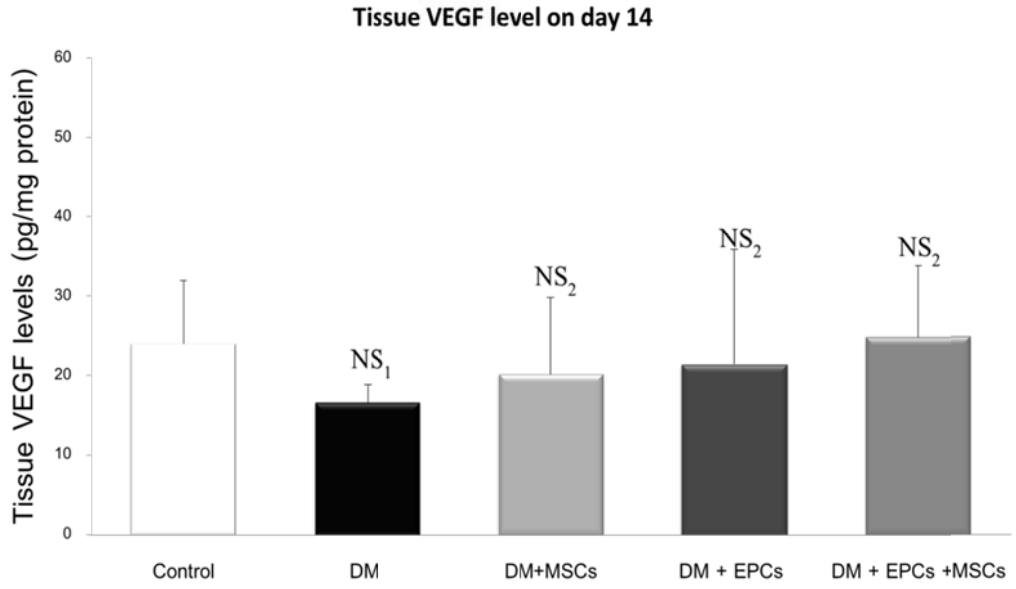


Figure 4.16 The tissue VEGF levels on day 14. The data is presented as means ± SEM.

NS_1 no significant difference as compared to Control group

NS_2 no significant difference as compared to diabetic group

CHAPTER V

DISCUSSION

The present experiment was conducted to evaluate the effects of combined endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) on angiogenesis and wound healing in diabetic mice model.

The effects of EPCs and MSCs on blood sugar and body weight

In the present study, the type 1 diabetes mellitus induction in nude mice was performed by using multiple low doses STZ (45 mg/kg body weight) injections daily for 5 day. STZ is chemically damaged pancreatic β cells by Deoxyribonucleic acid (DNA) damage, resulting in hypoinsulinemia and hyperglycemia (Lenzen, 2008: 216-226). In Table 4.2 and Figure 4.2, the blood glucose levels in DM, DM+MSCs, DM+EPCs, and DM+EPCs+MSCs groups were significantly higher than their age-matched Control groups, day 7- and 14- post wounded. The results also showed that there was no significant difference between DM and DM-treated groups. Therefore, it implied that hyperglycemia was not reduced by EPCs and/or MSCs treatments.

The diabetes-induced body weight lost is recognized in diabetic patient and in STZ-induced animal model. It also suggested that the BW lost in diabetes was caused by insulin deficiency which consequently promoted protein- and fat- catabolism (Rerup and Tarding, 1969: 89-96). However, some study reported that the BW did not change in multi-dose STZ induced diabetic mice (Yu et al., 2003: 31-42). Similarly our studies have shown that the body weights of all five groups were no significant difference on both day 7 and 14.

They so suggested that EPCs and MSCs have no effect on body weights in diabetic nude mice.

The effects of EPCs and MSCs on the wound closure

The characteristics of diabetic wounds are the frequent skin lesions and poor wound healing which commonly appear in diabetic patients (Reiber, Pecoraro, and Koepsell, 1992: 97-105). Therefore, several studies had pay attention to apply EPCs for enhancing wound healing in DM.

In our study by using the model of full-thickness wound, the results showed that on day 7- and day 14- post wounded the percentage of wound closure in age-matched control groups were significantly higher than DM groups. The %WC of DM+MSCs, DM+EPCs, and DM+EPCs+MSCs groups were significantly higher than %WC of DM group (Table 4.3-4.4 and Figure 4.4-4.5) ($P<0.05$). Therefore, it may be concluded that both EPCs and MSCs were able to stimulate the wound healing process in DM which that will be discussed later for the possible mechanism(s) in the following session.

The effects of EPCs and MSCs on the angiogenesis

Our obtained results of capillary vascularity (%CV) in Table 4.5 and Figure 4.7 showed that %CV of DM+MSCs, DM+EPCs and DM+EPCs+MSCs groups were significantly increased as compared to DM group. The %CV of DM+EPCs+MSCs was significantly increased than those of DM+MSCs and DM+EPCs groups on day 7. However, the %CV of DM+EPCs+MSCs group significantly increased more than DM group on day 14 (Table 4.6 and Figure 4.8). Therefore, it may be noted that both EPCs and MSCs could stimulate

wound neovascularization, however, the combined EPCs and MSCs seem to be the best.

It is proposed that the prolonged and incomplete diabetic wound healing is resulted by poor or reduced angiogenesis. Our study also confirmed that either EPCs or MSCs treatment could enhance angiogenesis in diabetic wound and that was very important for promoting successful wound healing (Debin et al., 2008: 106-115; Sivan-Loukianova et al., 2003: 368-377). Up to our knowledge, this study is the first time that indicates the *"synergistic"* effect of combined EPCs+MSCs treatment on enhancing angiogenesis in diabetic wound.

The effects of EPCs and MSCs on the re-epithelialization

The process of re-epithelialization in chronic wound like cutaneous ulcer and burn wound is an important process for the complete wound closure. In normal process of wound healing, the re-epithelialization represents the final state of proliferative phase which occurs during day 3 to day 14. In our study, the process of re-epithelialization was accessed on day 7 and day 14. The re-epithelialization was not significantly different between all groups on day 7 and 14 (Table 4.7-4.8 and Figure 4.10-4.11).

In fact, collagen is accounted as an important factor of extracellular matrix formation that extracellular matrix is an ultrastructure that supplies a substratum for cell adhesion. It also helps critically to regulate the growth, movement, and differentiation of cells through its networking. The present result provides an evidence to indicate the roles of fibrin gel in cell migration. The results on day 14 indicated that DM+MSCs, DM+EPCs and

DM+EPCs+MSCs effectively enhanced re-epithelialization up to 100%. A mechanism underling these observations may be associated with their effects on angiogenesis which is the other important process required for wound healing and it exists in the phase of proliferation similar to re-epithelialization.

The effects of EPCs and MSCs on the number of neutrophils infiltration

As shown in Table 4.9 and Figure 4.13, on day 7, the numbers of neutrophils in DM, groups were significantly increased more than that in Control group ($P<0.05$). DM+MSCs, DM+EPCs and DM+EPCs+MSCs showed no significant difference when compared to DM group. Interestingly, on day 14, the numbers of neutrophils in DM+MSCs, DM+EPCs and DM+EPCs+MSCs were significant less than DM group (Table 4.10 and Figure 4.14) ($P<0.05$). In the previous studies, many pro-inflammatory substances, such as TNF-α, IL-6 and IL-1, are elevated in the serum of type 1 and 2 diabetes patients. In addition, it also shown that this progressed increased pro-inflammatory substances appeared to link to the progression of diabetic state and insulin resistance. It believed that the pro-long increased pro-inflammatory induction caused the perpetuation of inflammatory responses, leading to a chronic inflammatory state, particularly in diabetes (Tellechea et al., 2010: 43-55; Zozulinska and Wierusz-Wysocka, 2006: S12-S16).

Our results suggested that neutrophils infiltration in diabetic wounded mice were reduced significantly when treated with MSCs and/or EPCs ($P<0.05$). It should be noted that EPCs and MSCs are able to reduce neutrophil infiltration concurrently with the enhancing effect on neovascularization in diabetic wounds.

The effects of EPCs and MSCs of the tissue VEGF level

The present study showed that on day 7, tissue VEGF level significantly increased in DM+MSCs, DM+EPCs and DM+EPCs+MSCs groups when compared to DM group ($p=0.0036$, $p=0.0122$, $p=0.0039$, respectively). However, at day 14, there was no significant difference between all groups. VEGF is an important growth factor particular for angiogenic process. In diabetes, the reduced production of VEGF caused poor circulation and impaired angiogenesis which appear to contribute to the poor wound healing. EPCs have been indicated for its important role in up-regulation of VEGF expression particular for hypoxic condition (Velazquez, 2007: A39-47). Therefore, our study has added new evidence that not only EPCs, MSCs can also increase in chemoattractive and mitogenic factors including VEGF. The previous studies reported that MSCs could increase numbers of cells positive for CD34, C-kit or Flk-1, which were markers for endothelial lineage cells and increased recruitment of endothelial cells and endothelial progenitor cells into the wound (Wu et al., 2007: 2648-2659; Wu, Zhao, and Tredget, 2010: 905-915). Therefore, these reported roles of EPCs and MSCs might be the reasons in explaining our best results of combined EPCs and MSCs treatment.

The correlations of wound closure, capillary vascularity, and VEGF level

Since it reported that the impaired diabetic wound healing is associated to decrease angiogenesis and endothelial dysfunction because defect the delivery of oxygen and nutrients to the wound (Singer and Clark, 1999: 738-746). The studies reported that angiogenesis play an important process of wound healing (Arnold and West, 1991: 407-422). In order to confirm our significant finding on roles of EPCs and MSCs treatment in diabetic wound

healing, the correlation between specific parameters from our study was then explored. As shown in Figure 5.1-5.2, the percentage of wound closure (y-axis) vs. capillary vascularity (x-axis) and between wound closure (y-axis) vs. VEGF level (x-axis) were plotted and defined the correlation using Pearson's Correlation method. The results showed that Pearson's Correlations of both are significant, r=0.784, (P=0.000); and r=0.844 (P=0.000). Therefore, it implied that the treatment of EPCs and MSCs could improve diabetic wound healing significantly related to its effect on enhancing angiogenesis through the increased VEGF expression.

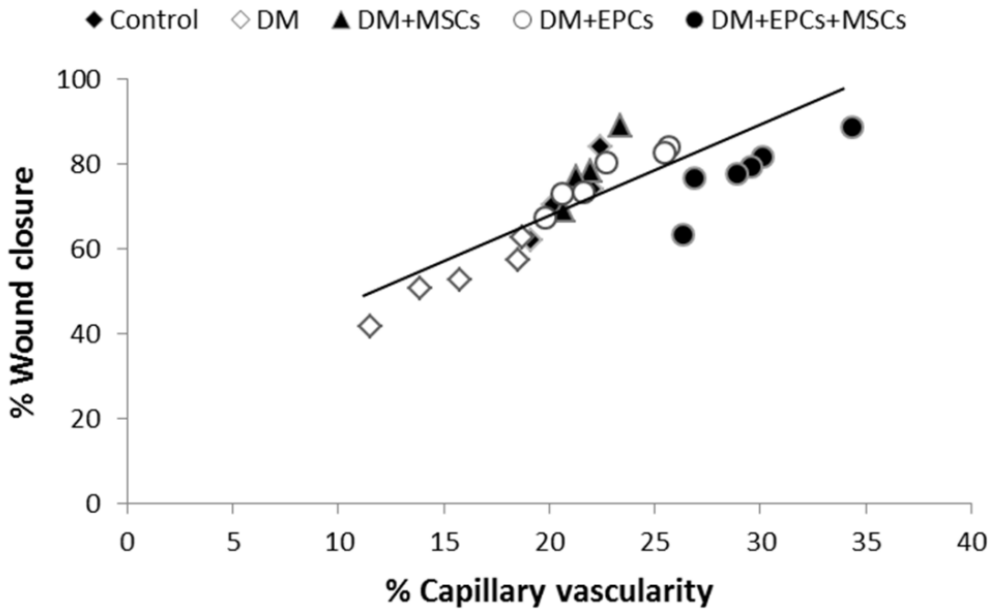


Figure 5.1 Correlation between the percentage of wound closure and the percentage of capillary vascularity in Control, DM, DM+MSCs, DM+EPCs and DM+EPCs+MSCs. The correlation analysis were examined by using Pearson's correlation and the best-fitting linear regression in data of each groups (n=4 to 6).

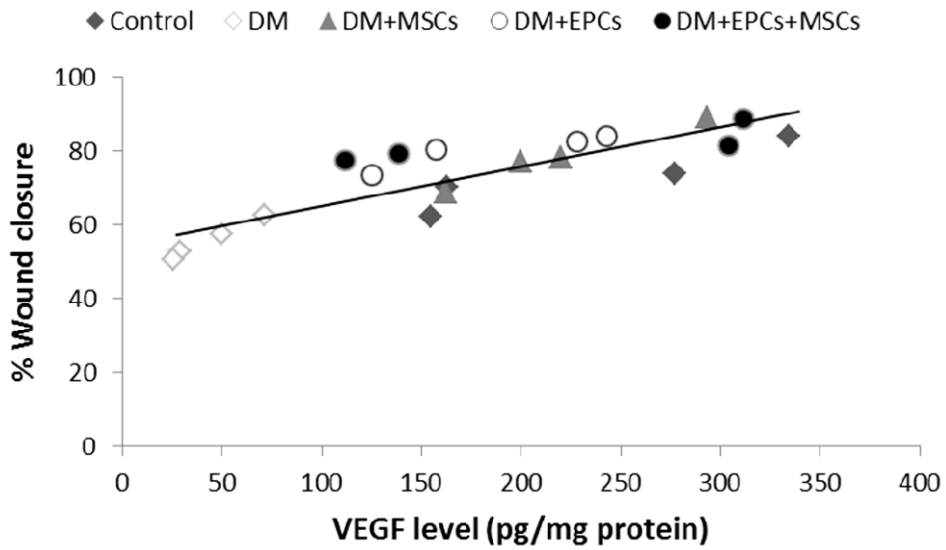


Figure 5.2 Correlation between the percentage of wound closure and the percentage of capillary vascularity in Control, DM, DM+MSCs, DM+EPCs and DM+EPCs+MSCs. The correlation analysis were examined by using Pearson's correlation and the best-fitting linear regression in data of each groups (n=4).

Propose mechanisms for effects of MSCs and EPCs on enhancement of the wound healing processes in diabetic mice.

A common characteristic of patients with diabetes is hyperglycemia which leads to increase oxidative stress (ROS production) (Tesfamariam and Cohen, 1992: H321-H326). Oxidative stress influences to endothelial cell function such as endothelial migration, proliferation and differentiation (Curcio and Ceriello, 1992: 787-790; Mascardo, 1988: 378-385). It is reported that DM-induced oxidative stress could decrease NO production and also result in increased leukocyte-endothelial cells interaction (Traub and Van Bibber, 1995: 439-445). In particular, in long-term or poor hyperglycemic control, it will lead to the development of diabetic complications i.e. vascular disease. Diabetes wound is characterized by prolong chronic inflammatory state, poor

circulation, impaired angiogenesis, disturbances in collagen metabolism and reduced production of growth factors such as TGF-β, EGF, VEGF, PDGF and KGF which contribute to delay wound healing (Volarevic et al., 2011: 5-10). In addition, endothelial progenitor cells were decreased number and dysfunction in diabetic patients (Loomans et al., 2004: 195-199), is one of cause to reduced growth factors production and angiogenesis which lead to prolonged and incomplete diabetic wound healing. The results of 7 days post-wound showed that EPCs and/or MSCs could improve angiogenesis, increase VEGF level because the effect of MSCs induced or released soluble cytokines. In previously study, moderate (TGF-β, KGF) or significant (EGF, PDGF and VEGF) in the production of growth factors involved in the repair of diabetic wound healing. These factors stimulated cell adhesion at the site of injury and induced cells to secrete more chemokine resulting in neovascularization (Volarevic et al., 2011: 5-10) and EPCs secreted many cytokines (VEGF and fibroblast growth factor) that could stimulate proliferation, migration and survival of endothelial cells which leaded to endothelial function recovery (He et al., 2004: 2378-2384). On day 14, the results showed EPCs or MSCs reduce neutrophil infiltration and increase wounded healing that due to MSCs has anti-inflammatory effects. The previous report showed that tissue injury activate MSCs can secrete anti-inflammatory factors including prostaglandin E_2 (PGE_2), TNF-α stimulated gene/protein 6 (TSG-6), and IL-1 and decrease proinflammatory cytokine activity which lead to decrease the proinflammatory signals and the recruitment of neutrophils (Prockop and Youn Oh, 2012: 14-20). However, angiogenesis was increased in combined EPCs and MSCs group on day 14. The results indicated EPCs or/and MSCs could increase angiogenesis and reduce neutrophil infiltration which help to improve diabetic wound healing.

Suggestion

Although, the result of this study indicated that EPCs and MSCs could enhance rapid wound healing in associated with the increased VEGF expression and consequently increased capillary vascularity. However, further research is needed to propose the possible mechanism(s) of MSCs on how it could stimulate VEGF expression and induce EPCs to homing and to differentiate into endothelial cells during proliferative phase in wound-healing process.

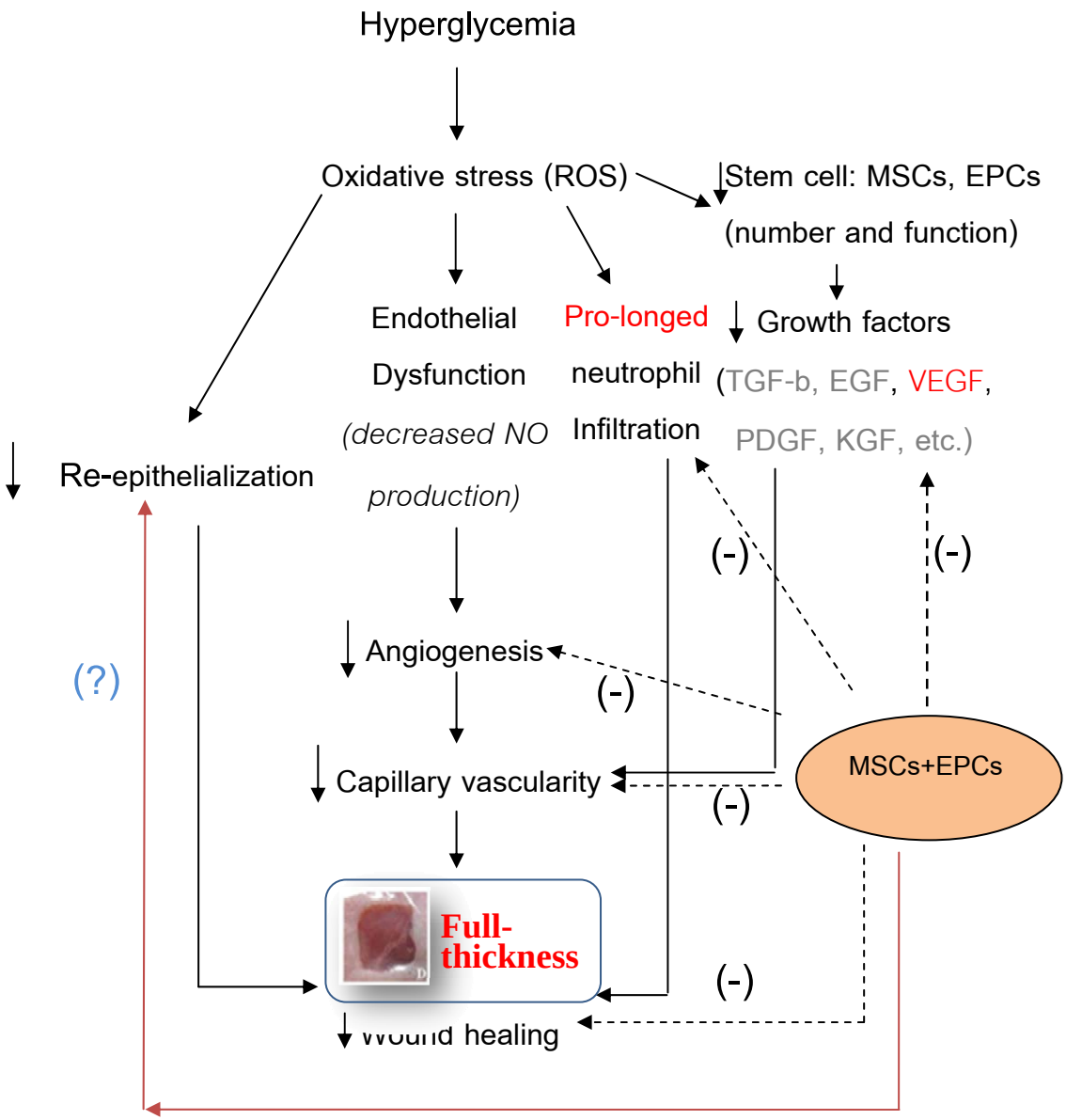


Figure 5.3 Proposed mechanisms for the effect of effects of MSCs and EPCs on enhancement of angiogenesis and wound healing processes in diabetic mice.

CHAPTER VI

CONCLUSION

The present study demonstrated the benefit effects of combined EPCs and MSCs treatment on angiogenesis and wound healing in diabetic mice (DM). The significant findings of this study are summarized as following:

1) On day 7, DM wound showed significant increase of neutrophil infiltration and decrease in VEGF, capillary vascularity, and wound closure as compared to control.

2) On day 14, DM wound showed increase of neutrophil infiltration and decrease in capillary vascularity, and wound closure as compared to control.

3) On day 7, EPCs or/and MSCs could rapidly increase VEGF level as well as increase in neocapillaries when compared to those parameters of DM group. However, it only showed trend of reduction of neutrophil infiltration when compared to 7-day DM significantly.

4) On day 14, EPCs or/and MSCs could increase in wound closure and reduction of neutrophil infiltration when compared to 14-day DM significantly.

5) On day 7, the combine EPCs and MSCs significantly increase angiogenesis when compared to single treatment of EPCs or MSCs alone.

6) Finally, it is suggested that the combination of EPCs and MSCs could enhanced wound healing in diabetic mice model. That might be used as therapeutic approach for treatment of chronic diabetic wounds, in particular for preventing diabetes amputation in the future.

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APPENDICES

APPENDIX A

Fibrin gel

Fibrin gel is use surgical hemostatic agent. In addition, it is used for cell delivery in tissue engineering and local delivery of factors or drugs. Fibrin glue consists of fibrinogen solution and a thrombin solution. The fibrinogen solution is obtained by cryoprecipitation method or ethanol fractionation method from human plasma. The thrombin solution uses human thrombin in commercial products. When the two solutions are mixed, the crosslinks of fibrin are produced by enzymatic action of thrombin and fibrinogen to from fibrin and fibrin and factor XIII to factor XIIIα (Spicer and Mikos, 2010: 49-55).

APPENDIX B

Principle of intravital fluorescence microscopy technique

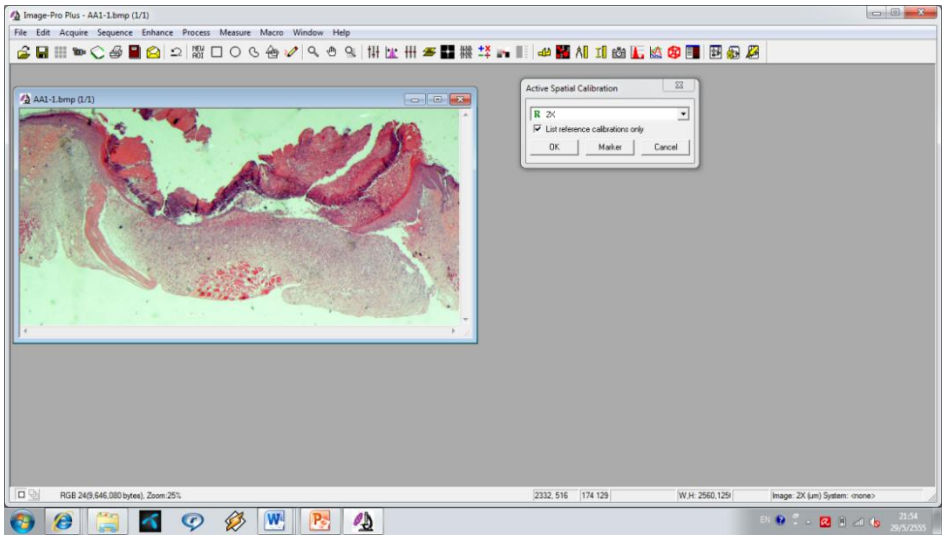
Intravital fluorescence microscopy has been used to observe the microvascular of many organ, such as, skin, brain, heart, lung, liver, pancreas, gut, and kidney.

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, cause to specimen emit light of longer wavelengths. The spectral emission is emitted fluorescence of illumination light which is separated. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp), the excitation filter, the dichroic mirror (or dichromatic beamsplitter), and the emission filter. The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen.

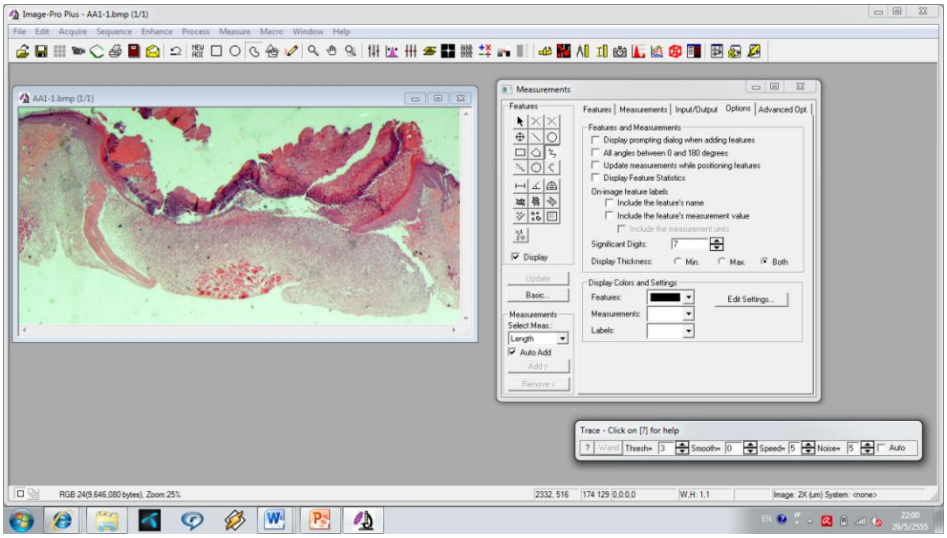
APPENDIX C

Method for measurement the re-epithelialiazation by using Image-Pro Plus 6.1.

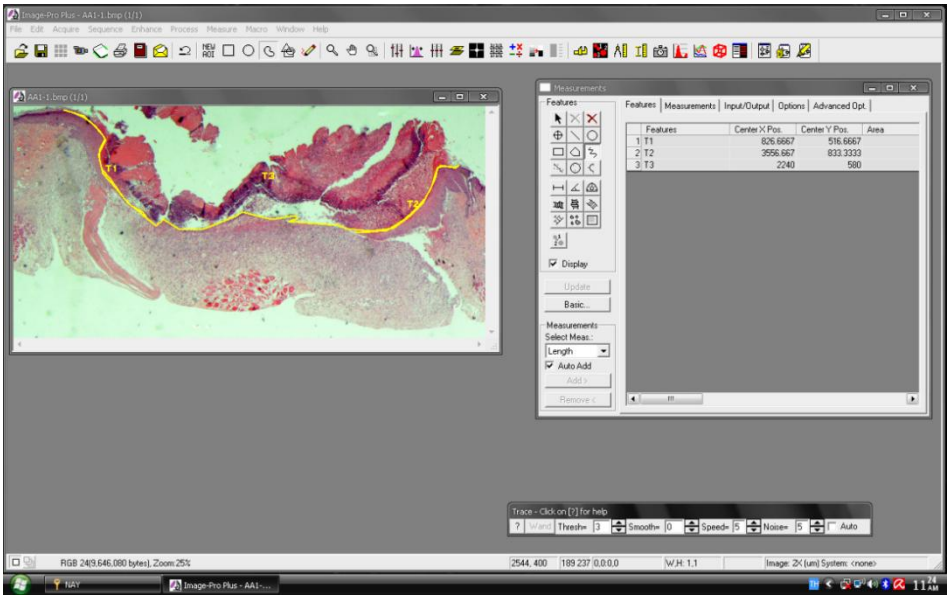
1. Calculated by selected at the objective lens at 2X in reference list of spatial calibration.



2. Choose the manual measurement and select in the trace feature.



3. Draw the line follow the distance of the wound edges and the distance covered by epithelium.



4. These values are calculated by follow:

$$\% \text{ Re-epithelialization} = \frac{\text{distance covered by epithelium (r)}}{\text{x } 100}$$

BIOGRAPHY

NAME	Miss Supakanda Sukpat
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INSTITUTIONS ATTENDED	Burapha University, 2004-2007: Bachelor of Science Chulalongkorn University, 2008-2012: Master of Science (Physiology)
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