EFFECTS OF MESENCHYMAL STROMAL CELL-CONDITIONED MEDIA AND HYPOXIA ON WOUND CLOSURE BY KERATINOCYTES

by

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A Thesis submitted to the

Graduate School-New Brunswick

and

The Graduate School of Biomedical Sciences,

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Biomedical Engineering

Written under the direction of

FRANCOIS BERTHIAUME

And approved by

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New Brunswick, New Jersey

MAY, 2016

ABSTRACT OF THESIS

EFFECTS OF MESENCHYMAL STROMAL CELL-CONDITIONED MEDIA AND HYPOXIA ON WOUND CLOSURE BY KERATINOCYTES

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Thesis Director:

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Chronic, prolonged, non-healing wounds remain a significant burden to patients, healthcare professionals, and the health care system. The hypoxic environment in such wounds is one of the major factors in the failure of cellular processes that normally repair damaged skin to form a scar. It has been suggested that human mesenchymal stromal cells (hMSCs) may be used to promote the healing of chronic wounds. However, the effect of hMSCs in hypoxia is unknown. This study aimed to develop an in vitro system to investigate the effect of hMSC-conditioned media (hMSC-CM) on wound closure by keratinocytes. Keratinocytes, the major cell component of the epidermis, play a significant role in dermal wound closure. Fetal bovine serum (FBS) has been reported to interfere with hMSC-derived products; therefore, the effect of serum concentration on keratinocyte proliferation and wound closure were first investigated. It was found that 1% v/v serum could be used with no adverse effects on keratinocyte proliferation. Keratinocytes (HaCaT) were grown to confluence and a scratch was used to create a model wound. The effect of a hypoxic environment (1% v/v O2 in the gas phase), in conjunction with hMSC-CM, on scratch wound closure was measured. The results suggest that hypoxia decreases wound closure rate by keratinocytes. Furthermore, hMSC-CM and 10 fold concentrated hMSC-CM increases wound closure rate under hypoxia by 18% and 36%, respectively. This suggests that hMSC-derived products could be beneficial in wound healing under prolonged hypoxia, such as in chronic wounds.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my advisor Dr. Francois Berthiaume for his guidance, continuous support of my study and research, and for his patience, motivation, and immense knowledge. I could not have imagined having a better advisor for this project.

I would also like to express my deepest appreciation to my graduate mentor Dr. Renea Faulknor for accepting me into the lab and for all her time and effort in mentoring me.

I am grateful to the following individuals: Dr. Nir Nativ, Mr. Aina Andrianarijaona, Dr. Melissa Przyborowski Olekson, Ms. Ileana Marrero - Berríos, Dr. Andrea Gray, Dr. Gabriel Yarmush, Ms. Paulina Krzyszczyk, and Dr. Serom Lee for their support, advice, and encouragement throughout the life of my research project and beyond.

Above all, I would like to thank all my family, my beloved husband and his family and everyone I know for loving, supporting, and encouraging me all through my life. Without them, I would not be where I am today.

The Garden State Louis Stokes Alliance for Minority Participation, the Norman and Ruth Feller Rosenberg Fellowship, the Douglass Residential College at Rutgers University, an Aresty award at Rutgers University, and The New Jersey Commission on Spinal Cord Research are all gratefully acknowledged for supporting this research.

DEDICATIONS

To all who contribute to making the world a better place to live.

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CHAPTER 1

INTRODUCTION

1.1. Motivation

Chronic wounds are an unsolved health care problem, which significantly affect a patient's quality of life. They challenge healthcare professionals and burden the health care system. Chronic wounds have a cost of care greater than $50 billion per year in the United States alone [1]. Common types of chronic wounds are arterial ulcers, venous ulcers, pressure ulcers, and diabetic foot ulcers. Diabetic wounds cause approximately 50,000 – 60,000 amputations each year in the USA [2]. The impaired healing of chronic wounds is pathologically complex, as it is often coupled with other health conditions such as diabetes, hypertension, and immobilization. Common characteristics of chronic wounds include persistent inflammation, hypoxia, and prolonged open wound [3].

A chronic wound, by definition, is a prolonged open wound in which re-epithelialization has stalled but remains hyper-proliferative, and as a result, is prone to infection, often taking three months or more to close which in turn slows the healing rate of the wound [3]. Keratinocytes form the epidermis and function as the barrier between the body and the environment [1]; thus, keratinocyte migration and proliferation are integral to the wound closure process. Common chronic wounds mentioned above stem from inadequate blood supply that leads to ischemia. This condition results in low oxygen tension in the wounds.

Chronic wound treatments include debridement, followed by cleansing, anti-inflammatory drugs if needed, and application of dressings to maintain moist wound

healing environment. Currently more novel treatments for chronic wounds such as vacuum assisted wound closure (VAC), scaffold or skin substitutes, topical oxygen therapy (TOT), and hyperbaric oxygen therapy (HBOT) are being utilized. Vacuum assisted wound closure uses negative pressure to remove wound fluid and locally increase blood circulation. Skin substitutes are used as scaffolds to provide temporary wound closure and promote tissue regeneration. Topical oxygen provides oxygen to the wound site to promote angiogenesis and decrease wound size [4]. HBOT, an FDA-approved treatment for chronic wounds, is known to provide oxygen to wounds and induce the formation of blood vessels [15]. These treatments, however, have variable clinical results, are expensive, and have adverse effects in some cases. VAC could cause uncontrollable bleeding under dressing, pain, and tissue death; while TOT can cause oxygen toxicity to tissue [15]. Skin substitutes are found to be only approximately 51% effective, are relatively expensive, and can cause wound exudate build up which further slows down the healing process [24]. HBOT has some side effects such as near-sightedness, seizures, trauma to lungs, and declination in cardiac function [16,17].

Prior studies have suggested that mesenchymal stromal cells (MSCs) secrete factors that promote wound healing [5,7,21]. One of the most common environments in which we find chronic wounds is hypoxia, low oxygen tension due to lack of vascularization in the wound [8]. Oxygen tension in chronic wounds is much lower compared to that of acute wounds, and has been measured to be 5-20 mmHg and 40 mmHg in chronic wounds and in acute wounds, respectively [18]. Hypoxia is known to decrease fibroblast-mediated contraction and α-Smooth Muscle Actin (α-SMA) expression in fibroblasts. MSCs were found to reverse the effect of hypoxia. MSCs

increase wound contraction by fibroblasts by increasing α-SMA expression under hypoxia [19]. It is unclear whether MSCs will be effective in promoting wound closure in chronic wounds under hypoxia. We hypothesize that MSCs secrete factors that enhance keratinocyte migration and proliferation under hypoxic conditions and therefore expedite wound closure. To explore this question, the in vitro system is set up where the effect of hypoxia and subsequently MSC-derived factors on wound closure by keratinocytes can be studied.

1.2 Project Scope

The primary focus of this project is to investigate the effect of MSC-derived factors in hMSC-conditioned media on wound closure under hypoxia using an i*n vitro* wound model, the *Scratch Assay*. In order to reduce variability of the keratinocytes and increase repeatability of the experiment, the HaCaT cell line is being utilized instead of primary cells [12]. Transforming growth factor-β (TGF-β) is known as one of MSC-secreted factors, but is also present in the fetal bovine serum (FBS) supplemented to the culture media for keratinocytes, Dulbecco's modified Eagle's medium (DMEM) [20-22]. Moreover, TGF-β has been found to increase HaCaT migration in a scratch assay [5]. Therefore, the serum concentration optimization was carried out to decrease the TGF-β background and allow the measurement of the effect of the MSC-secreted factors prior the wound closure study. Next, the wound closure in high serum (10% FBS) and low serum (1% FBS) under hypoxia and normoxia was examined. Finally, the effect of MSC-derived factors on keratinocyte wound closure under hypoxia was explored.

Wound closure of monolayer keratinocytes is propelled by cell migration and proliferation. Ridgway, et. al. investigated keratinocyte migration under hypoxia by seeding HaCaT in well plates and incubating overnight in either 1 ml of serum-free media (plastic control) or 1 ml of media containing human fibronectin (40 mg/ml). Then, a confluent monolayer of HaCaT cells was subjected to hypoxia (1% O_2) or normoxia (21% O_2) for 24 hours. Cell migration over time was measured using a scratch assay. The authors found cell migration increased under hypoxia compared to normoxia [9]. Another study found that hypoxia induced HaCaT cell migration compared with normoxia cells. It was also found that CD9 overexpression reversed the hypoxia-induced cell migration while CD9 silencing increased cell migration [9]. O'Toole, et. al. also conducted similar experiments to investigate keratinocyte migration on connective tissue. The effect of hypoxia on in vitro wound closure with and without collagen type I was investigated. The authors found that human neonatal keratinocytes cultured on collagen type I increased cellular migration under hypoxic conditions (0.2% and 2% O_2) compared with normoxic conditions (9% O_2 and 20% O_2) [11]. These studies were completed within 48 hours or less which emulates acute hypoxia, but not chronic hypoxia.

Chronic wounds are a type of wound, which does not heal and does not respond to standard treatments for at least thirty consecutive days [11]. The effect of prolonged hypoxic conditions on keratinocyte migration is unknown. Here, we first decreased the serum concentration so that it does not adversely affect cell proliferation. MSC secreted products have been reported to have therapeutic effects in wound healing and have been used as therapeutic cells in chronic wounds [7]. However, it has also been reported that the high serum (10% FBS) contains high levels of TGF-β1, which obscures the TGF-β1

secreted by the MSCs. Thus, decreasing serum is necessary to avoid the interference of serum when this system is used to investigate the effect MSC-derived products on keratinocyte migration. Once the serum was optimized the effect of hypoxia on wound closure by keratinocytes (HaCaT) was investigated.

CHAPTER 2

METHODS

2.1. Materials

2.1.1 Cells

Keratinocyte Cell Line (HaCaT)

The HaCaT cell line is derived from the spontaneous *in vitro* transformation of human keratinocytes from histologically normal adult skin. This cell line is immortalized but highly differentiated as indicated by specific and stable cytogenetic markers. Although it has a transformed phenotype in vitro, it is not tumorigenic and is noninvasive in vivo [12]. This cell line is used to represent the normal human epidermal keratinocyte because of its ease of manipulation *in vitro* over primary cells. The HaCaT cell line was purchased from AddexBio Technologies, San Diego, CA (Lot#0003798 Passage#14).

Human Mesenchymal Stromal Cells (hMSC)

Human bone marrow-derived MSCs were purchased from the Institute of Regenerative Medicine at Texas A&M and cultured as previously described [23,19]. Briefly, MSCs were cultured in alpha-minimal essential medium (α-MEM; Gibco), containing no deoxyribo- or ribo-nucleotides, supplemented with 10% v/v fetal bovine

serum (FBS; Atlanta Biologicals Flowery Branch, GA), 1% w/v penicillin-streptomycin (pen-strep; Life Technologies), 4 mM L-glutamine (Life Technologies), and 1 ng/ml basic fibroblast growth factor (bFGF; Life Technologies)[19]. hMSC passage 2-3 were cultured and passaged when the cells reached approximately 70% confluence and only hMSC passage 4 was used to prepared hMSC-CM for subsequent experiments.

2.1.2 Media

Dulbecco's modified Eagle's medium (DMEM; Gibco) was supplemented with Fetal Bovine Serum (FBS). DMEM + 10% v/v FBS and 1% v/v Pen/Strep (100 U/ml penicillin + 100 mg/ml streptomycin) was used to maintain the HaCaT cell line and used as 10%FBS media during experimentation. The different percentages of FBS (10%, 5%, 1%, and 0.01%) were prepared as follows:

Type of media	DMEM volume	FBS volume	Pen/Strep volume
10% FBS	445mL	50mL	5mL
5% FBS	470mL	25mL	5mL
1% FBS	490mL	5mL	5mL
0.1%FBS	494.5mL	0.5mL	5mL
Serum free	495mL	0.0mL	5mL

Table 1. Example calculation for preparation of various percentages of serum

These DMEM with different percentages of FBS were used for media optimization and the 10% FBS and 1% FBS media was used for subsequent experiments. α-MEM (α-MEM; Gibco) media was supplemented with premium select 10% FBS and 1% P/S was used for MSC culture.

MSC-CM Preparation

hMSCs P4 were seeded in α-MEM media at 1.33×10^3 cells/cm^2. At day 5 the media was removed and washed once with 1% FBS DMEM. MSC-CM was collected from 3.4×10^3 cells/cm^2 after 48 hours of incubation in 1% FBS DMEM.

2.1.3 Materials and Equipment

- 24-well plates (Falcon®, VWR)
- 10 µL plastic pipette tips
- Amicon® Ultra centrifugal filter Devices (3K Dalton MW cut off)
- Incubator with 21% O2, and 5% CO2 atmosphere at 37˚C
- Galaxy 14S Hypoxic chamber (New Brunswick, Eppendorf Company) automatically maintains 1% O2 and 5% CO2 at 37 ˚C
- AlamarBlue® (Life Technologies, Norwalk, CT)
- Fluorescence plate reader (set to 535 nm excitation/625 nm emission)
- Confocal Microscope with Olympus CKX31 camera and Magnifier Software

2.2. Experimental Design

2.2.1. Serum concentration optimization in culture media

In order to mitigate the interference due to serum in the culture medium on the effect of MSCs, the fraction of fetal bovine serum (FBS) was optimized. The keratinocyte HaCaT line was seeded in 24 well plates at $5x10^4$ cells/well in 500 µL/well of Dulbecco's modified Eagle's medium (DMEM; Gibco) + 10% v/v FBS and 1% v/v Pen/Strep (100 U/ml penicillin + 100 mg/ml streptomycin) for 24 hours in a 5% CO2 atmosphere at 37˚C. The medium was then switched to DMEM+10%, 5%, 1%, or 0.1% v/v FBS and incubated for 24 hours, 48 hours and 72 hours. The medium was then replaced to serum-free DMEM with 10% Alamar Blue® in total solution of 550 µL/well and the fluorescence (535 nm excitation/625 nm emission) measured after 4 hours of

incubation in a plate reader. The data were normalized to the average of the initial reading of each group.

Cell Proliferation Assay

Alamar Blue was used to estimate cell numbers during serum optimization. Alamar Blue® is a fluorescence-based method to noninvasively measure cell numbers. It is based on the fact that viable cells perform this reaction:

Resazurin + e- → **Resorufin**
(Non-fluorescent) (Fluorescent)

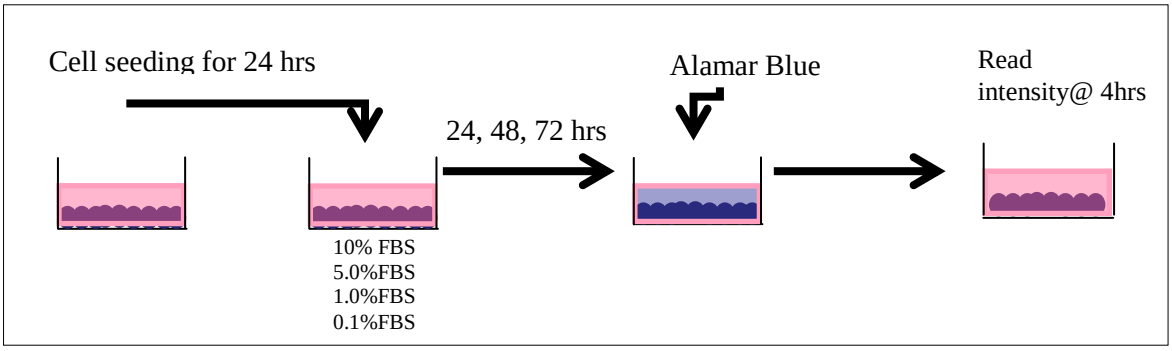


Figure 1. Cell proliferation assay using Alamar Blue to estimate cell numbers

2.2.2 The Effect of low serum on wound closure by Keratinocytes

A scratch assay similar to that described by Räsänen et. al. was set up to assess wound closure [6]. HaCaT cells were seeded in 24 well plates at 1.25×10^5 cells/cm^2 in 500 µL of DMEM media and grown until confluence. The old media was removed and replaced with 500 µL PBS/well. A 10-µL pipette tip was used to produce a scratch of uniform width across the cell monolayer thus creating an in vitro wound model (Figure 2). After wounding, cells were washed twice with PBS to remove cell debris, the medium was replaced with DMEM+1% FBS, and was cultured under hypoxia, in a 1%

O_2 chamber (5% CO_2, balance with nitrogen) from that point on. Images of the individual scratches were taken immediately after wound initiation and every 72 hours thereafter until day 9 with an Olympus CKX31 camera and Magnifier Software. Controls consisted of HaCaT monolayers cultured in regular DMEM with 1% v/v FBS under normoxia (21% O2). The wound area was measured using the NIH ImageJ software. Using the conversion factor of 960.00 pixel/mm (for 10X images) and 375.62 pixel/mm (for 4X images), the areas of the wounds were converted to mm^2. The measurement of the percent wound closure was calculated by subtracting the ratio of day 3, 6, or 9 to the area of day 0 from one hundred percent as shown in Figure 3.

In Vitro "Wound" (Scratch) Assay

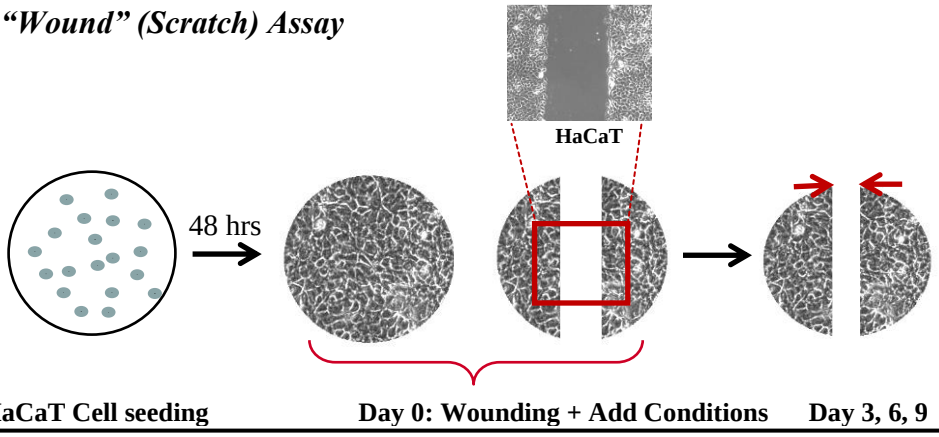
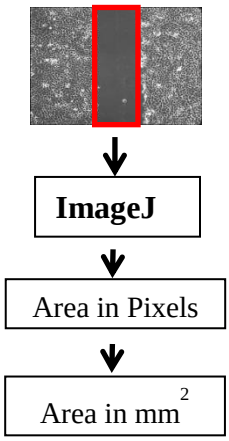


Figure 2. In vitro "wound" scratch assay to measure keratinocyte migration



$$\textbf{\% Wound Closure (\%)} = 100 - [(\frac{Wn}{W0}) * 100]$$

Where A_n is wound area of day n
A_0 is wound area at day 0

Figure 3. The measurement of wound width and percent wound closure from images.

2.2.3 The Effect of MSC-derived factors on wound closure by Keratinocytes

This experiment was conducted using the same protocol as described in 2.2.2 except the four groups being investigated are Normoxia (21%v/v O_2) with or without MSC-conditioned media (MSC-CM) and Hypoxia (1%v/v O_2) with or without MSC-conditioned media as shown in the table below.

	Normoxia	**Hypoxia**
Oxygen	21%v/v	1%v/v
Treatment	With or without MSC-CM	With or without MSC-CM

Table2: Experimental design for the effect of MSC-derived factors on wound closure

2.3 Data Analysis

Data shown are mean ±standard error of the mean (SEM) of N=3 or more replicates. P-value < 0.05 is considered statistically significant. Statistical analysis was evaluated using One-Way ANOVA or the Student t-test whenever appropriate.

CHAPTER 3

RESULTS

3.1 Serum Concentration Optimization in Culture Media

HaCaT cells cultured in DMEM with 1% to 10% FBS exhibited no significant difference in cell proliferation. However, the cell number in 0.1% FBS was clearly lower after 3 days incubation and there was a trend towards lower proliferation compared to those in 10% FBS, although the difference was not statistically significant (Figure 4).

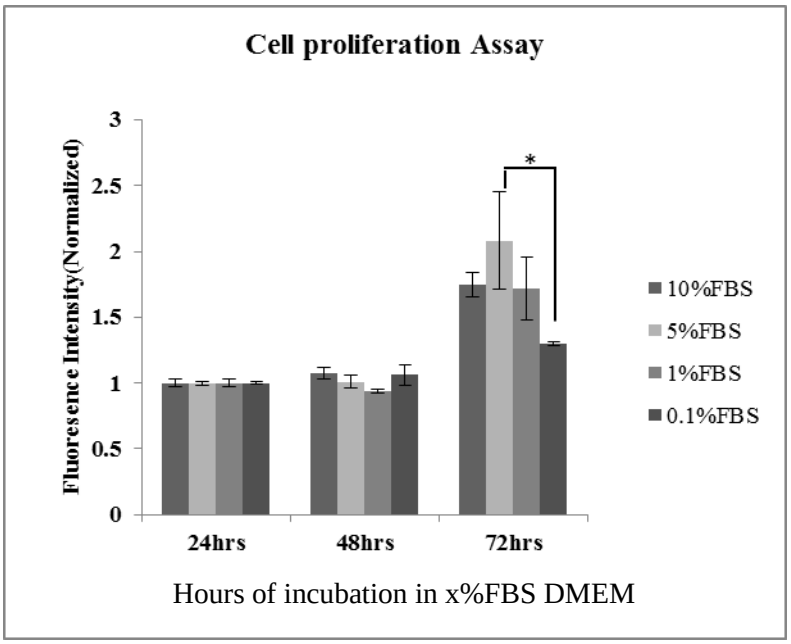


Figure4. HaCaT proliferation as a function of serum concentration. Relative cell number was estimated by the Alamar Blue assay at 4 h incubation with the dye. Data shown are normalized fluorescence intensities by the initial intensity expressed as mean ±standard error of the mean (SEM). N=3. * means $p<0.05$.

3.2 The Effect of low serum on wound closure by Keratinocytes

To evaluate whether low serum affect wound closure by keratinocytes under normoxia or hypoxia, a series of scratch assays were carried out under normoxia or hypoxia using 10%FBS(high serum) or 1%FBS(low serum) DMEM.

Under normoxia, HaCaT cells were seeded in 24 well plates with either high serum media or low serum grown until confluence. A scratch of uniform width across the cell monolayer was created. After wounding, cells were washed twice with PBS to remove cell debris. The medium was then replaced with either high serum or low serum DMEM from that point on. Images of the individual scratches were taken immediately after wound initiation and every 72 hours thereafter until day 9. The wound area was measured using the NIH ImageJ software. The measurement of the percent wound closure was calculated by subtracting the ratio of day 3, 6, or 9 to the area of day 0 from one hundred percent. The results shown in figure 5A demonstrated that low serum decreases wound closure rate in normoxia compared to high serum. This decrease is significant on day 9. The mean percent wound closure was 99% and 83% for the high serum, low serum respectively.

Similarly, scratch assays under hypoxia showed no difference in wound closure using low serum or high serum. The mean percent wound closure was 61% and 69% for the high serum and low serum respectively, as shown in figure 5B.

Together, these results showed that low serum decrease wound closure by keratinocytes under normoxia but this effect was not observed in hypoxia.

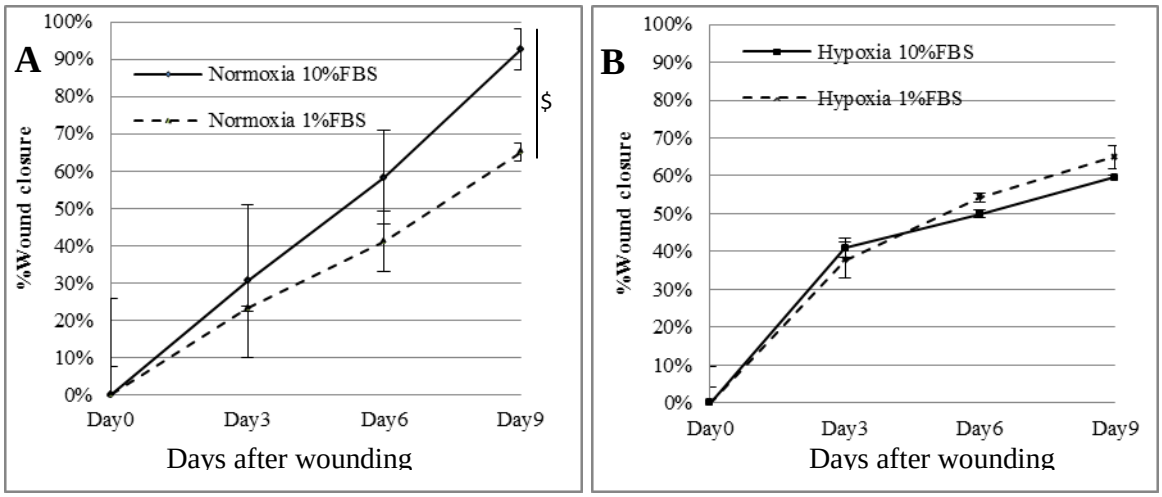


Figure 5. the effect of low serum on wound closure under normoxia(A) and The effect of serum concentration on wound closure under hypoxia (B)
Percent wound closure normalized by area of D0 in 10%FBS and 1%FBS concentration (A). Percent wound closure normalized by area of D0 in 1%FBS serum DMEM under hypoxia and Normoxia (B). Each data point is the mean ±SEM of N=3 replicate samples. $ means $p<0.001$

3.3 The Effect of MSC-derived factors on wound closure by Keratinocytes

3.3.1 The Effect of MSC-CM on wound closure by Keratinocytes

The representative images of the results showing that the MSC-derived factors increased wound closure by keratinocytes under both normoxia and hypoxia are shown in Figure 6. Top two rows of images in figure 6 show that MSC-derived factors increase wound closure more apparent during day 6 and day 9 under nomoxia. Bottom two rows of the images show that the wound closure enhanced by MSC-derived factors even under hypoxia. Together, all four rows of images show that the normoxia MSC-CM treatment group has the highest percent wound closure, followed by the normoxia control, the hypoxia MSC-CM treatment, and the hypoxia control group, respectively. It is noteworthy that the morphology of cells in the hypoxia control group appeared rounded

and are present in aggregates, whereas cells in the normoxia groups are more spread out. However, the use of MSC-CM on hypoxic cells seemed to reduce the presence of rounded cell aggregates, promoted a spread out cell morphology thus restoring the morphology towards that observed in the normoxic group.

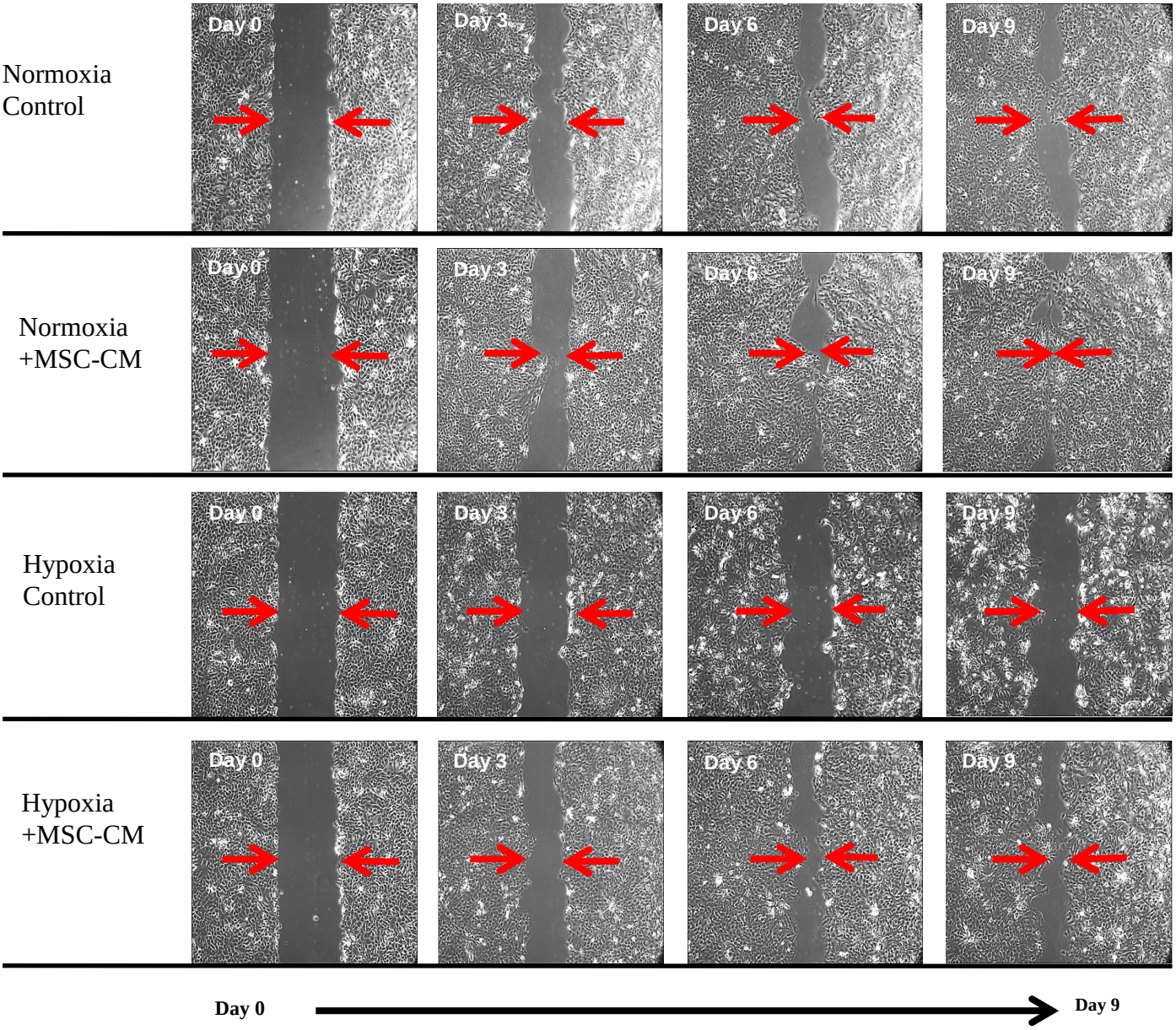


Figure 6. Representation images of wound closure under normoxia and hypoxia in1% serum concentration, with or without hMSC-CM at Day 0, 3,6, 9 after wounding.

The quantitative results confirmed that the normoxia MSC-CM treatment group has the highest percent wound closure, followed by the normoxia control, the hypoxia MSC-CM treatment, and the hypoxia control group, respectively. The wound closure in the hypoxia group showed slower wound closure than the nomoxic group for both the control and treatment group with hMSC-CM. The percent wound closure at day 9 were 80%, 68%, 50%, and 32%for the normoxia with hMSC-CM, normoxia control, hypoxia with hMSC-CM, and hypoxia control respectively, as shown in figure 7.

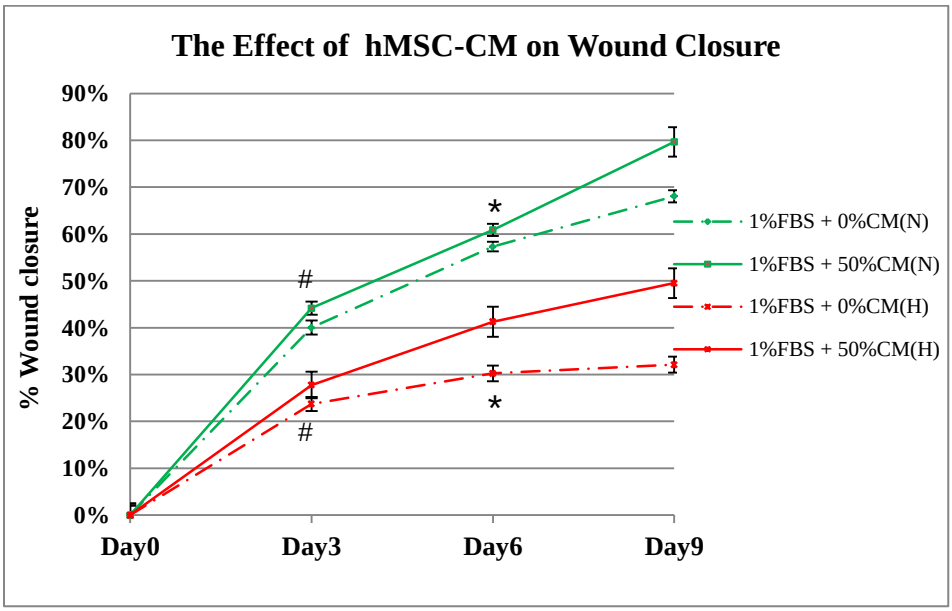


Figure 7. The effect of hMSC-derived factors on wound closure by keratinocytes(HaCaT) : Quantitative results, %Wound Closure at Day0,3,6,9 after wounding. Mean±SEM n=3 for normoxia, n=6 for hypoxia , # means $p<0.05$, *means $p<0.01$

The results showed a trend that a mixture of MSC-CM and fresh media at 1:1 ratio maybe an optimum concentration for enhancing the wound closure by keratinocytes. Comparing the mixture of MSC-CM and fresh media of 1:1 (50%CM) and 3:1(75%CM)

with the control, the 1:1 ratio has slightly higher percent wound closure than 3:1 ratio, and control respectively as shown in figure 8.

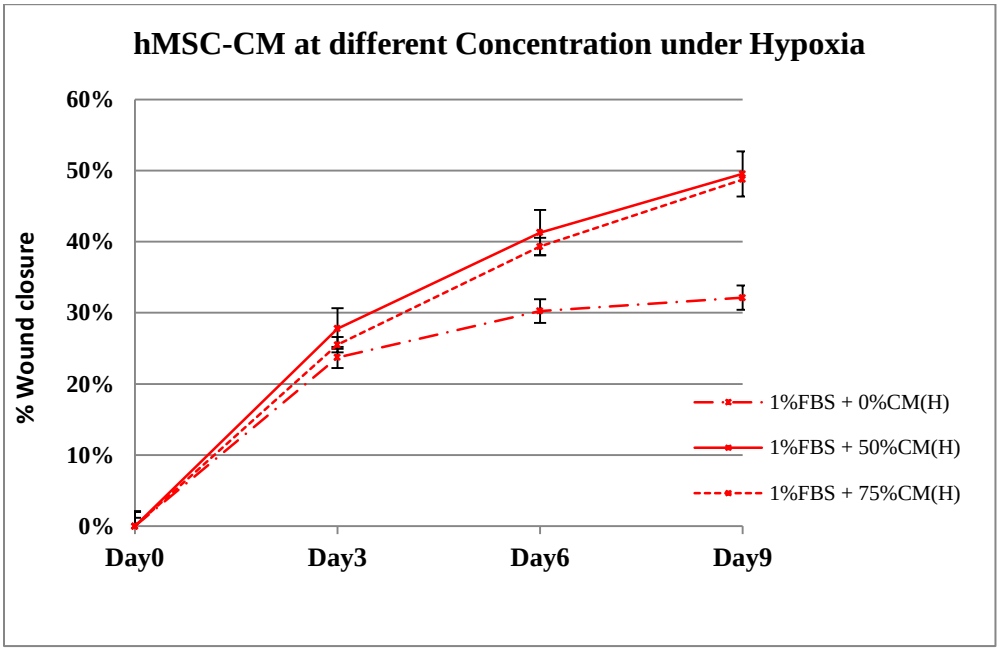


Figure 8. The effect of MSC-derived factors on wound closure by keratinocytes(HaCaT) at different concentrations: Quantitative results, %Wound Closure at Day0,3,6,9 after wounding. Mean±SEM n=3 for normoxia, n=6 for hypoxia 0%CM vs 75%CM Day 9=>p=0.06

3.3.2 The Effect of Concentrated hMSC-CM (cMSC-CM)on wound closure by Keratinocytes

Based on the hypothesis that MSCs secrete factors that enhance keratinocyte migration and proliferation under hypoxia, which in turn, enhanced wound closure, it would be reasonable to expect a more prominent wound closure enhancing effect to be greater when MSC-CM is concentrated. To test this, the experiment was conducted using concentrated MSC-CM. The experimental design is shown in the table below.

This experiment followed the protocol shown in Figure 2 and the condition being compared with the control was cMSC-CM.

The representative images in figure 9 show that the cMSC-CM enhanced wound closure in both normoxia and hypoxia. In normoxic group, the wound closed as soon as day 3 for the cMSC-CM treatment group while the wound still open as later as day 9 for the normoxic control. Similarly, in hypoxic group, the wound closed as soon as day 6 for the cMSC-CM treatment group while the wound stay open as later as day 9 for the hypoxic control.

	Normoxia	Hypoxia
Oxygen	21%v/v	1%v/v
Treatment	With or without cMSC-CM	With or without cMSC-CM

Table3: Experimental design for the effect of c MSC-derived factors on wound closure

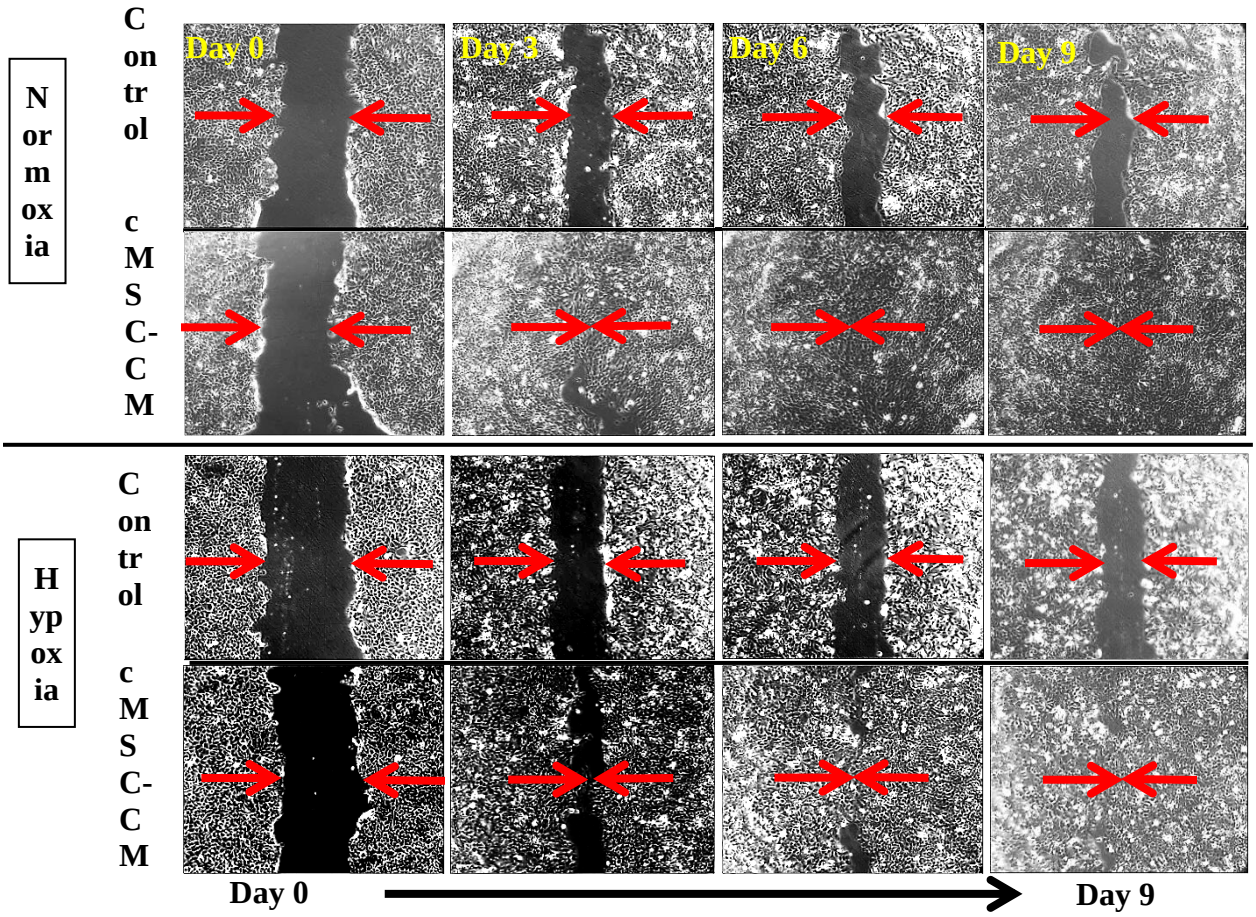


Figure 9. Representation images of wound closure under normoxia and hypoxia in1% serum concentration, with or without concentrated hMSC-CM at Day 0, 3,6, 9 after wounding.

Quantitative results in figure 10 show that concentrated hMSC-CM significantly accelerate wound closure by keratinocytes under both normoxia and hypoxia. The percent wound closure at day 6 was found to be 69%, 99%,54%, 90% for normoxia control, normoxia with cMSC-CM, hypoxia control, and hypoxia with cMSC-CM respectively. It is likely that this result is likely to be due to the higher concentration of MSC-derived factors after it was concentrated. In addition, using cMSC-CM as a treatment allows similar amount of fresh media in each group which may have contributed to the accelerate closure compared with the 50%CM group as shown in figure 7.

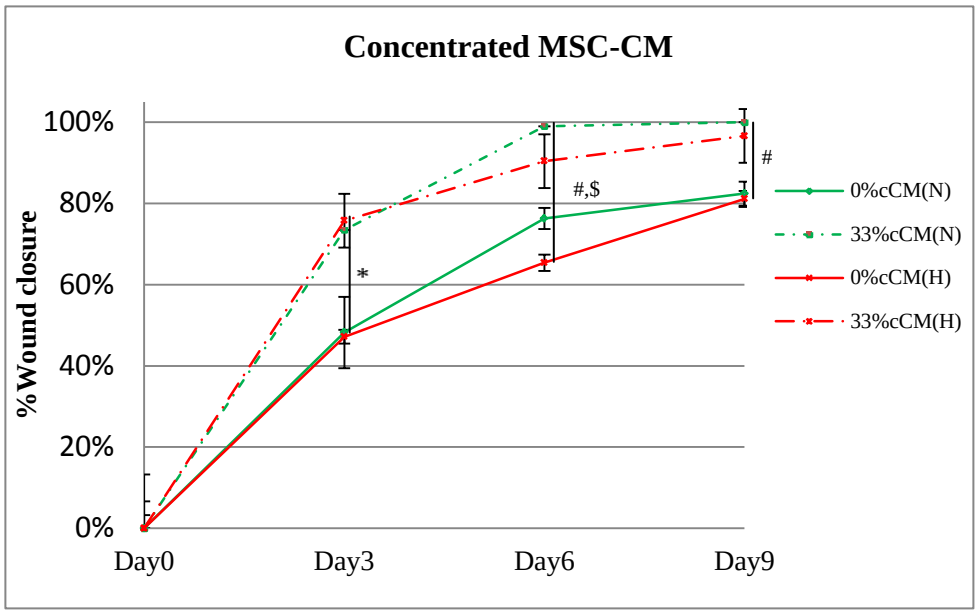


Figure 10. The effect of concentrated MSC-CM on wound closure by keratinocytes(HaCaT) : Quantitative results, %Wound Closure at Day0,3,6,9 after wounding. Mean±SEM n=3 for normoxia, n=6 for hypoxia* means $p<0.05$, #means $p<0.01$, $means $p<0.001$

The effect of hypoxia on keratinocyte morphology was also observed. Included in figure 11 are images from taken at day 9 of an actual experiment showing the difference in cell shapes of the cells from hypoxia control (Figure 11 A), normoxia

control (Figure 11C), and the hypoxia hMSC-CM treated (Figure 11B) groups. Hypoxia control appeared to be elongated shape compared with normoxia control and MSC-CM treated group appear to more similar to the normoxia group than that of hypoxia control. A number of dead cells were visually observed to be higher in the hypoxia control compared with the normoxia and the MSC-CM treatment groups.

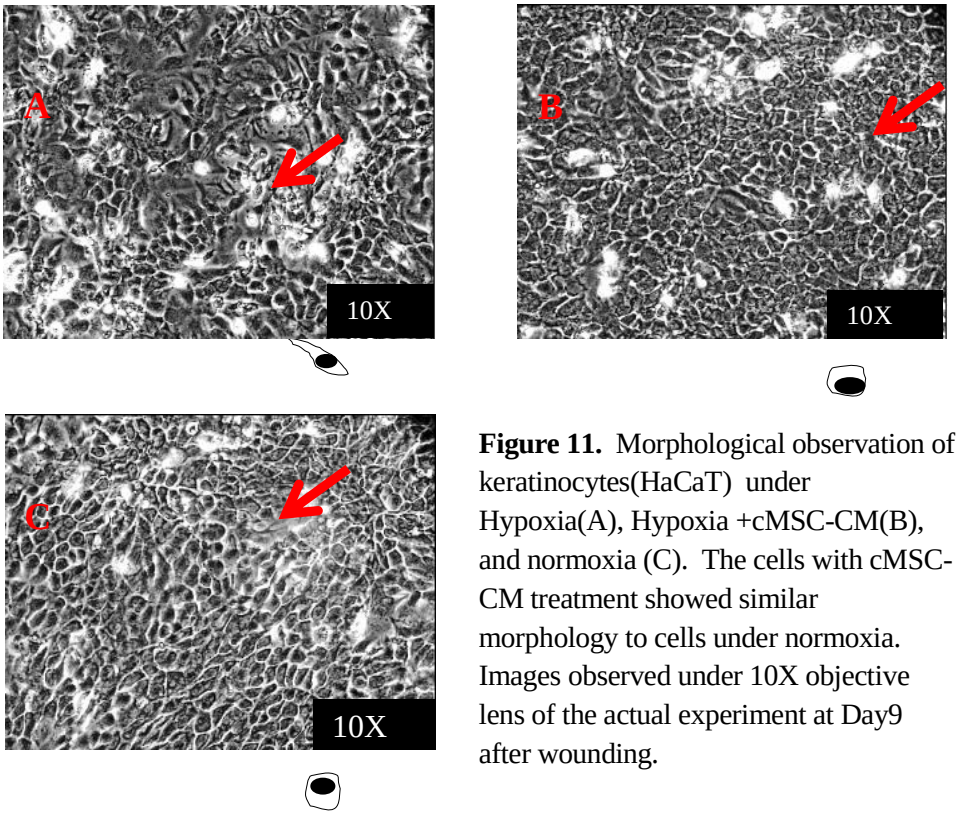


Figure 11. Morphological observation of keratinocytes(HaCaT) under Hypoxia(A), Hypoxia +cMSC-CM(B), and normoxia (C). The cells with cMSC-CM treatment showed similar morphology to cells under normoxia. Images observed under 10X objective lens of the actual experiment at Day9 after wounding.

To confirm this observation another study was conducted. Keratinocytes were seeded at 1.25×10^3 cells/cm^2 and were plated without MSC-CM treatments or scratch and grown under normoxia or hypoxia. The images were captured at day 9. The morphology of the keratinocytes at D0 of normoxic group(figure 12E) and D0 of hypoxic group(figure 12F) are very similar. However, at day 9, the morphology of the normoxic group(figure 12G) and hypoxic group(figure 12H) are very distinct. Further study is

needed to gain more information as to what might have caused these different morphologies and a possible relationship between these morphologies and decreased wound closure in hypoxia group.

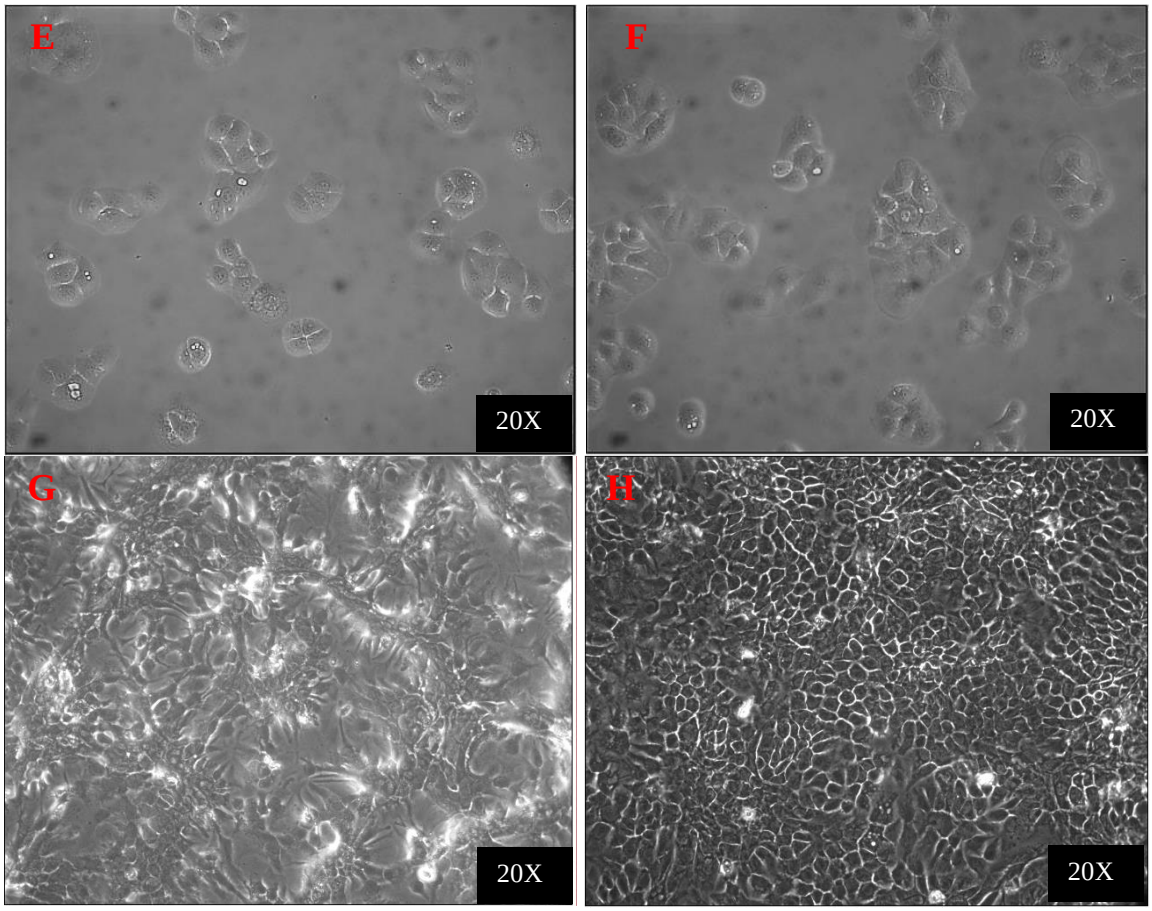


Figure 12. Morphological observation of keratinocytes (HaCaT) started at D0(A&B) with similar morphology. At day 9(G&H) cell morphology observed in normoxia and hypoxia group were different. Images observed under 20X objective lens at Day9 after wounding.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Discussion

The results suggest that hMSC-derived products could be beneficial in aiding wound healing under prolonged hypoxia such as occurs in chronic wounds.

Chronic wounds are an unsolved healthcare problem for which effective treatment options have not yet been identified. Tissue hypoxia, a common denominator among major types of chronic wounds, has been identified as a significant factor in delaying the healing of chronic wounds. This study established an in vitro system to explore the therapeutic potential of hMSC-derived products in wound healing under prolonged hypoxia. First, the serum concentration was optimized to reduce the interference of growth factors from media with the effect of hMSC-derived products. The results suggest that migration and proliferation occur in medium with 1% v/v FBS and that this low serum condition can be used to investigate wound closure by keratinocytes (HaCaT) under hypoxia. Then the effect of hMSC-derived products on wound closure by HaCaT was examined. It was found that hMSC-CM and concentrated hMSC-CM enhanced wound closure by keratinocyte under prolonged hypoxia.

A prior study on keratinocyte migration on collagen type I using an *in vitro* wound assay allowing keratinocytes to migrate for 16 hours under normoxic and hypoxic conditions suggested hypoxia increased cell migration [25]. However, here the keratinocytes (HaCaT) were exposed to prolonged hypoxia (9 days.) The results showed that under normoxia, low serum (1% FBS) decreases wound closure compared to high

serum (10% FBS.) However, the decrease in wound closure observed when moving from a serum concentration of 10% FBS (high serum) to a serum concentration of 1% FBS (low serum) was not observed in the hypoxic groups. This suggests that reducing serum concentration reduces would closure under normoxia but had no effect under hypoxia. Using high serum media to investigate wound closure under normoxia and hypoxia may introduce variability to the experiment by design. Therefore, using low serum media mitigates the interference of existing growth factors in FBS and lessens this variability.

These high serum conditions may result in an initial advantage of increased wound closure under normoxia versus hypoxia even without CM treatment; therefore, low serum condition was chosen for following studies in order to initiate normoxic/hypoxic wound closure at similar baselines. Here, wound closure by keratinocytes (HaCaT) was investigated using low serum media under normoxia and hypoxia. The data suggest that wound closure decreased under hypoxia using low serum media. The results demonstrated that the hypoxic condition in combination with serum concentrations alters keratinocyte migration, which in turn alters the wound healing process in cultured HaCaT monolayers. Prior research found that pre-conditioning keratinocytes in hypoxia for 12 hours decreases keratinocyte migration and it was predicted that prolonged exposure to hypoxia would delay wound closure [25]. The experimental results of this study are consistent with the prediction.

This *in vitro* model was designed to resemble chronic wounds by decreasing serum amount and prolonged exposure to hypoxia, which is more similar to real world clinical conditions. MSCs are known to have therapeutic effects in wound healing [7], as they have been used to aid the healing of burn wounds [6]. We have demonstrated that

this *in vitro* wound model can be used to measure wound closure using monolayer keratinocytes (HaCaT) under hypoxia. The effect of MSC-derived products on wound closure by keratinocytes was examined; in vitro under hypoxia without the interference of high serum. The results suggested hypoxia decreases wound closure by keratinocytes in low serum compared to normoxia and that that MSC-CM enhanced wound closure by keratinocytes. Based on the hypothesis that MSCs secreted factors affect keratinocyte migration and proliferation by increasing would closure rates; it was expected that concentrating MSC-CM would result in a prominent increase in wound closure rates by keratinocytes. As expected, cMSC-CM significantly increased would closure rates by keratinocytes even when using small amounts. This finding exemplifies the therapeutic effect of MSC-secreted factors, particularly in would closure under hypoxia.

For more accurate measuring of wound closure using this system, a few factors may be considered. One, the media could be pre-conditioned in hypoxia and the experiments may be conducted in a glove box hypoxic chamber [25]. This will mitigate the possibility of oxygen exposure during media change and imaging, and prevent the experiment from being affected by unwanted oxygen exposure in the hypoxic conditions. Two, culture flask coating material such as fibronectin, collagen type I may be used to mimic wound's nature microenvironment [10, 26]. Three, the age of the cell donor or number of passages may be contributing factors in wound closures. A study on human keratinocyte migration in which cells isolated from healthy elderly donors and healthy young donors were exposed to normoxia and hypoxia for 20 hours after which gold salt migration assays were used to observe individual cell tracks. The results of this study

concluded that the aged cells showed a significant decrease in cell migration [11]. The effect of cell passage on wound closure was not investigated in this study.

Conditioned media from other cell types could be compared with hMSC-CM and concentrated low serum media control with cMSC-CM, which was not included in this study. Therefore, it may be possible that other cell types could possess derived products that may alter wound closure. Additionally, serum in cMSC-CM may have been concentrated, which may have accelerated wound closure compared to the non-concentrated media controls.

4.2 Conclusion

This study demonstrated that low serum (1% FBS) does not affect cell proliferation but does decrease the wound closure rate under normoxia compared to high serum (10% FBS). Hypoxia affected the dynamics of wound closure in an *in vitro* assay. Low serum has no effect on wound closure by keratinocytes under hypoxia. Using low serum media to examine the effect of hMSC-CM on would closure, it was found that hypoxia decreases wound closure by keratinocytes compared to normoixia. Also, hMSC-CM shows a strong trend to enhance wound closure under hypoxia and normoxia. Concentrated hMSC-CM (cMSC-CM) significantly enhanced would closure by keratinocytes under hypoxia and normoxia. The ratio of hMSC-CM and fresh media was varied to investigate if there was an optimum hMSC-CM to fresh media ratio and it was found that 1:1 ratio was the best performance of the groups tested. When hMSC-CM was concentrated, 1:3 hMSC-CM to fresh media ratio was found to be the best formula.

4.3 Future Work

In order to provide more detailed explanations of how hMSC-CM enhanced wound closure by keratinocytes, further study is needed. It would be helpful to investigate the effects of hypoxia and MSC-CM on cell proliferation using LIVE/DEAD staining or similar viability assays to identify cell viability during the wound closure process under prolonged exposure to hypoxia. Future investigation on possible mechanisms could also be examined. The immunomodulatory role of hMSCs has been linked to their ability to secrete various trophic factors including TGF-β, and this factor has been reported to play a role in keratinocyte migration and proliferation [23,27,28]. Cells of older donors showed slower migration than those from young donors. It was also found that there is a difference in induction of TGF-β receptor expression by hypoxia in keratinocytes isolated from young and aged donors [30]. This suggests the slower migration of aged cells may be mediated by TGF-β. Examining the pathways involving the effect of hMSC-CM recovering wound closure under hypoxia could also yield the mode of action of hMSC-CM. TGF-β has been suggested as a potential mediator in the wound healing effect of hMSC-CM.

Another factor that may play a role in prolonging non-healing chronic wounds is oxidative stress. Oxidative stress is a consequence of an imbalance in the prooxidant-antioxidant homeostasis in chronic wounds, which adversely affects the progress of wound healing [29]. Increasing evidence in *in vivo* and *in vitro* studies suggesting that in the hostile microenvironment of a chronic wound, reactive oxygen species (ROS) play a prominent role in the pathogenesis and pathophysiology of a chronic wound [29]. This suggests that maintaining balance of ROS could be another path for wound healing

progress recovery. MSC-CM has been reported to decrease ROS in keratinocytes exposed to LPS & high glucose [13]. Also, hypoxia is known to increase ROS. It is possible that MSC-CM reduces the levels of ROS and therefore accelerates wound healing. Another interesting aspect of the effect of hypoxia on wound closure is the metabolic adaptation through increased HIF-1α expression and anaerobic metabolism. One could measure HIF-1α in parallel with an experiment similar to that described in section 3.3 to evaluate the level of HIF-1α profiles and determine whether that metabolic adaptation exists. A prior study demonstrated that Rat-MSC-CM and N-acetyl-L-cysteine (NAC) decreased the level of reactive oxygen species (ROS) in treated keratinocytes. This implied that MSC-CM was acting via the ERK signaling pathway and 2',7'-dichlorofluorescin diacetate (DCFH-DA) fluorescent staining method was used to determine whether high glucose (HG) and lipopolysaccharide (LPS) were able to induce ROS generation in keratinocytes in rats[14]. Similarly, this approach could be used to evaluate the ROS level change in hypoxic group with or without hMSC-CM treatment compared to normoxia control.

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APPENDIX I: DATA

3.2 The Effect of low serum on wound closure by Keratinocytes

	Normoxia 10%FBS				Hypoxia 10%FBS			
	Area mm^2	**Mean mm^2**	**%Closed**	**SEM**	**Area mm^2**	**Mean mm^2**	**%Closed**	**SEM**
Day0	0.656				0.375			
	1.007	1.07	0%	0.26	0.42	0.44	0%	0.04
	1.537				0.517			
Day3	0.39				0.236			
	0.733	0.74	31%	0.21	0.231	0.26	41%	0.02
	1.101				0.308			
Day6	0.201				0.227			
	0.616	0.45	58%	0.13	0.229	0.22	50%	0.01
	0.518				0.201			
Day9	0.053				0.181			
	0.186	0.08	93%	0.06	0.168	0.18	60%	0
	Normoxia 1%FBS				**Hypoxia1%FBS**			
Day0	1.023				0.51			
	0.894	1.02	0%	0.08	0.629	0.48	0%	0.1
	1.156				0.302			
Day3	0.799				0.326			
	0.776	0.79	23%	0.01	0.363	0.3	38%	0.05
	0.786				0.208			
Day6	0.755				0.206			
	0.574	0.6	41%	0.08	0.245	0.22	54%	0.01
	0.482				0.208			
Day9	0.311				0.106			
	0.383	0.36	65%	0.02	0.196	0.17	65%	0.03
	0.383				0.202			

3.3 The Effect of MSC-derived factors on wound closure by Keratinocytes

		Normoxia				Hypoxia			
		Area mm^2	Mean mm^2	SEM	%Closed	Area mm^2	Mean mm^2	SEM	%Closed
Day0	1%FBS	0.367 0.371 0.389	0.376	0.007	0%	0.370 0.351 0.405	0.375	0.020	0%
	50%CM	0.428 0.388 0.340	0.385	0.025	0%	0.332 0.362 0.433	0.376	0.021	0%
	75%CM	0.361 0.349 0.392	0.367	0.013	0%	0.394 0.360 0.362	0.372	0.009	0%
Day3	1%FBS	0.199 0.251 0.226	0.225	0.015	40%	0.271 0.272 0.316	0.286	0.015	24%
	50%CM	0.211 0.241 0.193	0.215	0.014	44%	0.236 0.240 0.338	0.271	0.029	28%
	75%CM	0.157 0.194 0.203	0.185	0.014	50%	0.286 0.267 0.278	0.277	0.012	26%
Day6	1%FBS	0.149 0.181 0.151	0.160	0.010	57%	0.249 0.241 0.296	0.262	0.017	30%
	50%CM	0.153 0.172 0.127	0.151	0.013	61%	0.188 0.177 0.297	0.221	0.032	41%
	75%CM	0.112 0.125 0.150	0.119	0.005	68%	0.232 0.222 0.223	0.226	0.011	39%
Day9	1%FBS	0.116 0.144 0.100	0.120	0.013	68%	0.239 0.235 0.291	0.255	0.017	32%
	50%CM	0.035 0.140 0.060	0.078	0.032	80%	0.147 0.153 0.269	0.190	0.032	50%
	75%CM	0.089 0.098 0.110	0.099	0.006	74%	0.186 0.196 0.191	0.191	0.012	49%

3.3.2. The Effect of concentrated MSC-conditioned media on wound closure by Keratinocytes

		Normoxia				Hypoxia			
		Area mm^2	Mean mm^2	SEM	%Closed	Area mm^2	Mean mm^2	SEM	%Closed
Day0	0%cCM(N)	0.865 0.641 0.407	0.638	0.13	0%	0.483 0.384 0.479	0.449	0.03	0%
	33%cCM(N)	0.766 0.460 0.737	0.654	0.10	0%	0.423 0.408 0.564	0.465	0.05	0%
Day3	0%cCM(N)	0.505 0.289 0.211	0.335	0.09	47%	0.225 0.214 0.269	0.236	0.02	47%
	33%cCM(N)	0.276 0.038 0.260	0.191	0.08	71%	0.101 0.069 0.180	0.117	0.03	75%
Day6	0%cCM(N)	0.251 0.166 0.184	0.200	0.03	69%	0.204 0.175 0.245	0.208	0.02	54%
	33%cCM(N)	0.000 0.000 0.022	0.007	0.01	99%	0.030 0.023 0.090	0.048	0.02	90%
Day9	0%cCM(N)	0.044 0.077 0.145	0.089	0.03	86%	0.181 0.159 0.226	0.189	0.02	58%
	33%cCM(N)	0.000 0.000 0.000	0.000	0.00	100%	0.013 0.046 0.042	0.034	0.01	93%

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