University of Reading

EXPLORING EFFECTIVE STRATEGIES TO IMPROVE WOUND CARE

By

KAWA A. OBEID

A thesis submitted to the University of Reading/ School of Pharmacy in partial fulfillment for the degree of

DOCTOR OF PHILOSOPHY

December 2016

Declaration

I, Kawa A. Obeid, confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged

……

Kawa A. Obeid

Abstract

Chronic wounds are those that fail to proceed through an orderly and timely reparative manner, typically characterised by persistent hypoxia and infection. Since oxygen is essential in every stage of wound healing and has bactericidal activity, wound oxygenation methods including hyperbaric oxygen (HBO) and normobaric oxygen (NBO) have been proposed as therapeutic modalities in the treatment of chronic wounds. These treatments can significantly elevate the oxygen partial pressure at poorly perfused wounded tissues. However, the molecular mechanisms whereby oxygen improves wound healing remain unclear.

The aims of this thesis were to explore the cellular and molecular impacts of high oxygen levels, elevated air pressure, chronic hypoxia and infection on re-epithelialisation as a critical step in wound healing. It was hypothesised that increasing oxygen tension in the wounded area would improve steps in re-epithelialisation process. This hypothesis was tested on human keratinocyte (HaCaT) cells where cell migration, proliferation and differentiation were examined at cellular and molecular levels using scratch assays, Western blotting, ELISA and MTT assays.

Data clearly demonstrated that a chronic hypoxic state had a deleterious effect on re-epithelialisation via attenuation of the rate of keratinocyte migration with increases in cellular adhesion, metabolic activities and hyperproliferation but no effects were seen on cell differentiation rates. In contrast, oxygenation of the cells via HBO and NBO resulted in a faster rate of cell migration with lower cell proliferation and metabolic

activities. However, elevating air pressure alone i.e. hyperbaric air (HBA), did not have any effects on the re-epithelialisation process.

Keratinocytes were stimulated with LPS and interleukins (IL-6 and IL-8) or co-cultured with THP-1 monocytes to create different inflammatory models under chronic hypoxic and normoxic conditions. Data revealed that IL-6, but not IL-8, attenuated keratinocyte's migration with a significant increase in cell adhesion and elasticity. Co-culture of monocytes with keratinocytes led to a marginal increase in cell migration under chronic hypoxic conditions. However, LPS stimulation did not lead to any significant effects on migration, proliferation and differentiation of keratinocytes.

These findings show that oxygen is critical in re-epithelialisation and the results presented here provide understanding of the cellular and molecular mechanisms by which HBO and NBO improve wound healing.

Table of contents

List of figures

List of tables

Acknowledgements

First and foremost, I would like to thank Professor Adrian C. Williams and Dr Wing Man Lau for providing me with the opportunity to carry out a PhD project under their supervision. I am deeply grateful to them for their continuous advice, guidance and support. They are great supervisors and teachers, always being encouraging and patient and I am looking forward to working with them in future.

Secondly, I would like to say a big thank you to Keng Wooi Ng, who was invaluable in training me in the lab, as well as offering his support and encouragement during my PhD journey. Most of all I would thank all the members of tissue culture lab in past and present; It is a joy to be part of a lab where everyone is so friendly, intelligent and helpful.

Thirdly, I would like to thank anyone who has helped me along the way; all help was always very much appreciated and never taken for granted. I would like to thank KRG and MHE for providing the funding for my project.

Finally, I would like to thank my wife, Arazw, and my daughter, Astana, my family and friends for their continuous love, prayers and support. I feel very blessed having them in my life.

Abbreviations

BSA	Bovine Serum Albumin
ddH2O	Ultra pure water
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco's minimum essential medium
ECL	Electrochemiluminescence
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Foetal bovine serum
HLPS	HaCaT stimulated with LPS under hypoxic condition
HRP	Horseradish peroxidase
HTHP-1	THP-1 cells under hypoxic condition
IL-6	Interleukin 6
IL-8	Interleukin 8
LPS	Lipopolysaccharide
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NLPS	Stimulation of HaCaT cell with LPS under normoxic condition
NTHP-1	Stimulation of THP-1 cell with LPS under normoxic condition
OPD	O- phenylenediamine dihydrochloride
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline + Tween 20
PCI	Protease Inhibitor Cocktail
PCNA	Proliferative cell nuclear antigen

RPMI	Roswell Park Memorial Institute
TBS-T	Tris buffered saline
TMB	Tetramethylbenzidine

Chapter I: General Introduction

1.1 Overview:

Hyperbaric oxygen (HBO) therapy is the clinical administration of pure oxygen under supra-atmospheric pressures (typically 1.4 up to 3 ATA) (Lee et al., 2012). It has traditionally been the mainstay treatment for gas embolism arising from an abrupt drop in ambient pressure (more commonly referred to as decompression sickness or 'the bends'), as can be experienced by deep sea divers or aviators who ascend too rapidly (Vann et al., 2011). More recently, HBO therapy has also been used as an adjuvant treatment to facilitate wound healing, particularly of chronic wounds (Gordillo and Sen, 2003).

Chronic wound costs patients and health organisations millions of pounds each year. A new study reveals that the annual NHS cost of managing wounds and associated comorbidities is around £5.3 billion (Guest et al., 2015). Chronic wounds occur when the normal wound healing process fails to bring about wound closure, which results in a chronic and open wound needing a continuous care. This has detrimental effects on the quality of life of the patients and their families.

Re-epithelialisation of the skin is a critical step in the wound healing process. During re-epithelialisation, skin cells proliferate and migrate to the site of injury to close the gap between the wound edges. Impaired re-epithelialisation is a major factor that leads to chronic wounds. Therefore, studies targeting the re-epithelialisation process and insight into its mechanism are of great value in understanding chronic wounds and informing treatment strategies.

HBO is a promising strategy in the treatment of chronic wounds. However, its mechanism in the wound healing process is not fully understood. In particular, the

influence of HBO on re-epithelialisation has received little attention. Investigations into the influence and mechanisms of HBO on re-epithelialisation could help construct a better understanding of the role of HBO in the wound healing process overall, and provide a stronger evidence base for its clinical application.

1.1.1 The Skin

The skin is the largest organ of the human body. It covers the entire body, has a surface area of 1.2 to 2.2 m^2, and weighs 4 to 5 kg. It is also called the integument, which is simply means " covering" (Marieb, 2000).

1.1.2 Functions of the skin

The functions of the integumentary system include physical protection, hydro-regulation, thermoregulation, cutaneous absorption, synthesis, sensory reception, and communication (Martini et al., 2012). The skin is a physical barrier to microorganisms, water, and ultraviolet (UV) light. Although certain toxins and pesticides may enter the body through cutaneous absorption, the acidic surface (pH 4.0-6.8) retards the growth of most pathogens (Van De Graaff, 2011).

1.1.3 Structure of the skin

The skin comprises three layers: the epidermis, dermis and hypodermis (Figure 1-1)

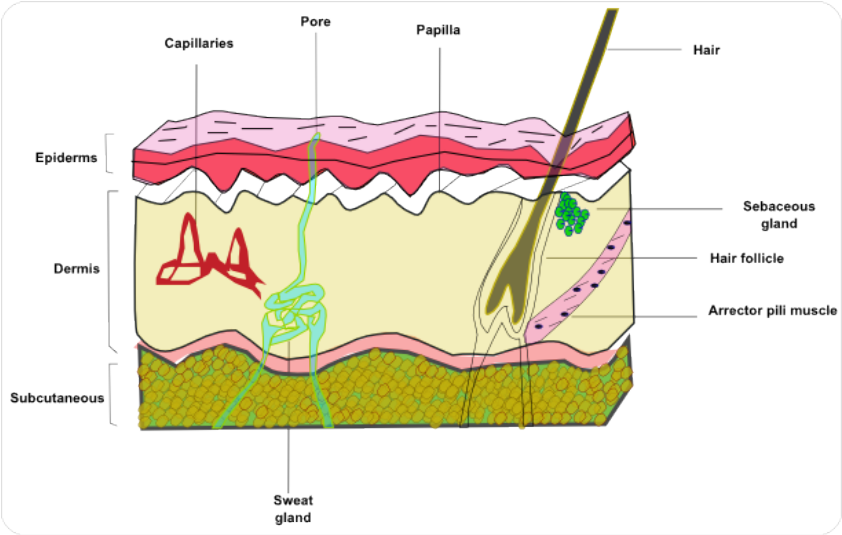

Figure 1-1.The structure of human skin.

1.1.3.1 Epidermis

The epidermis is composed of stratified squamous epithelium, which is 30 to 50 cells in thickness. The layered cells are avascular (without blood vessels). Epidermis is consisted of five layers as shown in figure 1-2: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale (Van De Graaff, 2011).

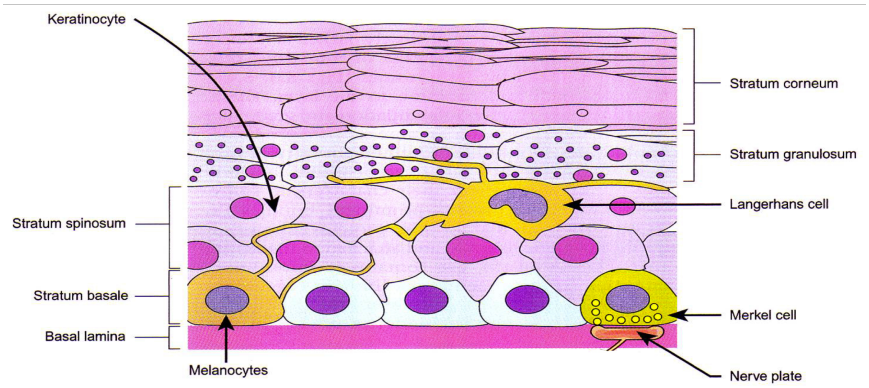

Figure 1-2 Epidermal structure

The stratum corneum, outer layer the epidermis are dead, keratinized, and cornified (Van De Graaff, 2011). Four cell types are present (Pack, 2001):

A. Keratinocytes produce keratin, a protein that hardens and waterproofs the skin. Mature keratinocytes at the skin surface are dead and filled almost entirely with keratin.
B. Melanocytes produce melanin, a pigment that protects cells from ultraviolet radiation. Melanin from the melanocytes is transferred to the keratinocytes.
C. Langerhans cells are phagocytic macrophages that interact with white blood cells during an immune response.
D. Merkel cells occur deep in the epidermis at the epidermal-dermal boundary. They form Merkel discs, which, in association with nerve endings, serve a sensory function.

The epidermis undergoes a constantly renewing process. Stem cells (SCs) residing in the epidermis and hair follicle microenvironment called the bulge that functionally replenish the epidermal cells during the regeneration steps, these cells also actively participate in the cutaneous wound healing. Bulge SCs are more quiescent than other cells within the follicle. However, the bulge SC is considered as a rich source of multipotent SCs that can be recruited during wound healing to help the regeneration of the epidermis. The two major properties of SCs are their abilities to self-renew and to differentiate along multiple lineages (Blanpain and Fuchs, 2006).

The epidermal SCs located in the basal layer, attached to the basal lamina. In order to differentiate, these cells undergo several rapid divisions, and then stop dividing and move out toward the surface of the skin producing an outer layer of dead keratinized cells that are continually shed from the surface (Albert et al., 2008).

1.1.3.2 Dermis

The dermis consists of strong, and flexible connective tissue that is found below the epidermis, supporting the epidermis structurally and nutritionally (Pack, 2001). It is composed of cells that found in any connective tissue proper: fibroblasts, macrophages, and occasional mast cells and white blood cells, and its semifluid matrix is heavily embedded with collagen, elastin, and reticular fiber (Marieb, 2000). The dermis consists of two layers (Pack, 2001):

A. The papillary layer is a thin, outer layer with fingerlike projections called dermal papillae that protrude into the epidermis. In the hands and feet, the dermal papillae generate epidermal ridges (sweat from the epidermal ridges leaves fingerprints).
B. The reticular layer is a thick layer, below the papillary layer, that makes up most of the dermis.

The dermis contains huge number of blood vessels extended from the branches of the subcutis artery. These branches form a superficial artery plexus in the papillary and reticular dermal boundaries. These blood vessels carry 8–10% of the total blood flow in a resting adult (Tortora and Grabowski, 2003).

1.1.3.3 Hypodermis

The hypodermis is composed of a layer of subcutaneous fat cells and forms the link between the skin and the rest of the body. Through it pass all the blood vessels and nerves supplying the skin layers above it. It also provides a cushioning layer and some thermal insulation when the external environment is cold (Tortora and Grabowski, 2003).

1.2 Wounds

The term wound has been defined as a disruption of normal anatomical structure and, more importantly, function. Wound healing is the complex, protracted and dynamic process that results in the reestablishment of anatomical continuity and functional integrity (Diegelmann and Evans, 2004). There are several causes of wounding, including traumatic (mechanical, chemical, physical), intentional (surgery), ischemia (e.g. arterial leg ulcer) and pressure (e.g. pressure ulcer) (Dealey, 2012). Wounds can be classified as acute or chronic.

1.2.1 Acute wounds

Acute wound refers to a wound which has a short healing period, typically 3-7 days (Sussman and Bates-Jensen, 2007). They may be cuts, abrasions, lacerations, burns or other traumatic wounds (Bryant and Nix, 2006). The wounds occur suddenly, move rapidly and predictably through the healing process. They usually respond to treatment and heal without complication. (Rovee and Maibach, 2004). The postoperative wounds also can be considered as an acute intentional wounds and they are usually heal by first intention (Dealey, 2012)

1.2.2 Chronic wounds

The mechanistic definition of chronic wounds are those which have failed to proceed through an orderly and timely reparative process to produce anatomic and functional integrity over a period of 3 months (Werdin et al., 2009). From pathophysiological point of view chronic wounds can be classified into three major types: pressure ulcers, venous ulcers, and diabetic ulcers(Mustoe et al., 2006). Less common are

inflammatory ulcers, ulcers secondary to malignancy and ulcers caused by an abnormality of red blood cells (hemaglobinopathy), and ulcers caused by impaired immunity (Rovee and Maibach, 2004)

1.2.3 Wound healing process

Wound healing is a complex physiological process whose intricate details are not well understood. Nonetheless, it is clear that it generally consists of four continuous, overlapping phases, i.e. haemostasis, inflammation, granulation and remodelling (Thackham et al., 2008) (Figure 1-3). Each phase is regulated by a variety of mediators, e.g. platelet-derived growth factor (PDGF), interleukins (ILs), tissue necrotic factor (TNF), transforming growth factor (TGF), prostaglandins (PGs), nitric oxide (NO) and leukotrienes (LTs) (Bellavia et al., 2014; Martins-Green et al., 2013; Pakyari et al., 2013) (Figure 1-4). Chronic wounds fail to follow through this healing process due to insufficiency in key of tissue repair mechanisms, or sustained activation of tissue degradation mechanisms that outstrip reparative processes (Chen and Rogers, 2007).

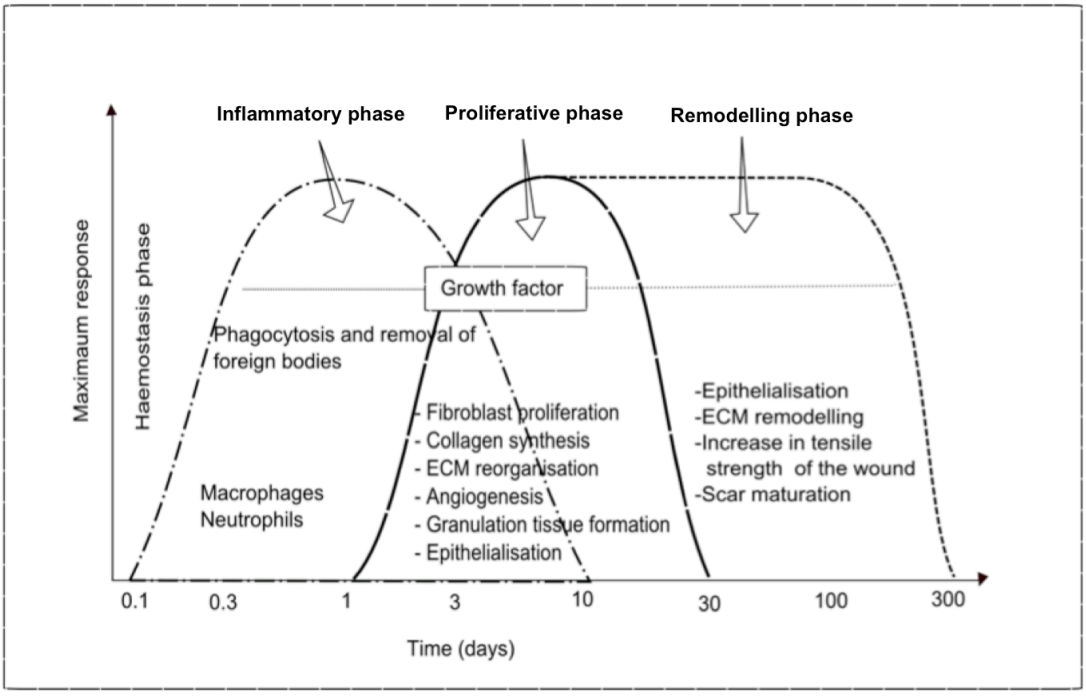

Figure 1-3.The four phases of wound healing process (Adapted from Enoch, 2006).

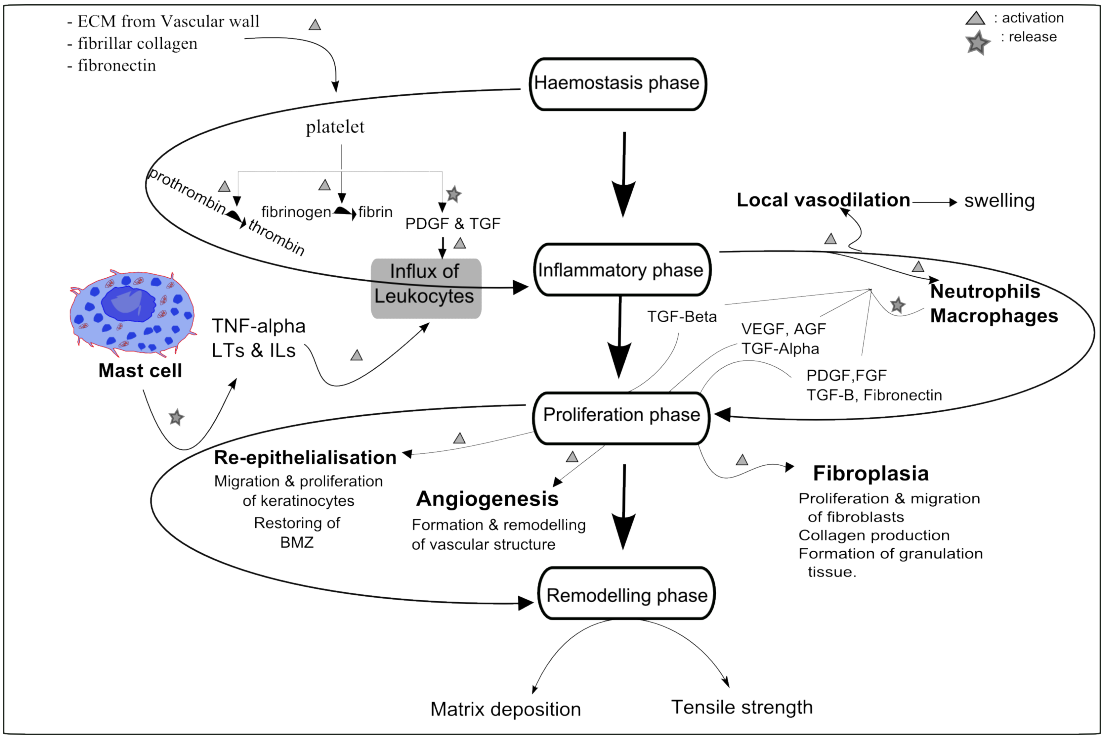

Figure 1-4.Regulation of wound healing by cytokines and growth factors.

1.3 HBO IN WOUND HEALING

1.3.1 Historical context of HBO

HBO therapy originated from compressed air therapy, which dates back to the seventeenth century. Nathaniel Henshaw, a British physician, was said to have created the first hyperbaric chamber to treat those patients suffering from acute conditions (Jain, 2009a). In the early days of hyperbaric medicine, compressed air (rather than pure oxygen) was used, as oxygen was not discovered until the late-eighteenth century (Priestley, 1775). The possibility of using HBO to treat decompression sickness had been considered shortly after the discovery of oxygen, but clinical application of HBO did not materialise until the early 1900s, for fear of oxygen toxicity (Jain, 2009a).The hyperbaric chambers were developed more rapidly during the first half of nineteenth century by Junod and Pravaz, and the first mobile hyperbaric chamber was created by Fontaine in 1877.

In 1878 Paul Bert discovered the scientific basis of oxygen toxicity and recommended normobaric, but not hyperbaric, oxygen for decompression sickness (Jain, 2009b). Historically, the potential benefits of using oxygen under pressure for the treatment of decompression sickness were first realized by Drager in 1917, Drager designed a chamber for using pressurized oxygen in the treatment of diving accidents and by the end of 1937 hyperbaric oxygen had become an important tool as well as focus in medical research (Jain, 2009b). In 1965, it was reported that HBO could assist in cardiopulmonary surgery and prevent anaerobic infections and it

shows some effects in the treatment of problematic wounds. (Kuffler, 2011). HBO is mainly used as an adjuvant therapy with conventional treatment strategies of the chronic wounds (Boykin and Baylis, 2007).

1.3.2 Principles of HBO in wound healing

Oxygen is well recognised as a key element in modulating the series of events involved in wound healing, including the production and regulation of wound healing mediators, particularly during the inflammatory phase (Davidson and Mustoe, 2001; section 1.3.2 below). HBO is thought to help wound healing by increasing the amount of oxygen in the skin and peripheral tissues (Nazario and Kuffler, 2011). It is also known to stimulate cellular proliferation, inhibit necrosis and help in the prevention and treatment of infections (Kindwall et al., 2008).

Tissue trauma simultaneously increases the oxygen demand of damaged tissue to accelerate tissue repair, and significantly decreases the oxygen supply to the wounded area mainly due to oedema (Gajendrareddy et al., 2005). Hypoxia caused by destructed vasculature, is a major rate limiting factor for wound healing (Khanna and Wallace, 2002). This mismatch in oxygen demand and supply creates hypoxic conditions in the wound microenvironment impeding the wound healing process (Eming et al., 2007) . Therefore, to correlate the balance of oxygen demand and oxygen supply some efforts have been done, one of them is HBO, which is designed to overcome hypoxic conditions in the wound and to increase the oxygen level even upper than normal level. It can greatly enhance oxygen delivery to tissues by increasing oxygen concentration in the plasma, via a mechanism governed by fundamental gas laws (Zamboni et al., 2003). The pO2 is almost zero in the central

area of a wound due to the extended intercapillary distance and the devascularisation process. Transport of oxygen to the hypoxic center of a wound, or to a wound with impaired perfusion, is often only possible with hyperbaric oxygen (Rollins et al., 2006).

Under normal physiological conditions, the majority of oxygen carried in the blood is bound to haemoglobin in red blood cells which is equal to 97%, while a relatively small proportion is unbound and dissolved in the plasma (Tibbles and Edelsberg, 1996) and (Nazario and Kuffler, 2011). Henry's law states that, at constant temperature, the amount of a given gas that is dissolved in a liquid at equilibrium is proportional to the partial pressure of the gas (Gill and Bell, 2004). Therefore, the plasma concentration of oxygen would increase with increasing partial pressure of oxygen. Under normobaric conditions, at equilibrium, the arterial oxygen tension is about 100 mmHg, whereas tissue oxygen tension is about 55 mmHg, Under clinical HBO conditions, these values can reach up to 2000 mmHg and 500 mmHg, respectively (Tibbles and Edelsberg, 1996). Accordingly, oxygen concentration increases from 3 ml/L under normobaric conditions to 60 ml/L under HBO conditions, the latter allowing for adequate tissue oxygenation even in the case of severe anaemia (Leach et al., 1998). Throughout the course of HBO treatment at 2.8 ATA, the dissolved oxygen level in blood will be 6 mL in 100 mL of plasma. Consequently, the haemoglobin is also fully saturated with oxygen. The increased oxygen tension in blood in compare to the surrounding tissues provides enough force to drive oxygen from blood to the deep tissues by simple diffusion (Mortensen, 2008).

Increasing the dissolved oxygen concentration in the plasma has the direct effect of increasing the oxygen gradient between the oxygen-rich plasma and hypoxic tissue, thus facilitating oxygen diffusion down the concentration gradient to cells that suffer from reduced oxygenation (Hollander et al., 2000). There is also a notable difference between oxygen concentration in the center of a wound (≤3 mmHg) and its periphery (20 mmHg), and this oxygen gradient appears to stimulate wound healing (Knighton et al., 1981). In addition, it has been noted that HBO reduces oedema, and increases the diffusion of oxygen and nutrients to injured tissue (Mychaskiw et al., 2005).

In a study that included 20 patients with chronic wounds, the role of HBO in increasing tissue oxygen tension and improving wound healing was examined; Tissue oxygen tensions of below 10 mmHg were increased dramatically in patients treated with HBO. Patients in whom tissue oxygen tension did not exceed 50 mmHg did not show any improvement in wound healing via clinical observations, while those in whom tissue oxygen tension rose to above 100 mmHg showed significant improvements (Wattel et al., 1990).

1.3.2.1 HBO in the haemostatic phase

Haemostasis is the first step in the wound healing process. This provides an immediate physiological response to stop the bleeding from blood vessels destroyed during tissue injury. Haemostasis consists of two key events, i.e. the formation of fibrin clot and coagulation (Kirsner and Eaglstein, 1993). Platelets are exposed to and activated by the extracellular matrix in the vascular wall, such as fibrillar collagen, fibronectin, and other adhesive matrix proteins. Upon activation, platelets undergo adhesion as well as aggregation and at the same time release important

mediators (eg, serotonin, adenosine diphosphate, and thromboxane A2) and adhesive proteins (eg, fibrinogen, fibronectin, thrombospondin, and von Willebrand factor VIII) (Velnar et al., 2009). These mediators and locally generated thrombin induce further platelet aggregation and secretion and form the platelet plug. With the conversion by thrombin of fibrinogen to fibrins during platelet aggregation, a fibrin clot is formed to stop the bleeding (Li et al., 2007). Integrins are cell adhesion molecules that mediate cell-cell, cell-extracellular matrix, and cell-pathogen interactions. Integrin regulation and signaling play a central role in the haemostasis process (Luo et al., 2007). Reactive oxygen species (ROS) have emerged as an important mediator both transducing the signals associated with integrin activation and modulating integrin function (Gregg et al., 2004).

New studies unveiled that in addition to macrophages, every cell involving in the wound healing process is fitted with a specific enzyme to convert O2 to ROS that participate as a cellular messenger to promote haemostasis and the entire wound healing process (Sen, 2003). Recent evidence has demonstrated that apart from its oxidative killing activity, reactive oxygen species (ROS) such as H2O2 and superoxide (O2-) play an important role in the activation of the clotting cascade, coagulation, as well as the secretion of PDGF and TGF during haemostasis (Rodriguez et al., 2008). Respiratory burst which is a rapid release of ROS from macrophages mediated by an oxygen-dependent enzyme, nicotinamide adenine dinucleotide phosphate (NADPH)-linked oxygenase (Davidson and Mustoe, 2001). Defects in NADPH results in impaired healing in humans (Eckert et al., 1995). Acute hypoxia is a potent stimulant of ROS release but chronic hypoxia decreases the ROS production. Although, acute hypoxia stimulates initiation of haemostasis phase,

oxygen is needed to maintain it (Rodriguez et al., 2008). Apart from Enzymatic activation of NADPH, Evidences from both in-vitro and in-vivo studies suggesting that HBO directly increases the ROS levels in the different body compartments (Jamieson et al., 1986). The oxidative stress resulted from HBO is fundamental for its action on haemostasis cascades (Thom, 2009).

1.3.2.2 HBO in the inflammatory phase

During the inflammatory phase, leukocytes infiltrate the wounded area under the effect of PDGF, TGF from platelets, kallikrein and fibrinopeptide from fibrin degrades, as well as TNF, LT and IL from mast cells (Kirsner and Eaglstein, 1993). Platelets release huge numbers of chemo-attractants and growth factors (e.g. TGF-β and PDGF), in responding to these mediators; leukocytes including neutrophils and macrophages infiltrate the wounded area to remove damaged tissue debris and foreign particles (Velnar et al., 2009). Other important chemotactic signals include the tumor necrosis factors (TNF), leukotrienes (LTs) and interleukins (ILs), which are released by mast cells, macrophages and neutrophils (Noli and Miolo, 2001). Also the degradation product of fibrin of the clotting has a similar effect. Neutrophils are the first leukocytes to arrive at the injured area in a large number. During the inflammation phase, the number of neutrophils decreases gradually and macrophages become the predominant cells (Li et al., 2007).

Throughout the inflammatory phase of wound healing, macrophages and T-cells secrete polypeptides (glycoproteins) known as cytokines. Cytokines are a common name for a large group of signaling proteins, for instance lymphokine, monokine, chemokine and interleukines (cytokine from leukocytes). They act by binding to

membrane receptors and exert their activity through a second messenger to change the biological activity of cells (Al-Waili and Butler, 2006). Macrophages have been reported to express a wide range of cytokines including interleukin-I (IL-1), platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF), colony-stimulating factors (CSFs) and transforming growth factor-β (TGF-β) (Rappolee et al., 1988). Neutrophils are responsible for releasing VEGF and IL-8. These cytokines stimulate angiogenesis through activation of endothelial cells (Schruefer et al., 2005). PDGF is responsible for the mitogenesis of fibroblasts. In addition to cytokines, macrophages also release Prostaglandins (PGs) which exert a broad function and activity throughout the course of inflammatory stage of wound healing (Al-Waili and Butler, 2006) PGs play a key role in the initiation and maintenance of inflammatory responses after injury, including edema, pain, hyperalgesia, decreasing immunity and increasing the production of substance P. IL-1 and IL-2 increase PG production (Inamoto et al., 1991). The influence of HBO on the inflammatory stage of wound healing can be divided into two categories, the first one is the effect on the production of PGs and cytokines from various cells involved in the wound healing and the second one is its antimicrobial activity.

Firstly, early in the wounding process, local vasodilatation, blood and fluid extravasation into the extravascular space, and blocking of lymphatic drainage can produce cardinal signs of inflammation, including redness, swelling, and heat (Li et al., 2007). HBO2 treatment causes a marked decrease in IL-1 and IL-2 release that resulted in a significant decrease in PGE2 production by macrophages (Inamoto et al., 1991), (Benson et al., 2003). Prostaglandin I2, PGD2, PGE2, and PGF2a are potent substances for vasodilatation, whereas PGD2, PGE2, and PGF2a also

increase vascular permeability, which can cause edema (Sapirstein and Bonventre, 2000). Therefore, the reduction of PGs production may play an important role in the anti-inflammatory effect of HBO and may ultimately result in enhanced local or general immune system (Al-Waili and Butler, 2006), (Inamoto et al., 1991). HBO can reduce oedema and preserve microcirculation due to hyperoxic vasoconstriction (Bilic et al., 2005). The mechanism of vasoconstriction effect of HBO is not well understood. However, evidences suggested that HBO can induce vasoconstriction through inhibition of vasoactive PGs and increased production of the endothelium-derived vasoconstrictor endothelin (Mouren et al., 1997). PDFG possess a synergistic effect on up-regulating of PDGF B-Receptor when combined with HBO. This is can rationalise the synergistic effect of combination of HBO2 and PDGF on wound healing process (Bonomo et al., 1998). HBO and pressure alone can suppress INF- γ but not hyperoxia alone or normoxia which means that the anti-inflammatory effect of HBO is partly may be due to inhibition of INF-γ (Granowitz et al., 2002). HBO2 exerts a vasoconstricting effect in brain by increasing oxidative stress and decreasing NO bioavailability. This might be due to increasing in cytosolic Ca2+ concentrations (Efrati et al., 2009).

Secondly, massive data from both experimental models and human subjects suggesting that pO2 level is a major determinant of susceptibility to infection (Hunt and Aslam, 2004). Infectious wounds might be considered as one of the major problems that associated with wound, in particular in case of diabetic patients which is associated with a high prevalence rate of amputation. In term of infection, O2 play the same important effects as antibiotics and those modalities that combining both of them provided additional beneficial effects (Knighton et al., 1984). A study conducted

by (Greif et al., Outcomes Research Group, 2000) revealed that increased oxygen level can improve host immune responses. Hyperoxygenation of the wound under a high pressure eliminates bacterial infection from the wounded area, while hypoxic wounds are more susceptible to bacterial infection (Kuffler, 2011).

NADPH-linked oxygenase is an enzyme which is considered as one of the most important factor for wound healing process in particular during the inflammatory phase, this is due to its role in respiratory burst that occurs in leukocytes. The activity of this enzyme and production of higher amount of oxidants to prevent infection are dependent on oxygen. Increasing oxygen tension from 80 mmHg to 400mmHg leads to an increase in maximum enzymatic velocity from 50% to 90% (Davidson and Mustoe, 2001). Hyperoxygenation of wounded tissues has a bactericidal effect on anaerobes and microaerophilic aerobes; and it also enhances microbicide effects which are provided by oxidative pathways, regulating macrophage functions and covering oxygen consumption at infected tissues (Aydın et al., 2014). The anti-infectious activity of oxygen is mediated by ROS production in neutrophils and macrophages via NADPH oxidases pathway. The maximum level of ROS generation can be achieved at pO2 at > 300 mmHg (Allen et al., 1997). The macrophages and neutrophils utilize the majority of the consumed oxygen for respiratory burst and production of ROS at the wound area (Sen, 2003).

If the wounds are hypoxic this leads to inactivation of oxygen dependent pathway and increases the rate of wound infection (Jönsson et al., 1988). The bactericidal activity by leukocytes is dependent on oxidative phosphorylation which needs a sufficient amount of oxygen to be work. Therefore, re-establishment of oxygen level

induces wound healing (Kuffler, 2011). The bactericidal effect that mediated by neutrophils was significantly decrease by hypoxia (Knighton et al., 1984). Activation of NADPH-linked oxygenase in leukocytes causes respiratory burst which is a rapid release of superoxide radicals and H_2O_2. That leads to oxidizing of bacterial cell membrane and killing of bacteria (Zamboni et al., 2003).

Moreover, one of the problems regarding bacterial infection is the development of bacterial resistance to antibiotics, but in case of oxygen, bacteria cannot create resistance this is due to the fact that most of them are anaerobic pathogens (Stewart and Costerton, 2001). HBO exerts a direct bactericidal effect on anaerobic bacteria including Clostridium perfringens and also, it exerts bacteriostatic effect for certain species of Escherichia and pseudomonas (Tibbles and Edelsberg, 1996). Oxygen free radicals increase under HBO which play a key role in oxidation of protein and lipid membranes, as well as inhibition of bacterial metabolic condition. HBO is active specifically against anaerobic bacteria and it act as an assistant for oxygen dependent peroxidase system through which leukocytes kill bacteria (Knighton et al., 1984). Generally, wounds are hypoxic and they do need increasing in tissue oxygen tension in order to get enough ROS from the phagocytes to kill bacteria by oxidative killing (Davidson and Mustoe, 2001). On the other hand another study find out that hypoxia may stimulate ROS generations initially. While later on it does need oxygen to sustain it. So, chronic hypoxia is key factor in suppression of this process (Rodriguez et al., 2008).

The first step in the treatment of acute infection is via increasing of tissue oxygenation, enhancement of white blood cell oxygen dependent killing and

reduction of edema (Escobar et al., 2005). In order to understand the advances in wound healing, first of all we should start with the role of ROS and disclaim that ROS is usually harmful. It is harmful only in very high concentrations while the H_2O_2 concentration in wound fluid is normally about 5 to 15μM. Even though wounds are hypoxic by nature, it is clear they heal in an oxidative condition (Hunt and Aslam, 2004). Staphylococcus is the major wound pathogen and oxidants produced by leukocytes are considered as the only bactericidal mechanism against this pathogen. However, tissue hypoxia is probably the most significant background for susceptibility to wound infection (Hunt and Aslam, 2004). A study conducted by Oztas et al (2001) demonstrated that penicillin exerts a synergistic effect in combination with HBO in the treatment of murine model infected with streptococcal myositis (Oztas et al., 2001).

1.3.2.3 HBO in the granulation phase

The granulation phase consists of three major events, i.e. re-epithelialisation to restore the permeability barrier, angiogenesis to ensure adequate blood supply to the wounded area, and fibroplasia to reinforce the injured dermal tissue (Li et al., 2007).

Re-epithelialisation:

During this process the intact epidermis is restored after cutaneous injury. It involves the migration and proliferation of keratinocytes and restoration of basement membrane zone (BMZ) connecting the epidermis to the dermis (Laplante et al., 2001). TGF-β, secreted from macrophages, is considered a main stimulant for the activation of re-epithelialisation (Schreml et al., 2010), (Falanga, 1993). Many other mediators also play an important role during this process, for instance, in the case of proliferation and migration of keratinocytes, integrin receptor and ECM proteins such

as metalloproteinases, collagen V, fibronectin and fibrin. For the restoration of the BMZ, collagen 1V, VII and XII and lamininis are important mediators (Li et al., 2007). Patel et al. (2005) conducted a study in which the human keratinocytes were subjected normobaric and hyperbaric conditions. In the case of normobaric conditions, two different concentrations (20% and 90%) of oxygen were used; in the case of hyperbaric environment, only 90% oxygen was used. The results showed that cell growth increased in 20% higher than 90% at normobaric condition, while there is no growth under hyperbaric condition. However, normoxia and hyperoxia stimulate VEGF expression by keratinocytes (Patel et al., 2005). In a separate study, angiogenesis and re-epithelialisation were significantly increased when exposed to HBO in an intervention group compared to control group due to elevation of TNF-α level, which increases the supply of metalloproteinases in the wounded area (Sander et al., 2009). Re-epithelialisation is fundamentally depends on the keratinocytes motility. Cell motility decreases as available cell energy production via oxygen decreases to or below 10mmHg pO2 (Nazario and Kuffler, 2011).

Another study demonstrated that HBO had not a significant effect on melanocyte proliferation and Glycosaminoglycans GAG synthesis, while 30% oxygen at 1ATA led to a significant increase in the proliferation of fibroblasts. In contrast, the proliferation of fibroblasts and keratinocytes were inhibited when they were exposed to HBO at 2 ATA, while HBO at up to 3 ATA stimulated keratinocyte proliferation significantly. Fibroblast proliferation was significantly increased at 1 and 2.5 ATA (Dimitrijevich et al., 1999). However, some other experiments stated that HBO at 1-2.5 ATA did not have a negative or positive effect on the proliferation of keratinocytes (Hollander et al., 2000) and (Kairuz et al., 2007).

Angiogenesis:

A critical step in wound healing process is restoring the blood supply to the wounded tissues through angiogenesis, which involves formation and remodelling of the vascular structures (Eming et al., 2007). Newly formed vessels participate in granulation tissue formation and provide nutrition and oxygen to growing tissues (Folkman, 1982). Angiogenesis is activated by some mediators secreted from macrophages that include VEGF, TGF-β and angiogenic factor (Li et al., 2007). It involves 9 consequence steps, which include: endothelial cell activation, lysis of matrix, migration of endothelial cells, mitosis, tube formation, matrix synthesis, establishment of circulation and recruitment of pericytes (Knighton et al., 1983). It is well established that VEGF possess an angiogenic effect at the wound area and the HBO induces VEGF mRNA in both endothelial and macrophage cells (Deaton et al., 1994).

Many studies have been conducted to examine whether hypoxia or oxygen is responsible for the initiation of angiogenesis. Although it is commonly believed that hypoxia stimulates angiogenesis initially but oxygen is needed to maintain it (Patel et al., 2005). Hypoxia serves as a physiologic cue to drive an angiogenic response via HIF-dependent mechanisms and HBO with its oxidative stress at sites of neovascularization will stimulate growth factor synthesis in particular VEGF by augmenting synthesis and stabilizing hypoxia-inducible factor HIF-1α (Hunt et al., 2007). Although, the degradation of HIF occur when the cells replete with oxygen, ROS can stabilize HIF via production of thioredoxin which has shown to promote the expression and the activity of HIFs (Ema et al., 1999).

Most of the research in the area of the relationship between oxygen and angiogenesis has been done by using animal models. However, the application of the results from animal studies to the human being is controversial because human being is much more complex as well as diseases and nutrition may affect the results and should be read with caution (Bishop, 2008). FGF and VEGF are responsible for angiogenesis but they provide their activity at different points of time, for instance, FGF stimulates angiogenesis during the first 3 days of wound healing process, while VEGF stimulates angiogenesis during the granulation phase in human surgery wounds (Nissen et al., 1998). The level of VEGF rises significantly (40%) if macrophages are exposed to HBO for 5 consequent days (Al-Waili and Butler, 2006). The stimulation of VEGF production by HBO might be able to explain some aspects of its angiogenic effects. It has been observed that the amount of VEGF significantly increased after 7 days exposure to HBO (Sheikh et al., 2000). The production of VEGF by fibroblasts and macrophages as well as angiogenesis can be stimulated by hypoxia (Zhao et al., 1994). Another study investigated the impact of oxygen concentration on the rate and density of capillary growth during the angiogenesis phase of wound healing. In the study different concentrations of oxygen were used, including 12%, 20%, 40% and 70%, for the rabbit ear wound. The higher oxygen concentrations significantly increased the rate and density of capillary growth. Also when the ear wound was exposed to hypoxic conditions, capillary growth was inhibited. The result of the study demonstrated that oxygen play a key role in terms of angiogenesis and higher oxygen level resulted in more significant angiogenic effects (Knighton et al., 1984). once tissue pO2 drops below 10mmHg, there is no

angiogenesis, which leads to further oxygen deficiency, preventing tissue granulation and blocking tissue healing (Nazario and Kuffler, 2011).

Another study conducted by (Hopf et al., 2005) investigated the impact of oxygen on angiogenesis in which a mouse subcutaneous wound model subjected to hypoxia, normoxia, hyperoxia (either normobaric or hyperbaric) conditions. Mice in the hypoxic (13%) and normoxic (21%) conditions were exposed to oxygen constantly. While those in the hyperoxic conditions exposed to oxygen twice daily for 90 minutes. After 7 days of the study, a substantial increase in angiogenesis was observed in the hyperoxic group, while angiogenesis was significantly decreased in the hypoxic group, there was no significant change in angiogenesis in the normoxic group. The result of this study demonstrated that hyperoxia played an important role in wound healing process via the stimulation of angiogenesis (Gordillo and Sen, 2003). HBO produced and increased oxygen gradient between the center and peripheries of the wound, which stimulated revascularization of the ischemic tissues (Knighton et al., 1981). HBO increased the oxygen gradient which leads to formation of angiogenesis and wound healing for chronic wounds (Lee et al., 2006). Oxygen plays an important role in the deposition of perivascular collagen which is needed for the formation of endothelial cells, specifically through enhancing of the hydroxylation of collagen proline residues (Hopf et al., 2005).

On the other hand in case of acute hypoxia, the lactate level increases in the wounded area which stimulates angiogenesis but chronic hypoxia may result in inhibition of angiogenesis, collagen formation and oxidative killing of bacteria by macrophages (Knighton et al., 1983). Another study unveiled that ROS activate

VEGF mRNA in macrophages and keratinocytes, and consequently increase the secretion of VEGF (Sen et al., 2002). Nitric oxide (NO) has been considered as an active molecule involved in the wound healing process, it is responsible for the activation of a number of enzymes via increasing the concentration of free calcium ions (Garthwaite, 1991). NO plays an important role in the regulation of the different phases of wound healing, for example, angiogenesis, formation of granulation tissue, migration of epidermal cells and collagen deposition (Fukumura et al., 2001), (Pollock et al., 2001). PG which come from arachidonic acid as a result of the destruction of cell membrane is responsible for the production of NO (Al-Waili and Butler, 2006). It has been noted that HBO increases the concentration of NO which contributes to wound healing through stimulation of angiogenesis and inhibition of neutrophils integrin-2 function (Thom, 1993). NO stimulates the production of VEGF and proliferation of endothelial cells. Furthermore, NO donors stimulate fibroblast to produce a large amount of collagen (Al-Waili and Butler, 2006). It has been observed that HBO elevates the level of NO significantly in the wound fluid after 1-4 weeks of the exposure to the therapy (Boykin and Baylis, 2007). Also HBO stimulates angiogenesis and revascularization by stimulation of TNF-α release (Lahat et al., 1995).

Fibroplasia:

Fibroplasia consists of several processes, which include: proliferation and migration of fibroblasts, production of collagen and matrix proteins. The last two are participating in the formation of granulation tissue. Fibroplasia is activated by PDGF, FGF, TGF-β and fibronectin (Li et al., 2007). Cytokines, in particular PDGF and TGF-

β1 by interaction with extracellular matrix (ECM) to enhance wound healing especially by stimulating of fibroblasts proliferation (Xu and Clark, 1996). The relationship between oxygen levels and TGF-β release has been studied. Fibroblasts were exposed to two different oxygen conditions, 2% oxygen at 14 mmHg (hypoxia), and 15% oxygen at atmospheric pressure (normoxia) for 72 hours. The result revealed that the production of TGF-β under hypoxic conditions was increased nine-fold in compared to the normoxic environment. Likewise, TGF-β mRNA increased eightfold in the similar manner. The researchers concluded that a low oxygen tension may be considered a stimulant for the initiation of the granulation phase (Falanga et al., 1991). A later study however concluded different results; fibroblasts from healthy individuals were exposed to 1% oxygen and 20% oxygen. Fibroblast proliferation was three times higher in 20% oxygen compare to 1% oxygen. Additionally, chronic hypoxia (more than six passages) induced a 3-fold decrease in TGF-β1 mRNA. The researchers concluded that, although acute hypoxia may stimulate release of growth factor, chronic hypoxia inhibited it (Siddiqui et al., 1996).

Niinikoski (1969) has investigated the effect of oxygen on wound healing for the first time. His experiment was based on using of 35-70% oxygen inhalation in rats. The result of his experiment showed a significant increase in tensile strength of wound tissues. Few years later, another study was conducted to investigate the effect of oxygen level on rate and density of collagen synthesis in rabbits. Hypoxic, normoxic and hyperoxic environments were used on 6 rabbits for the duration of 25 days. The results revealed that hyperoxia significantly stimulated collagen synthesis (Hunt and Pai, 1972). Further investigations were performed to evaluate the impact of oxygen on cell proliferation and collagen synthesis in murine fibroblasts; the cells were

exposed to a series of oxygen tensions including 1788 mmHg, 722 mmHg, 160 mmHg, 80 mmHg, 38 mmHg (normal tissue level) and 15 mmHg for 4 days. The result shows that maximum proliferation and collagen synthesis were seen at ≤80 mmHg. This might be due to the fact that higher oxygen tensions exerts a toxic effects on cells (Mehm et al., 1988). TGF-β stimulates formation of the procollagen and fibrinogen and some research has shown that hypoxia and hyperoxia can alter the production of the growth factors (Semenza, 2001).

The hydroxylation of proline and lysine results in the formation of mature collagen which is responsible for the collagen fibril cross-linking. At 250 mmHg oxygen tension the production of collagen reaches to the maximum level, while in case of severe hypoxia was completed halted (Kuffler, 2011). Macrophages attract fibroblasts to the injured area and fibroblasts starts collagen synthesis, which also needs oxygen. Proline, lysine and glycine create non-helical procollagen via protein synthesis. Procollagen formation requires hydroxylation of proline and lysine. In this process oxygen assists the hydroxylation process. Subsequently, procollagen is converted to tropocollagen through lysyl oxidase. Formation of mature collagen involves two important processes, i.e. aggregation of collagen molecules and cross-linking. These processes require glycosaminoglycan as a matrix and two enzymes, including prolyl-hydroxilase and lysyl-hydroxylase, both oxygen dependent (Zamboni et al., 2003). The secretion of collagen and angiogenesis are stimulated by the high level of lactate in the wound environment (Zamboni et al., 2003). It has been observed that HBO at 1 ATA and 100% oxygen stimulates collagen synthesis better than higher pressure (2-2.5 ATA 100% oxygen). A possible explanation for this is that increased pressure causes suppression of glycosaminoglycans (GAG) which is the matrix of mature

collagen (Dimitrijevich et al., 1999). In contrast to the general notion, accumulation of lactate in wounds is not due to hypoxia. Lactate is generated mainly in the oxidative burst and it chelated iron which leads to more oxidant production by reaction between Fe^{++} and O2 that releases H2O2. The H_2O_2 is changed somewhat to produce relatively more hydroxyl radicals. This is known to enhance collagen gene transcription and control the activity of VEGF (Hunt and Aslam, 2004).

In contrast to the common conception of the beneficial effects of HBO on collagen synthesis, Dimitrijevich et al., demonstrated that HBO has an inhibitory effect on collagen synthesis by fibroblasts (Dimitrijevich et al., 1999). Hypoxia stimulates collagen synthesis via increasing collagen mRNA prevalence, procollagen synthesis and prolyl-hydroxylase activity (Hopf et al., 2005). However, other studies revealed that a critical level of oxygen is needed for fibroblasts to secrete collagen molecules (Stücker et al., 2002). Both in vitro and in vivo studies demonstrate that oxygen has an important role in collagen synthesis and the amount of deposited collagen is directly proportional to the measured tissue oxygen tensions (Jonsson et al., 1991). The results of an in vitro study showed that proliferation rates were not significantly changed by using HBO, while it had a stimulatory effect on cell differentiation and elevation the expression levels of cytokeratin and involucrin (Hollander et al., 2000). An in vivo study recorded a tissue oxygen tension of 5-15 mmHg between the epicentre of the wound and granulation tissues, active proliferation of fibroblasts was only seen in the area where the tissue oxygen tension was greater than 15 mmHg (Hunt et al., 1972). Fibroblasts proliferation was increased in a dose-dependent manner when the cells were exposed to HBO for 24 hours (Tompach et al., 1997).

In another in vitro study, HBO stimulated fibroblast proliferation in a dose-dependent manner, and the maximum proliferation was seen at 2.4 ATA (Brismar et al., 1997). Acute hypoxia activates the proliferation of human dermal fibroblasts, whose rate of proliferation increased by 71% when the cells were exposed to 1% oxygen for 72 hours (Siddiqui et al., 1996). Pro-collagen gene transcription occurs upon exposure of fibroblasts to H_2O_2 and the presence of oxygen at this step consolidates the collagen deposition and tissue integrity (Hunt and Aslam, 2004). Collagen production is maximal when pO2=250mmHg and falls to almost zero in severe clinical hypoxia (pO2=25 mm Hg) due to the failure of collagen fibril cross-linking, which requires the hydroxylation of pro- line and lysine to synthesize mature collagen (Nazario and Kuffler, 2011). HBO2 or HBO2 combined with PDGF treatment down-regulating the Type III collagen/Type I collagen content, which could result in mechanically stronger collagen fibrils (Chan et al., 2007).

1.3.2.4 HBO in the maturation phase

The maturation phase of wound healing starts with the deposition of matrix and associated changes. In fact, the maturation phase starts early in the wound healing process and overlaps with the haemostasis phase, when the fibrin clot is replaced by granulation tissue (Li et al., 2007). One of the most important steps during the tissue repair and angiogenesis is the breakdown of the extracellular matrix. MMP-9 and MMP-2, secreted by macrophages, degrade collagen IV. HBO induces an up-regulation of TNF-α, which leads to acceleration of MMP-9 expression (Sander et al., 2009). HBO has been shown to induce the re-deposition of collagen IV, a main component of the basal membrane, when a sample of human dermal equivalent is exposed to HBO for 5 days (Kairuz et al., 2007). Oxygen is one of the key

component in cross-linking of collagen (Eyre et al., 1984). The elevation of oxygen level at the wounded area results in increased collagen deposition and tensile strength; this effect is directly proportional to the level of tissue oxygenation and it can be maximized by supplemental oxygen (Hunt and Pai, 1972).

1.4 HaCaT cell line

HaCaT cells are a spontaneously immortalized (greater than 140 passages, human keratinocyte line that has been widely used for in-vitro studies of skin biology and wound healing. This HaCaT cell line has unlimited growth potential in vitro (clonogenic on plastic and in agar) but remains nontumorigenic. HaCaT cells have similar biological behaviour as normal keratinocytes. Differentiation markers including involucrin and filaggrin are expressed and regularly located in this cell line. Moreover, HaCaT is the first permanent epithelial cell line from adult human skin that exhibits normal differentiation and provides a promising tool for studying skin biology and epidermis. (Boukamp et al., 1988)

1.5 Objectives and Aims

The principal aim of this project was to investigate the influence of hyperbaric oxygenation on wound healing process focusing on the re-epithelialisation of skin as a critical step in wound healing. The specific objectives were:

1. To develop an in-vitro model represents epidermis from immortalized human keratinocytes HaCaT cells and to evaluate epidermal wound healing using scratch assay.

2. To develop techniques that allows utilization of hyperbaric oxygen for in-vitro models.
3. To examine the hypothesis that hyperbaric oxygenation can induce the re-epithelialisation of the skin
4. To test in the influence of hypoxia and hyperbaric conditions on the keratinocyte's migration and proliferation profiles
5. To investigate the effect of infection on re-epithelialisation process under hyperbaric hyperoxia, normobaric hypoxia and normobaric normoxic conditions.

Chapter II: Methods development and Techniques

2.1 Materials and Equipment

HaCaT cells were purchased from Cell Lines Service (Eppelheim, Germany). Dulbecco's Modified Eagle's Medium (with 4.5g/L Glucose and L-Glutamine) (DMEM), fetal bovine serum (FBS) and trypsin were purchased from Lonza Ltd (Slough, UK). Roswell Park Memorial Institute (RPMI) medium and Low endotoxins FBS were purchased from Life Technologies (Paisley, UK). THP-1 was obtained from ATCC (London, UK).

A digital camera (DEM130E 1.3M pixel CMOS ship) fitted to a light microscope (Nikon, Eclipse.TS100) operated with Scopephoto software (63X) used in scratch assay was obtained from Hangzhou Scopetek Opto-Electric Co., Ltd. O-phenylenediamine dihydrochloride (OPD), bovine serum albumin (BSA) and lipopolysaccharide (LPS) were purchased from Sigma (Poole, UK). ELISA plate (Microplate Immuno MaxiSorp 96 well flat bottom polystyrene), sodium hydrogen carbonate and sodium carbonate anhydrous were purchased from Fisher Scientific (Loughborough, UK). Phosphate-citrate buffer and sodium perborate were obtained from Lonza group Ltd (Slough, UK). Avidin HRP, purified anti-human TNF-α Monoclonal Antibody (TNF-α MAb1), biotinylated anti- human TNF-α (TNF-α MAb1), anti-human IL-8 capture and detection antibodies, recombinant IL-8, IL-6-capture and detection antibodies, recombinant IL-6 and Tetramethylbenzidine (TMB) solution were purchased from eBioscience (Hatfield, UK). Epoch BioTeck spectrophotometer and Gen5 software were obtained from Epoch BioTeck Ins. (Winooski, USA)

RIPA buffer, Protease Inhibitor Cocktail (PIC), Pierce^{TM} BCA Protein Assay Kit were purchased from ThermoFisher Scientific (Loughborough, UK). Mini-Protean TGX

Precast Gel, Mini-Protean Tetra electrophoresis system apparatus, HC power pack, Laemmli loading buffer, 2-mercaptoethanol, running buffer, transfer buffer, tris buffer saline, mini trans-blot cell apparatus, nitrocellulose membrane, precision plus protein and electrochemiluminescence ECL were purchased from Bio-Rad Laboratories Ltd (Hertfordshire, UK). PCNA (primary and secondary antibodies), Involucrin (primary and secondary antibodies), E-cadherin (primary and secondary antibodies) were purchased from Abcam (Cambridge, UK). β-actin (primary and secondary antibodies) was achieved from Sigma (Poole, UK). Dry block heater was purchased from Bibby Scientific Ltd. (Staffordshire, UK) and a cold centrifuge was obtained from Eppendorf (Stevenage, UK).

The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Sigma-Aldrich Ltd (Gillingham, UK). DMSO was purchased from Fisher Scientific (Loughborough, UK). Oxygen combustion vessel was obtained from Parr Instrument Company (Moline Illinois, USA). The gas mixtures containing various O_2 concentrations and the gas regulators have been achieved from BOC (Guildford, UK). Modular Incubator Chamber MIC-101 purchased from Billups-Rothenberg Inc. (Del Mar, CA. USA). A portable dissolved oxygen meter (Jenway 970) obtained from Jencons Scientific Ltd (Grinstead, UK)

2.2 Methods

2.2.1 HaCaT cell culturing

2.2.1.1 Preparation of cell culture media

FBS was thawed at 4°C overnight and then warmed in a 56°C water bath for 30 minutes to inactivate complement proteins. For routine subculture and growth experiments (cell counting and scratch assay), the "routine culture media" was used which contained DMEM with 10% v/v FBS and is termed "media" throughout this thesis. In the case of thawed cells, "enriched FBS media" was used (DMEM with 25% v/v FBS) while for freezing down cells, enriched medium+ %10 v/v DMSO was used.

2.2.1.2 Thawing frozen cells

Frozen HaCaT cells (1.5ml per vial at 2 million cells/ml of enriched culture media with 10% DMSO) were thawed rapidly with gentle agitation in 37ºC water bath over 1-3 minutes. As soon as the cells were completely thawed, they were resuspended in 8ml of enriched culture media. The cells were centrifuged at 300 × g for 5 minutes, and the supernatant containing the cryoprotectant (DMSO) was removed. The cells were then re-suspended in fresh full FBS media, transferred to a T25 cell culture flask at a density of 12 X 10^4 cells/cm^2, and incubated at 37ºC in 5% CO_2/95% air.

2.2.1.3 Changing culture media and cell passaging

The culture media was changed regularly at two days intervals. The medium was gently removed by a pipette strip and then 5mL fresh full culture media was added to the culture flask. During media changes, the cells were viewed under the microscope

to ensure that there were no signs of microbial contamination or abnormal debris from dead cells. When the cells were 70-90% confluent, the subculturing procedure was performed to replenish the nutrients, remove metabolic waste and create space for continuous cell growth.

To subculture the cells, the full culture media was removed and the cells were washed twice with 5ml phosphate buffered saline (PBS, pH=7.4). The cells were then incubated with 1 ml of trypsin for 10 min to ensure complete cell detachment from the culture flask. The cells were resuspended in 5ml of full culture media, neutralizing the trypsin. Typically the cells were diluted 1:4 with culture medium and adjusted to a final density of approximately 800 cells/cm^2 in a T25 cell culture flask (culture surface area 25cm^2).

2.2.1.4 Freezing HaCaT cells for long term storage

Before freezing down any cell aliquot, the cell pellet was re-suspended in freezing media (enriched media + DMSO) at 1 × 10^6 cells mL^{-1}. A 1.5 mL aliquot of the suspension was placed in a 2mL cryotube. The tubes were then placed in a polystyrene box and stored at -80^oC overnight. After that, the frozen cells were placed in liquid nitrogen for long-term storage.

2.2.2 HaCaT cell growth study

HaCaT cells were seeded in 24 well plates at 2 × 10^4 cells/well. Cell count was performed twice daily. To prepare the cells for counting, the media was removed from the wells and the cells were washed twice with 1ml PBS, before adding 200µl of trypsin (0.05% v/v). The cells were incubated for 10 minutes with trypsin to ensure complete cell detachment. Fresh routine culture media (800µl) was added to

neutralize the trypsin, and a 20µl aliquot of the resuspended cells from each well was loaded onto a haemocytometer for counting. For cell counting, 10µl of cell suspension was loaded into the two counting chambers. The counting areas were filled by capillary action and the haemocytometer slide put under a light microscope at 10X magnification. Cells located at the lower and right margins of the 5 squares were ignored. The total number of cells in two chambers (10 squares) provided the cell number per 1µl (Figure 2-1)

This process was repeated over seven days. The cell media was changed every other day during the experiment. The lag time, time to reach stationary phase and doubling time were calculated from the linear portion of the growth curve by plotting log cell numbers/ ml against time.

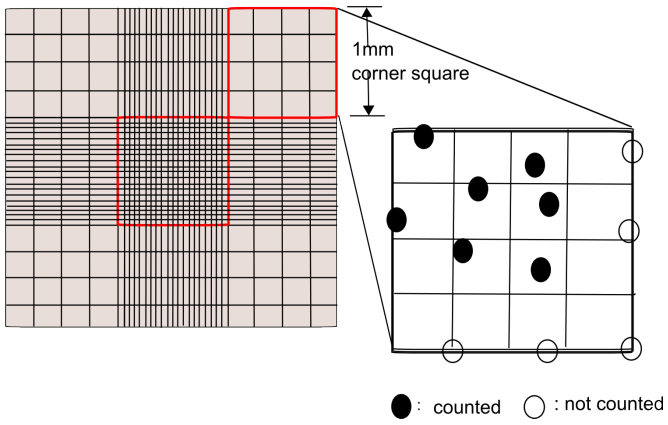

Figure 2-1 A schematic diagram of a hemocytometer counting area. The cells that located on the top left boundaries and within the $1mm^2$ square were counted, but those that located on the bottom or right boundaries were not counted.

HaCaT cell growth showed the expected phases including the lag phase, log phase and stationary phase (Figure 2-3). In the lag phase, the cells were adjusting to their new growth environment. Although, the cells increase in size, there was no net cell division and therefore no increase in numbers. The log phase was shown when the cells were dividing rapidly, and the cell numbers increased exponentially. During the stationary phase, cell death occurred at an equal rate as cell division, resulting in a constant cell count. A fourth phase, i.e. the death phase, may exist after the stationary phase due to continuing depletion of nutrients and build-up of metabolic waste. This phase was not observed here, which indicating that HaCaT cells can remain viable for a longer period than 7 days.

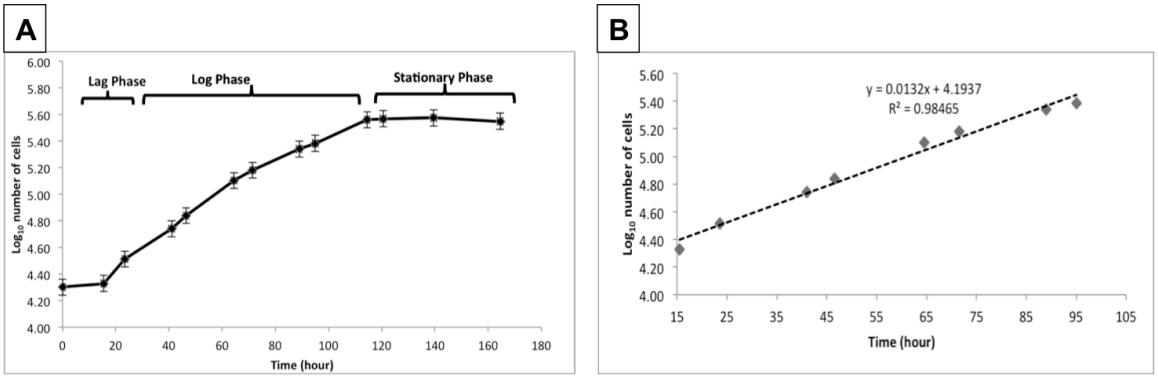

Figure 2-2 Figure 2-3 HaCaT cell growth characteristics under routine culture conditions (Mean ±SD, n = 4). A) The lag phase, log phase and stationary phase. B) The linearity of the exponential phase.

From the log phase of the growth curve, the linear portion was used to estimate the lag time through extrapolation, which was 8.05 hours. The time to reach the stationary phase was approximately five days and the doubling time was 22.9 hours, and these results are in agreement with previous studies performed on HaCaT cells, the doubling time was 23 hours (Boukamp et al., 1988) or 24 hours (Scheitza et al., 2012).

2.2.3 Hyperbaric oxygen and pressure control treatments

The hyperbaric chamber consisted of an oxygen combustion vessel with O_2 fill connection. The cells were put into the chamber and flushed with the gas mixture for 3 minutes. The inlet valve was closed tightly and then the gas mixture can be pressurized to 3 atmospheres absolute (ATA) (Figure 2-).

Two conditions were investigated using the hyperbaric chamber. One was the hyperbaric-hyperoxic condition (HBO) where the chamber was flushed with a gas mixture containing 95% O_2 and 5% CO_2 under 3 ATA, and the other condition was hyperbaric air (HBA), where 21% O_2, 5% CO_2, and 74% N_2 under 3 ATA was used. The dissolved oxygen concentrations were measured using a Jenway portable dissolved oxygen meter. The pH of the media was checked and maintained at 7.4. The dissolved oxygen level in the media after two hours was 1.89 mg/L and 9 mg/L under hyperbaric-normoxic and HBO conditions respectively (**Error! Reference source not found.**). The testing conditions were maintained for 2 hours before the cells were returned to an incubator at ambient condition (5% CO_2, 37^{o}C).

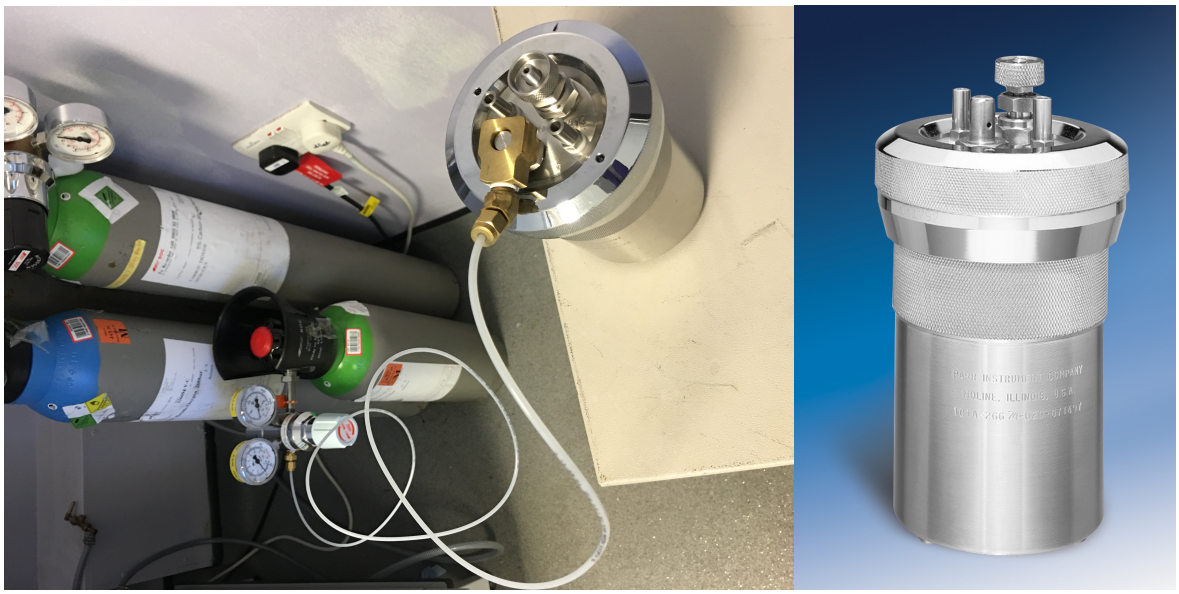

Figure 2-3 The oxygen combustion vessel and the connection unit that connected to the gas cylinder.

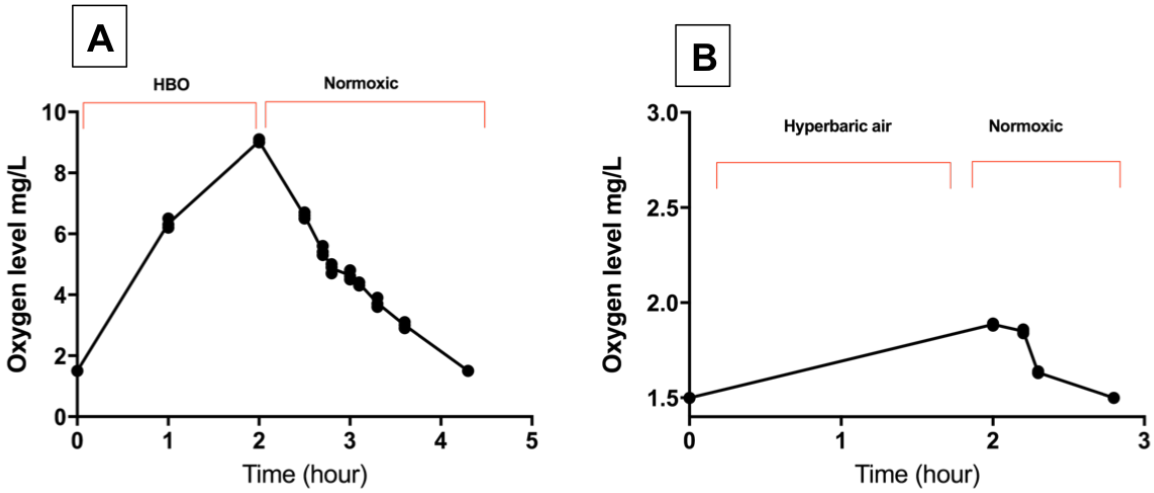

Figure 2-4 The levels of dissolved oxygen (mg/L) in cell culture media at different time points. The measurement performed prior to and after exposure of the cells to the treatment models i.e. HBO and HBA treatments. A. under HBO condition (95% O_2, 2ATA). B. under HBA conditions (21%O_2, 3ATA).

2.2.4 Pressure controlled and oxygen changing models (Hypoxic and hyperoxic conditions)

HaCaT cells were seeded at $2x10^4$ cells/ml and the culture flasks were placed in the experimental chamber. To achieve hypoxic and hyperoxic condition, a Modular Incubator Chamber MIC-101 was used. The modular chamber utilizes a surface-type seal where all portions of the O-ring are uniformly compressed by a stainless steel ring clamp for a reliable air-tight seal (**Error! Reference source not found.**).

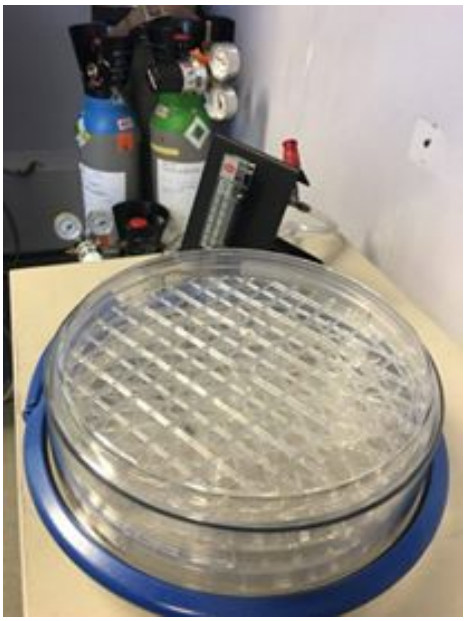

Figure 2-5 The Modular Incubator Chamber MIC-101 connected to gas cylinders with a two-stage regulator.

The hypoxic condition was created via flushing the chamber with a gas mixture containing 5% CO_2 and 95% N_2 for 3 minutes under 1ATA to achieve medium containing 0.1mg/l of dissolved oxygen (Figure 2-6). This oxygen level is close to the hypoxic state of an in vivo wound that ranged from 0.08-0.012 mg/L (Davidson and Mustoe, 2001). Then, the outlet and inlet valves were closed tightly to prevent gas

leaking. The chamber was then placed in an incubator at 37°C. A portable dissolved oxygen meter was used to measure oxygen tension in the media at different time intervals. The result demonstrated that the hypoxic condition was achieved within two hours following exposure of the culture media to the gas mixture and it reached to the full hypoxic state in five hours. The data also indicated that the oxygen level recovered to the normal level within two hours of removing of the culture plate from the chamber (Figure 2.7).

To achieve a normobaric hyperoxic condition (NBO), the modular chamber was flushed with a gas mixture containing 95% O_2 and 5% CO_2 for 3 minutes under 1ATA. The dissolved oxygen level was measured indicating a significant elevation in the dissolved oxygen level in the cell culture media (Figure 2-7). The hyperoxic condition was achieved within one hour (3.6mg/l) and reached to the maximum level in two hours (6.5mg/l). The oxygen level recovered two hours after removing the culture plate from the chamber.

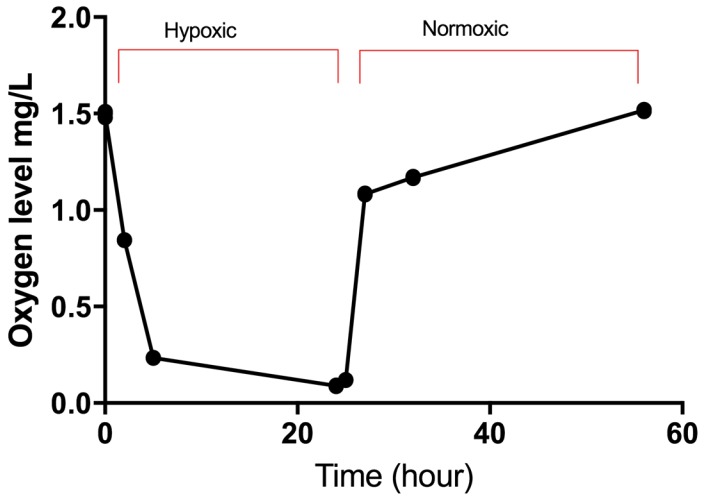

Figure 2-6 The change in the dissolved oxygen level in the cell culture media following exposure of HaCaT cells to hypoxic condition (95%N_2+5% CO_2 under 1ATA).

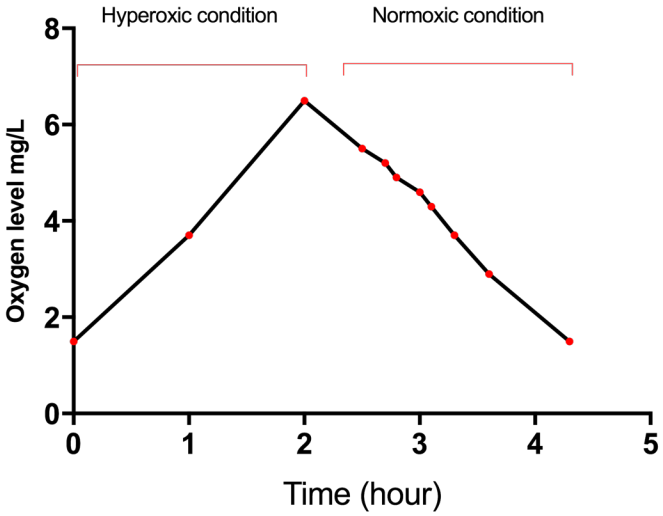

Figure 2-7 The change in the dissolved oxygen level in the cell culture media following exposure to hyperoxic condition (95%O_2+5% CO_2 under 1ATA).

2.2.5 Inflammatory model

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria. Theoretically, when keratinocytes are stimulated with LPS, they respond by releasing large numbers of cytokines. In the wounds, the primary infectious agents are Gram-negative bacteria. Therefore, this model aims to represent in-vivo inflammatory wounds. The physiological effects of these cytokines and their individual roles in wound healing processes have received very little attention. In the inflammatory model, the THP-1 monocyte cell line from acute monocyte leukaemia served as a positive control because monocytes are well known for their high response with LPS stimulation (Rossol et al., 2011).

IL-6 and IL-8 are the most common type of cytokines released from keratinocytes upon stimulation (Jiang et al., 2012, Song et al., 2002). The levels of these cytokines in the cell supernatants have been monitored via Enzyme-Linked Immunosorbent Assay (ELISA).

The inflammatory model was used to investigate proliferation, migration and differentiation of HaCaT cells under these conditions. The inflammatory model was sub-categorised into three different models. Firstly, the HaCaT cells were stimulated with a culture medium containing 100 µg/ml LPS. Secondly, the HaCaT cells treated with recombinant IL-6 and IL-8 separately as the primary cytokines detected in the HaCaT cell supernatant following LPS stimulation. Finally, the HaCaT cells were treated with the supernatant collected from THP-1 cells following stimulation with LPS, which contains various cytokines including TNF-a, IL-8, IL-6, etc.

2.2.6 ELISA

2.2.6.1 Antibodies

Three different cytokines in the HaCaT cell supernatants were analysed using ELISA (Table 2-1).

Specificity	Type	Clone
TNF-α	Capture	MAb1
	Detection	MAb11
IL-8	Capture	Monoclonal, clone name confidential
	Detection	Polyclonal
IL-6	Capture	MQ2-13A5
	Detection	MQ2-39C3

Table 2-1 The details of antibodies used in ELISA.

2.2.6.2 ELISA Protocol

Flat-bottomed 96-well maxisorp plates were coated overnight, at 4°C, with 100µl of purified monoclonal antibody. The primary antibody was removed, the plate was washed three times with 250µl of washing buffer (0.05% Tween 20 in phosphate buffered saline, PBS-T), then blocked with the blocking buffer (5% BSA) for 2 hours at room temperature. The plate was washed three times with 250µl PBS-T, and then 100µl of the standard (recombinant protein). The samples were added to the wells and incubated for 2hour at room temperature. Then, the plate was washed three times with washing buffer, 100µl of detection antibody was added to each well, and the plate was incubated for 1hour at room temperature. Next, the solution was

removed from the wells, the plate was washed three times with 250µl washing buffer, and 100µl of avidin-HRP was added to each well. The plate was incubated for 30 minutes at room temperature. Then the avidin-HRP solution was removed, and the plate was washed seven times with 250µl BPS-T. Afterward, 100µl of the substrate (TMB) was added to each well, and the plates were kept for 20 minutes at room temperature for color development. The colorimetric reaction was quantified at 450nm on an Epoch BioTeck spectrophotometer and the sample concentrations were determined from the absorbance with reference to a standard curve using Gen5 software Bio-Tek.

2.2.7 MTT

5 mg of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was dissolved in 1ml PBS. The MTT solution (20µl) was added to the wells containing 200µl of the culture medium and incubated for 3 hours at 37 °C. The MTT is reduced by metabolically active cells to generate intracellular purple formazan. Following the incubation, the media was aspirated from the well by a syringe. 100µl of DMSO was added to each well and incubated for 30 minutes at room temperature to dissolve the water insoluble purple MTT formazan product. Then, the plate was read on a Bio-Rad Benchmark Microplate Reader at 570nm.

2.2.8 Western Blot

2.2.8.1 Sample preparation

HaCaT cells were seeded and exposed to different treatment models. When the treatment ended, the media were removed and the cells were washed twice with ice cold PBS. Afterward, 150µl of ice-cold lysis buffer (RIPA +PIC) (Table 2-3) was

added to the wells and the cells were scraped and suspended in the lysis buffer. The samples were placed on ice for 5 minutes before centrifuging them with a cold centrifuge (13000 rpm for 15 minutes at 4^{o}C). Then, the supernatant (lysate) was collected and stored at -20^{o}C.

2.2.8.2 Protein quantification assay

To normalize the amount of the samples loaded onto the gel, a total protein quantification was performed using Pierce^{TM} BCA Protein Assay Kit in which, bovine serum albumin (BSA) was used as the standard with a calibration curve from 2000µg/ml to 25µg/ml.

Standard solutions	ddH_2O µl	BSA stock µl	Conc. µg/ml
A	0	300	2000
B	125	375	1500
C	325	325	1000
D	175	175	750
E	325	325	500
F	325	325	250
G	325	325	125
H	400	100	25

Table 2-2 BSA standard calibration curve dilutions (n=2).

20µl of the standard and samples were added to 96 wells and then 200µl of the Bradford reagent was added to each well to develop a colorimetric reaction. The plate was read on a Bio-Tek spectrophotometer at 562nm (Figure 2-8).

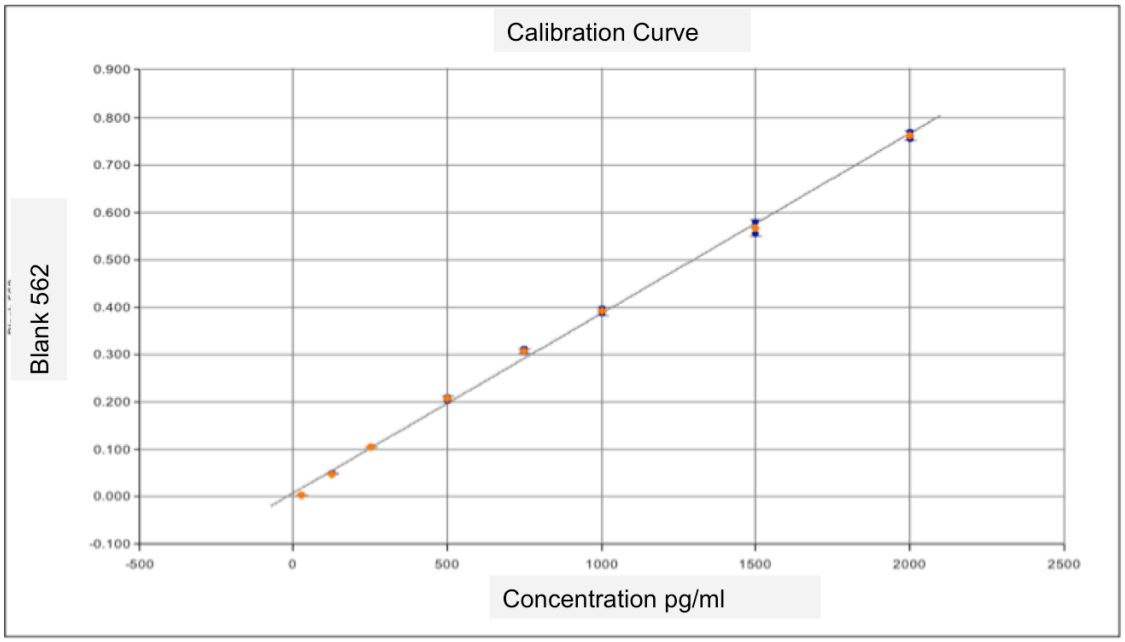

Figure 2-8 A representative standard calibration curve for protein quantification (R^2=0.99).

2.2.8.3 Gel electrophoresis

Mini-protean TGX precast gels containing 4-20% gel were used to perform electrophoresis with a Bio-Rad mini-protean tetra electrophoresis system apparatus (Figure 2-9). The system was powered by an HC power pack.

Figure 2-9 The components of the Bio-Rad mini-protean tetra electrophoresis apparatus. A. running tank B. comb and backing gel plates C. casting stand and frame D. mini-protean tetra cell casting frame and casting stand.

Cell lysate was mixed with an equal volume of loading buffer (Laemmli loading buffer 950µl+ 2-mercaptoethanol 50µl) (Table 2-3) and boiled for 5 minutes at 100 ^{o}C. Afterward, the sample was loaded on the gel alongside 5µl of Precision Plus Protein (Bio-Rad Lab Ltd, UK). The tank was filled with the running buffer to the marked level and the system connected to the power pack. The electrophoresis was performed at 0.03A for 40 minutes.

Buffers	Components
RIPA	150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0.
Laemmli	62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue
Running Buffer	25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3

Table 2-3 The components of the buffers used in the sample preparation and running process.

2.2.8.4 Protein transfer (Electroblotting)

The gel was removed from the cast and soaked with the transfer buffer for 5 minutes before putting it into a transfer cassette. The transfer cassette was made of two sponge pads, two filter papers, a nitrocellulose membrane and the gel (Figure 2-10). The nitrocellulose membrane was soaked in the transfer buffer for 5 minutes and the cassette was assembled and, placed in the Bio-Rad Mini Trans-Blot cell apparatus in a direction that allow the protein to migrate from the black negative cathode towards the red positive anode (Figure 2-11). The tank was then filled with transfer buffer to the marked level and an ice pack placed in the tank to prevent build up of heat. The system was connected to the power pack and electroblotting was performed at 100V for 1 hour.

Figure 2-10 The Mini Trans-Blot cell Apparatus including the transfer tank, transfer blot, cooling pack and transfer cassette.

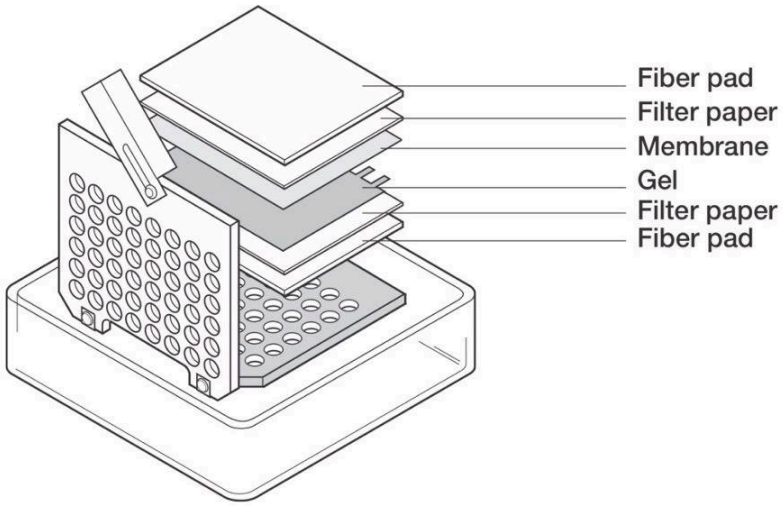

Figure 2-11 Assembling the gel transfer cassette.

2.2.8.5 Blocking and Immunoblotting

Once the transfer was completed, the membrane was removed from the cassette and washed once with ultrapure water. The membrane was blocked with 5% dry milk for 1hour at room temperature with constant gentle shaking.

Reagents	Components
Blocking solution	5% in TBS-T
Washing Buffer	Tris Buffer Saline+ Tween 20
ECL	1:1 of Clarity western peroxide reagent and Clarity western luminol/enhancer reagent

Table 2-4 The components of the reagents used for washing, blocking and colour development

Following the blocking step, the membrane was washed three times with washing buffer, 5 minutes each with vigorous shaking. Afterward, immunoblotting was performed by adding the primary antibody. The membrane was placed in a universal tube with the primary antibody (Table 2-5) and incubated overnight in a cold room with gentle shaking.

Once the incubation time completed, the membrane was transferred to a clean container and washed five times with washing buffer, 5 minutes each with vigorous shaking. Then, the secondary antibody was added to the membrane and incubated at room temperature for 1 hour with gentle shaking. Following the incubation period, the secondary antibody was aspirated, and the membrane was washed seven times with washing buffer at room temperature with vigorous shaking, 5 minutes each. Then, electrochemiluminescence (ECL) was added in a sufficient amount to cover the membrane and incubated at room temperature without shaking for 3 minutes.

After this, the membrane was removed from the container and any excess of ECL was removed through touching a corner of the membrane with a piece of tissue, The membrane was placed into a clear bag and sealed with a vacuum sealer. The colour developed was immediately read on the ImageQuant LAS 4000, which is a camera system that produces digital images of the chemiluminescent dyed membrane (Figure 2-12).

Primary antibody	Species raised in	Primary antibody Dilution	MW (kDa)	Secondary antibody dilution
PCNA	Rabbit	1:1000	35	1:1000
β- actin	Mouse	1:2000	45	1:1000
Involucrin	Rabbit	1:1000	140	1:1000
E-cadherin	Rabbit	1:10000	120	1:1000

Table 2-5 The details of the primary antibodies with their dilution rates. All antibodies were purchased from Abcam (Cambridge, UK) except β- actin that was obtained from Sigma Aldrich (Gillingham, UK).

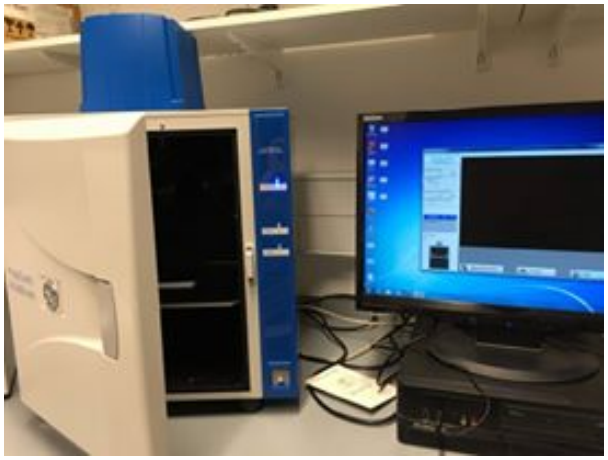

Figure 2-12 The ImageQuant LAS 4000, CCD camera-based quantitative biomolecular imager for quantitative imaging of gels and blots by chemiluminescence.

2.2.9 The sensitivity of the Elisa and Western blotting assays

Different proteins have been evaluated using Elisa and Western blotting. The sensitivity of the Elisa assay depends on the properties of the primary antibodies, the sensitivities of IL-6 and IL-8 were ranged from 8 pg/ml to 1000 pg/ml. However, the sensitivity of TNF-α ranges from 2-250 pg/ml. On the other hand, the protein expression of PCNA, e-cadherin and involucrin has been measured using Western blotting. The PCNA, e-cadherin and involucrin antibodies detect PCNA, e-cadherin and involucrin proteins in a total protein from 10ug with a predict band size of 29kDa, 97kDa and 68kDa, respectively.

2.2.10 Dissolved oxygen measurement

A portable dissolved oxygen meter (Jenway 970) was used to measure the dissolved oxygen level in the cell culture media. Before using the oxygen meter, it was calibrated with zero oxygen solution (sodium sulphite) and the membrane probe was polarized with the electrolyte solution (5% KCl) and placed in a beaker containing deionized water for 20 minutes. Afterwards, the oxygen level in the medium was measured at different time intervals.

Figure 2-13 Jenway 970 Dissolved oxygen meter.

2.2.11 Optimization of Scratch assay

2.2.11.1 General protocol

A reference line was etched into the bottom of each well on the outer surface of the plate using a cutter. HaCaT cells were suspended in routine culture media and seeded in 24-well plate. The cells were grown to a confluent monolayer over 2-3 days. A scratch was created in the cellular monolayer in each well, by pressing a sterile pipette tip (10µl, 200µl, 1ml or 5 ml) against the bottom of the well and dragging it across the cells in a smooth motion.

Following the creation of the scratch, the media was removed, and the wells were washed twice with 1 ml PBS per well to remove floating cells and debris. The cells were incubated with routine culture media at 37^{o}C under 5% CO_2/95% air. Images of the scratch were captured at 0hour, 8hour, 24hour, 36hour, 50hour & 60hour with a digital camera (DEM130E 1.3M pixel CMOS ship) fitted to a light microscope (Nikon, Eclipse.TS100) operated with Scopephoto software (63X). The scratch area was

measured, and the mean was determined for each time point. The scratch area covered by cells was determined by subtracting the final scratch area from the initial scratch area.

2.2.11.2 Role of media on the closure rate and wound consistency

To investigate the effect of changing the media on the rate of scratch closure, HaCaT cells were seeded in 12-well plates. Once the cells reached confluence, a scratch was created. The seeded wells were divided into three groups—group 1: media changed daily, group 2: media changed every two days, and group 3: media changed every three days. The scratch areas were measuring using ImageJ on photomicrographs of the scratches taken at 0hour, 8hour, 24hour, 36hour, 50hour & 60hour, as described previously. Further, the effect of media presence during the scratching was also investigated. A 24-well plate was divided into two sections. In one section, the scratching was carried out while the cells were immersed in routine culture media; in the other, the media was removed before scratching.

2.2.11.3 Optimization of the scratch width

Initially, the scratch was made using a 10uL, 200 and 1mL pipette tips. The scratches obtained were deemed too narrow to allow an accurate determination of the rate of wound closure since the “wounds” were fully closed within 2 days. Consequently, 5mL pipette tips were used. This created the widest scratch observable under the microscope at 4x magnifications, Table 2-6 and Figure 2-14. The width of the scratch area and the diameter of the pipette tips were also measured in ImageJ from photomicrographs and it showed a linear relationship between the tips diameters and the width of the scratch area.

Tip volume	Tip diameter ± SD (µm)	Initial scratch width ± SD (µm)
10µL	802 ± 18	568 ± 47
200µL	818 ± 19	584 ± 41
1mL	1274 ± 12	1107 ± 247
5mL	2182 ± 67	1805 ± 110

Table 2-6. Different tips diameters and their corresponding scratch widths.

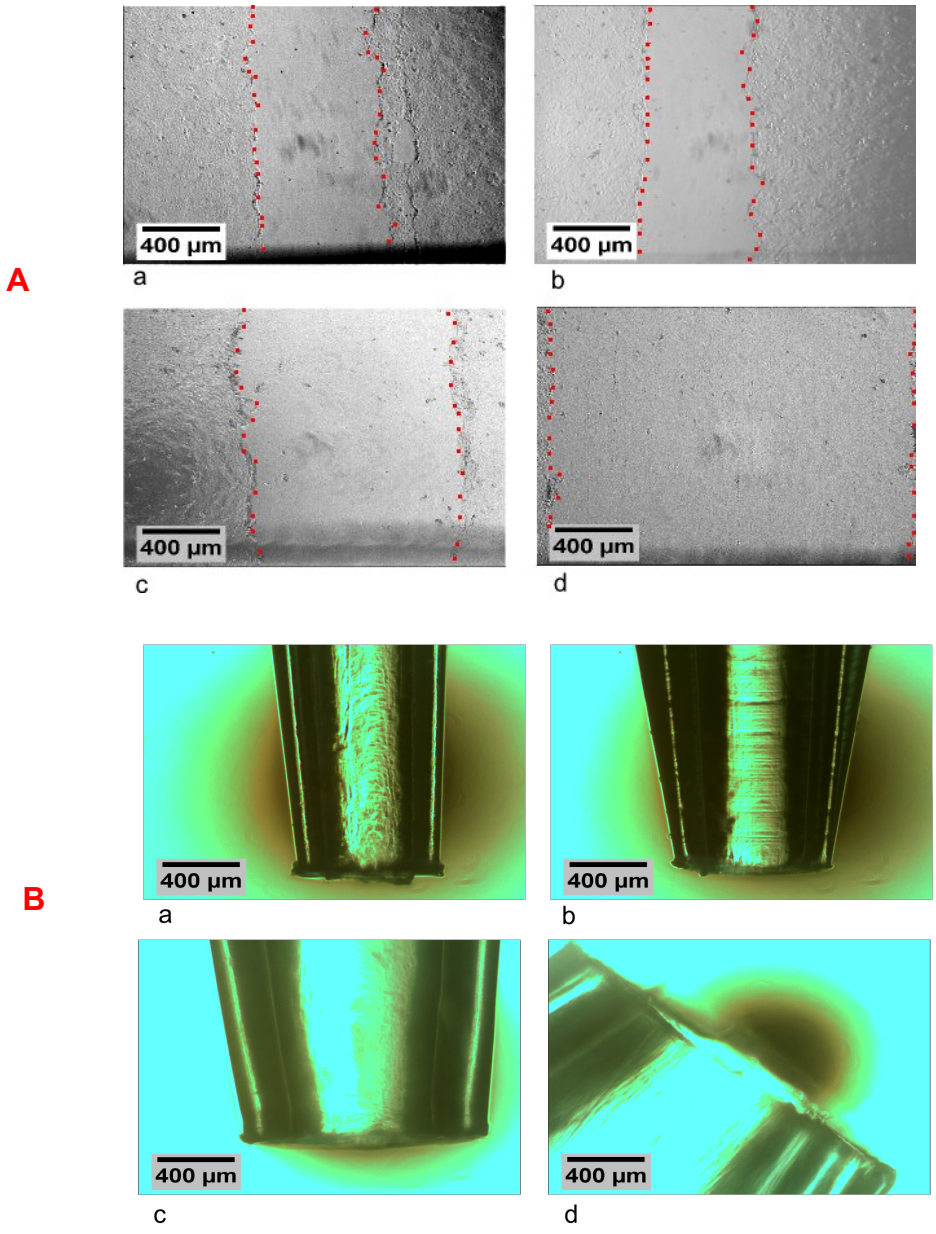

Figure 2-14 Different pipette tips and their corresponding scratch width. **A**: Scratch widths created by different tips a) 10µl tips, b) 200µl tips, c) 1mL and d) 5mL. **B**: Different tips images a) 10uL, b) 200uL, c) 1mL and d) 5mL.

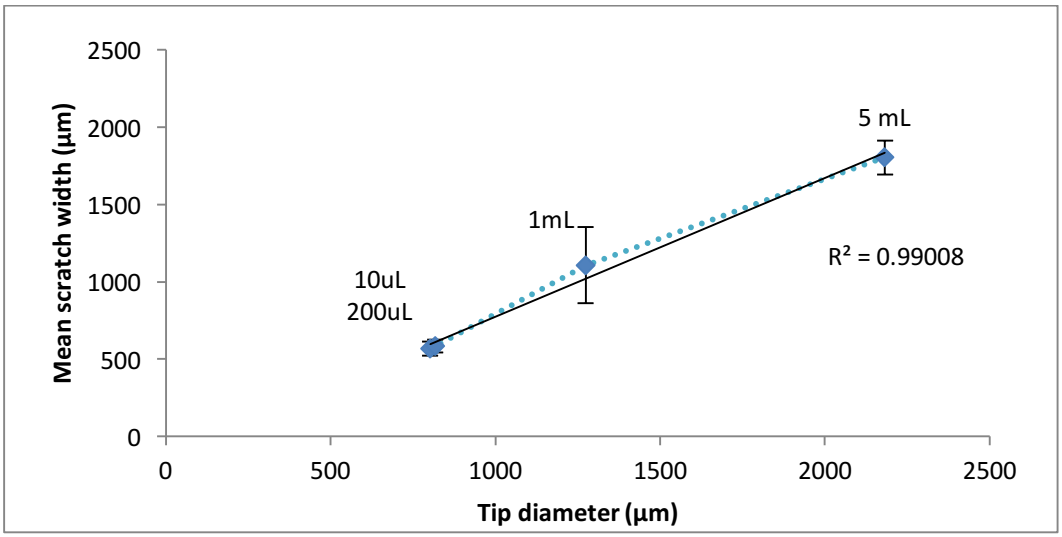

Figure 2-15 The relationship between pipette tip diameter and scratch width (10µl: 568µm±47, 200µl: 584µm ±41,1ml: 1107µm ± 247 and, 5ml: 1805µm±110., n=12).

Creating a scratch with culture media in the well resulted in greater well-to-well variations in scratch width (1543µm ± 238). The greater variation was likely due to the presence of culture media making the bottom of the wells more slippery, resulting in inconsistent traction between the pipette tip and the bottom of the wells. On the other hand, performing the scratching without media gave a more consistent scratch (1823 µm ±111) as shown in (Figure 2-16).

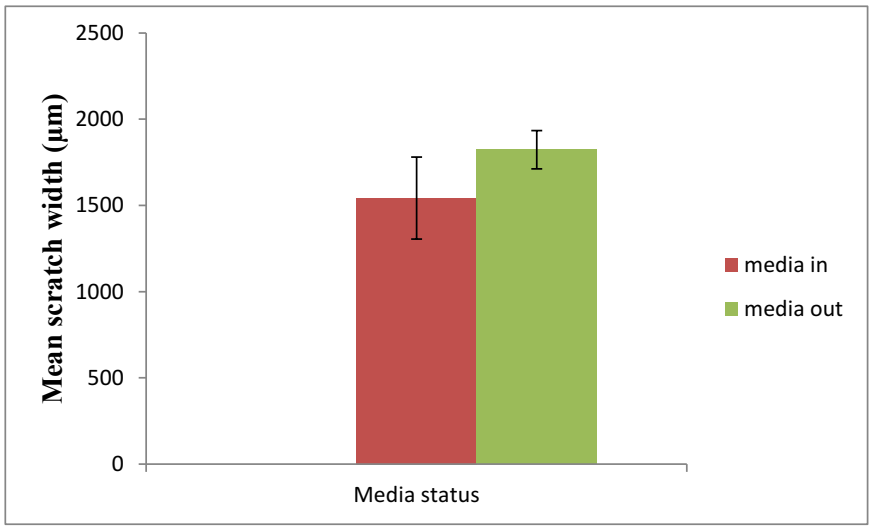

Figure 2-16 The role of media on the consistency of wound size (media in= 1543 ±238 µm and media out= 1823 ±111 µm, n = 3)

To determine the influence of media change on the rate of scratch closure, the area covered by cells was plotted against time. A linear trendline was forced through the origin and the associated R^2 value computed. The rate of scratch closure was determined from the generalised equation $y = mx$, where m = rate of scratch closure (Figure 2-17). The rate of scratch closure within each time segment was determined and shown in (Figure 2-18).

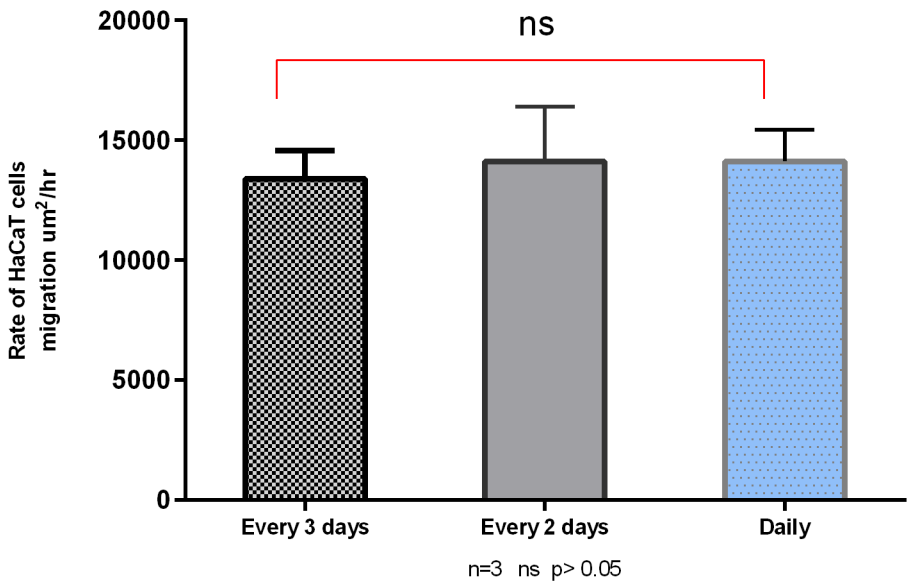

Figure 2-17 Effect of media change on the rate of scratch closure (micron^2/hour).

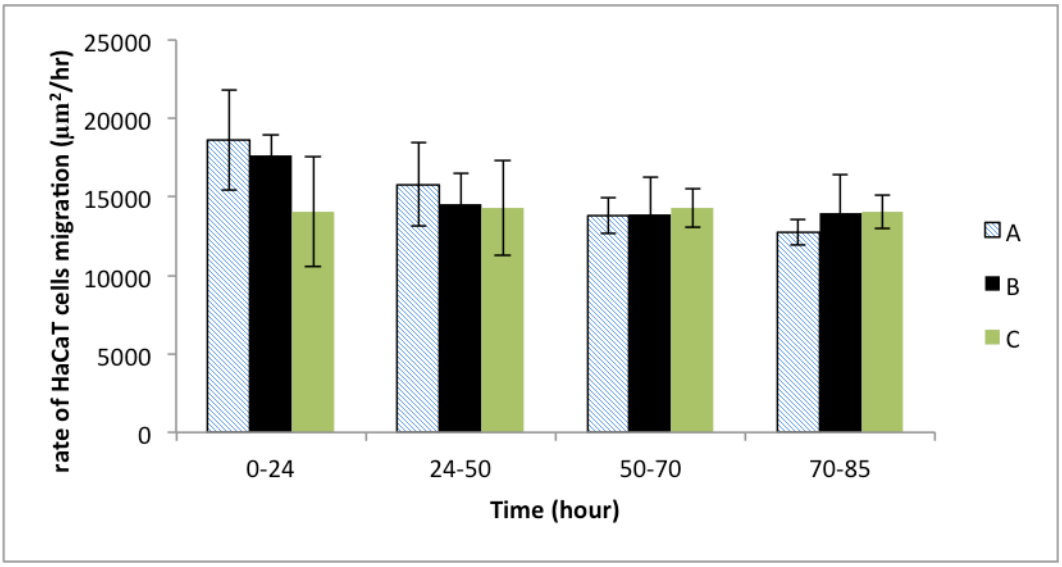

Figure 2-18 The rate of wound healing micron/hour. at different time intervals. A: media changed every 3 days. B: media changed every 2 days. C: media changed daily.

The rate of scratch closure when changing media daily is more consistent throughout the period of closure while fluctuation could be seen in the case of changing media every two or three days, most notably for days 1-2, suggesting a decrease in the rate of healing with time. However, the rate of wound healing was not statistically significant as p-value > 0.05 for all groups (media changing every three days, every two days and every day).

From the above, an optimized protocol was developed and can be summarized in the following steps:

1. Prior to seeding the cells in the well plate, a reference line was etched at the bottom of the plate on the outside surface.
2. Once the cells became confluent, media was removed prior to scratching
3. A scratch line dragged throughout the cells using 5ml tips.
4. The media was changed every two days because there was no difference between changing the media every day and changing the media every two days and also, to be in line with general protocol.
5. The scratched area photographed and analysed by ImageJ software.
6. The scratch area covered by cells was determined by subtracting the final scratch area from the initial scratch area.

2.2.12 Statistical analysis

Where appropriate, one-way ANOVA and the student's t-test were performed using the Prism software (GraphPad Software, Inc., San Diego, CA). Statistical significance was defined as $p < 0.05$.

Chapter III: The role of chronic hypoxia on HaCaT cells

3.1 Introduction

Wounds with blood vessel damage disturb blood supply to the microenvironment of the wound. Vascular disruption and high oxygen consumption by metabolically active cells rapidly cause tissue hypoxia (Bishop, 2008). In wounds where oxygenation is not restored, healing is impaired, and the wounds become chronic and difficult to heal. The inability of the vasculature to deliver enough O_2-rich blood to the wound tissue leads to, among other consequences, hypoxia. Tissue hypoxia reflects a reduction in oxygen delivery below tissue demand, which is one of the main characteristics of ischemic wounds (Biswas et al., 2010). Ischemic wounds are known to be associated with high treatment costs and can result in low quality of life.

Skin is naturally mildly hypoxic (Bedogni et al., 2005). The hypoxic nature of the skin becomes more severe once the skin suffers an acute injury with vascular disruption and from the elevated oxygen consumption by cells located at the edge of the wound (Ninikoski et al., 1972; Varghese et al., 1986). Prolonged tissue hypoxia makes a wound more vulnerable to bacterial infection that can also be associated with a prolonged inflammatory response, and eventually leads to tissue necrosis (Xia et al., 2001). Apart from ischemia, several systemic conditions, including advancing age and diabetes, can also reduce vascular flow creating a notably hypoxic wound with an oxygen tension of 5 to 20 mmHg (Guo and Dipietro, 2010). Although oxygen represents 21% of ambient air, oxygen concentrations within the human body range from 13.5% in the lungs, and 5%–12.5% in the circulation, to 0.6%–5% in tissues (Nauta et al., 2014).

Hypoxia is unavoidable in wounded tissue and delivery of adequate oxygenated blood to the wounded area is essential to promote the wound healing process. Following disruption to local blood vessels as a result of tissue injury, cell metabolism and oxygen consumption is increased, creating a hypoxic microenvironment (Davidson and Mustoe, 2001). However, it is believed that this hypoxic microenvironment then acts as a stimulus to tissue repair by creating an oxygen gradient between the hypoxic wounded tissue and the nearby adequately perfused tissue. It is this oxygen gradient that is thought to promote the diffusion of oxygen to the hypoxic tissue (Bishop, 2008). Throughout the course of the wound healing process, the wound center is generally hypoxic with an increasing oxygen gradient towards the intact tissue (Gordillo and Sen, 2003). The tissue oxygen tension ranges from 0-20mmHg at the wound center to 60-70mmHg at the periphery, while the oxygen tension in the arteries is approximately 100mmHg. This hypoxic environment remains until the end of the proliferative phase (Bishop, 2008). These temporary differences in oxygen tension are believed to act as a stimulus for migration and proliferation of endothelial cells and fibroblasts, and adequate angiogenesis to restore normal blood supply (Niinikoski et al., 1972a). If this process fails to occur, the prolonged insufficient vascular supply of oxygen leads to non-healing or chronic wounds, such as diabetic ulcers (Nauta et al., 2014). It is proposed that the gradient driving oxygen diffusion from the oxygenated blood is directly proportional to the cellular oxygen consumption at the wound site (Niinikoski, 1969).

According to some studies and review articles, although prolonged hypoxia delays the rate of wound healing, acute hypoxia is needed to serve as a stimulus and to trigger the wound healing process. It has been proposed that temporary hypoxia

induces cytokines and growth factors release especially from macrophages, including PDGF, TGF-β, VEGF, TNF-α, and endothelin-1. These cytokines and growth factors promote different stages of wound healing such as angiogenesis and re-epithelialisation (Guo and Dipietro, 2010). However, these studies did not explore the differences between re-epithelialization and overall wound closure (Rodriguez et al., 2008).

Keratinocyte migration and proliferation both play a crucial role in skin wound closure by the process of re-epithelialization. Impairments in this process are associated with the clinical phenotype of chronic non-healing wounds. Hypoxia as a key feature of ischemia is known to limit re-epithelialization (Biswas et al., 2010). Following injury, the keratinocytes start to migrate to the provisional wound bed within several hours. Within a few days after injury, these migratory keratinocytes proliferate to re-build the epithelium along with the basement membrane (Xia et al., 2001).

The migration, proliferation and protein synthesis of the endothelial cells and fibroblasts are active even though the wound is hypoxic. Later on, the oxygen level returns to normal levels via revascularization of newly formed granulation tissues. Thus, there is a growing interest in the influence of low oxygen tension on individual steps of the wound healing process. (Davidson and Mustoe, 2001). The main aim behind in-vitro studies of wound healing under chronic hypoxic environment was to explore the influence of both oxygen and ischemia on that process. (Guo and Dipietro, 2010). The need for experimental models representing the characteristics of ischemic wounds is compelling. Therefore, to investigate the pathological mechanism of wounds, the migration, proliferation, and differentiation of skin keratinocytes was

studied along with associated biochemical changes of wound healing using an *in vitro* chronic hypoxic model.

3.2 Results

3.2.1 The influence of hypoxia on migration and adhesion of keratinocytes

Scratch assays and Western blotting were used to evaluate the effect of chronic hypoxia on keratinocyte migration. HaCaT cells were seeded in six well plates (section 2.2.1) and scratch assay (section 2.2.10) was performed on the confluent cells before being exposed to hypoxic condition for 72 hours (section 2.2.4). The cell migration was measured and compared with cells treated in normoxic conditions. As shown in Figure 3-1, the hypoxic condition significantly reduced keratinocyte migration (hypoxic: 10641 ± 2105 μm^2/hr and normoxic: 16083 ± 2284 μm^2/hr, $p \leq 0.05$). Hence, delayed re-epithelialization was observed (Figure 3-2).

The pro-migratory activity of the cells was further assessed by measuring the expression of E-cadherin as a tight junction protein using Western blotting. The samples were collected from the scratched cells, and Western blotting was performed as explained in section 2.2.8. The result clearly indicated that prolonged low oxygen tension significantly up-regulated the expression of E-cadherin as an abundant protein tight junction in the keratinocytes, causing strong cell-cell and cell-matrix adhesion (Figure 3-4).

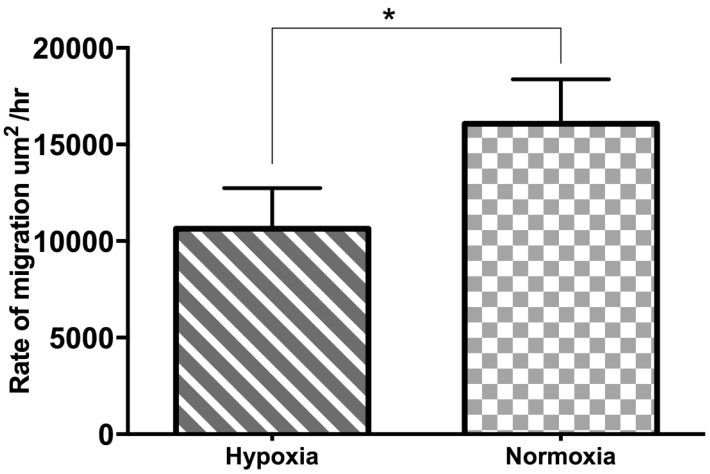

Figure 3-1 The rate of HaCaT cells migration (μm^2/hr) from scratch assay. HaCaT cells exposed to hypoxic condition in modular incubator chamber for 72 hour with normoxic condition being the control. The data presented as mean ± s.d. of three independent experiments (hypoxic: 10641 ± 2105 μm^2/hr and normoxic: 16083 ± 2284 μm^2/hr, n=3. *p ≤ 0.05)

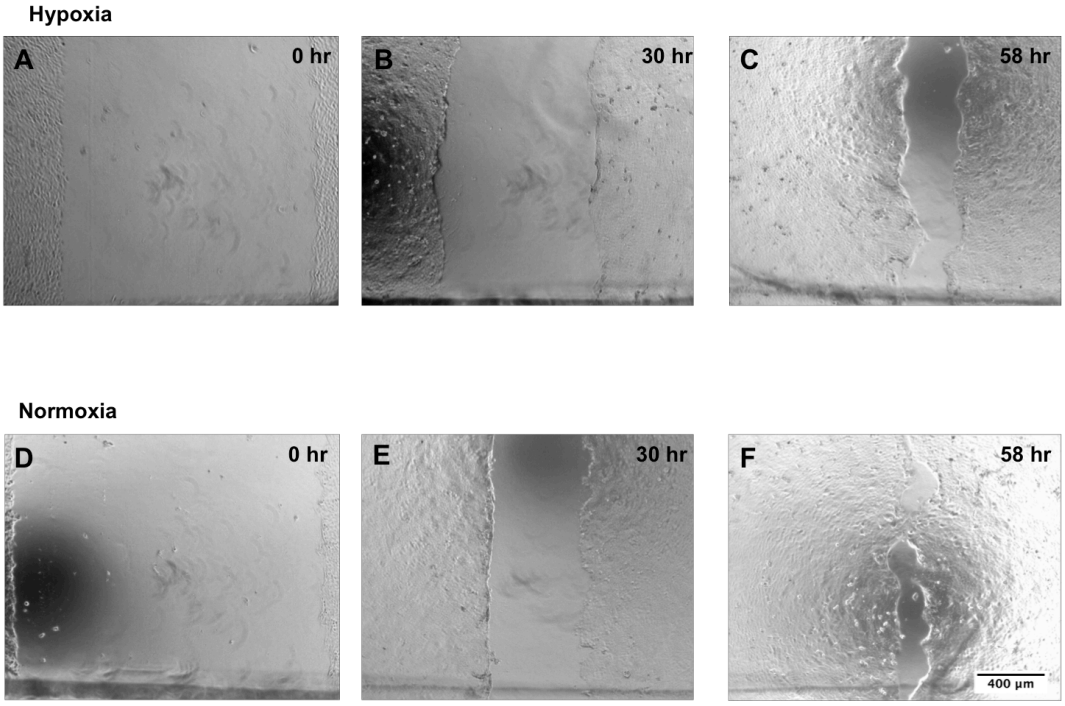

Figure 3-2 Representative images of the rate of HaCaT cell migration from the scratch assays at 0, 30, 58 hours. A-C representing HaCaT cell under chronic hypoxic condition and D-F representing HaCaT cells under normoxic condition.

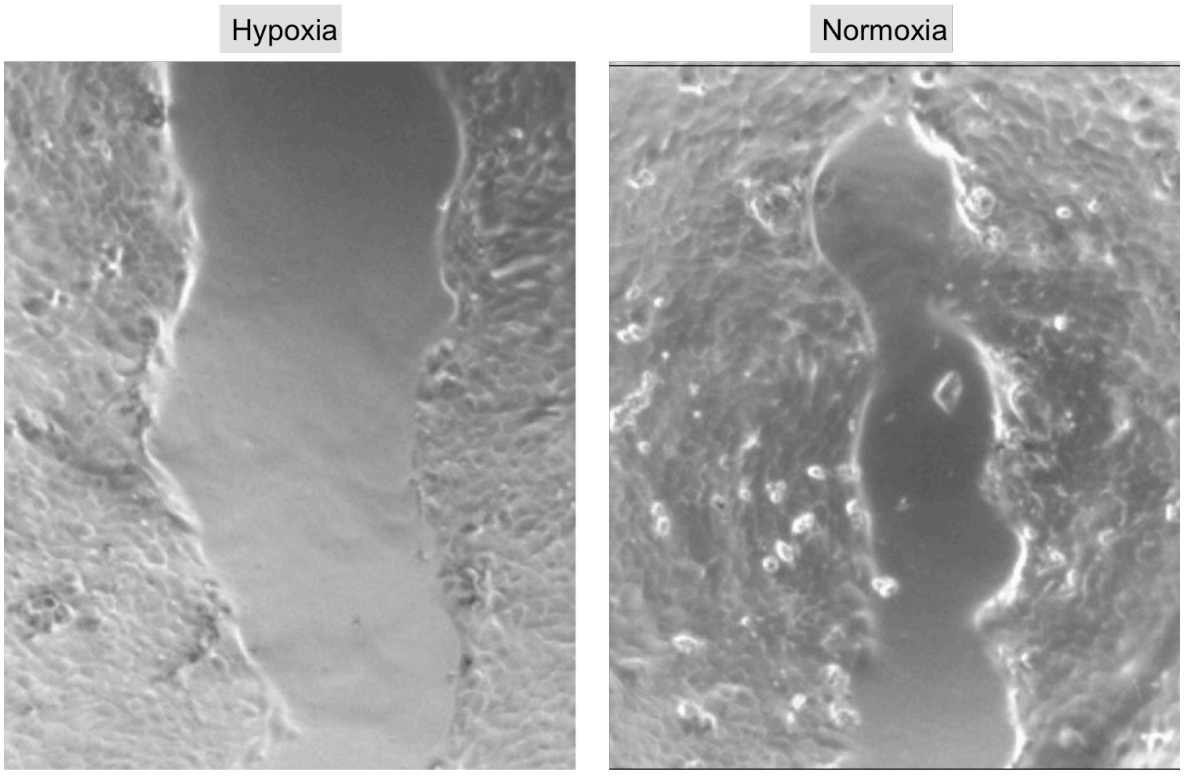

Figure 3-3 Representative images of scratch wound edges in hypoxia and normoxia models at 48hr.

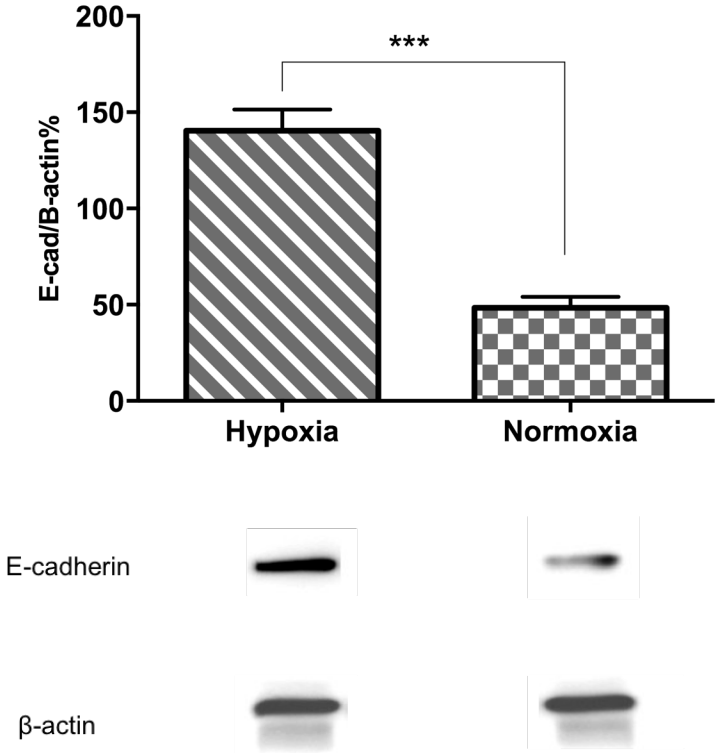

Figure 3-4 E-cadherin expression analysed by Western blot. HaCaT cells were exposed to hypoxic and normoxic conditions at 1 ATA for 72 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control. The histogram represents the numerical data of E-cadherin normalized against β-actin from the quantification of the bands and presented as mean ± s.d. of three independent experiments. (Hypoxic: 140.33±11.02% and normoxic 48.33±5.77% E-cadherin/β-actin%. n=3, ****p*Value<0.001).

3.2.2 The influence of hypoxia on proliferation and viability of keratinocytes

Apart from the pro-migratory activity, the proliferation profile of HaCaT cells was examined under the chronic hypoxic condition. To determine if these two wound healing processes i.e. migration and proliferation occur independently, MTT assay was used to determine metabolic activities and Western blotting for PCNA was used as a marker for cell proliferation. In contrast to the lower pro-migratory activity of

keratinocytes under chronic hypoxic condition, HaCaT cells metabolic activity (Figure 3-5) and proliferation increased with an over expression of PCNA (Figure 3-6).

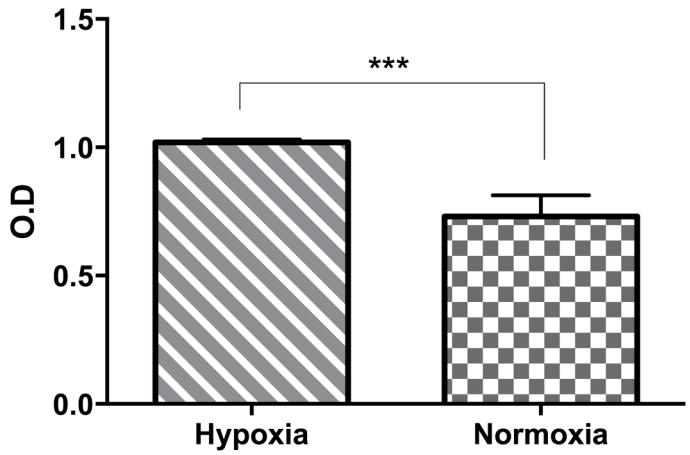

Figure 3-5 The viability of metabolically active HaCaT cells under chronic hypoxic and normoxic conditions using MTT assay (n=4, ***p≤0.001).

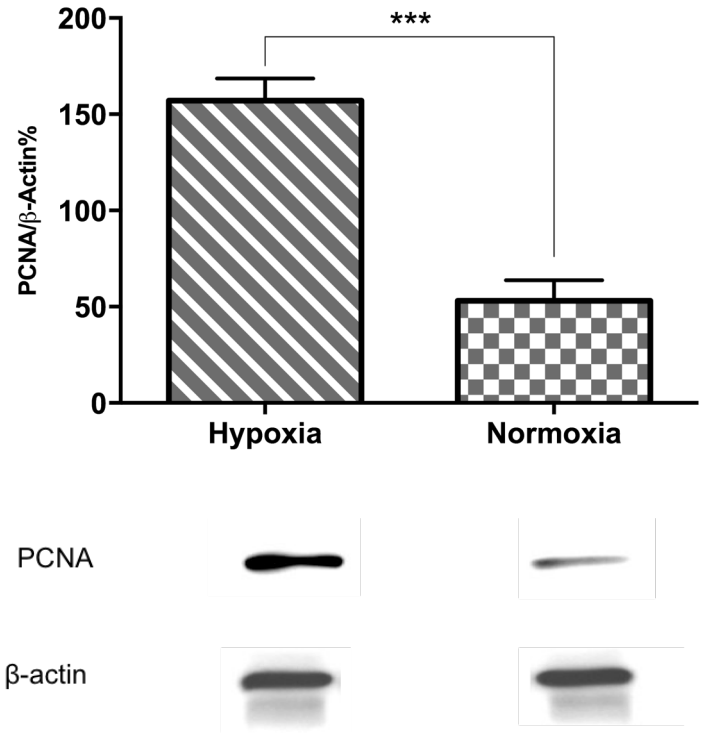

Figure 3-6 Expression of PCNA by HaCaT cells under chronic hypoxic and normoxic conditions at 1 ATA for 72 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control. The histogram represents the numerical data of PCNA normalized against β-actin from the quantification of the bands and presented as mean ± s.d. of three independent experiments., (hypoxic: 157% ± 11 and normoxic 53% ± 10 PCNA/ β-actin%, n=3, ****p*Value<0.001

3.2.3 The effect of hypoxia on differentiation of keratinocytes

In order to evaluate the influence of chronic hypoxia on the differentiation rate of keratinocytes, cultured HaCaT cells were exposed to the chronic hypoxic condition for 72 hours. Following the treatment, the cell lysates were collected and examined to evaluate the expression of involucrin as a terminal differentiation marker using Western blotting. Under chronic hypoxic condition, involucrin expression was 224.33

± 8.02% against 241.00± 21.07% in normoxic condition, showing no significant differences (Figure 3-7).

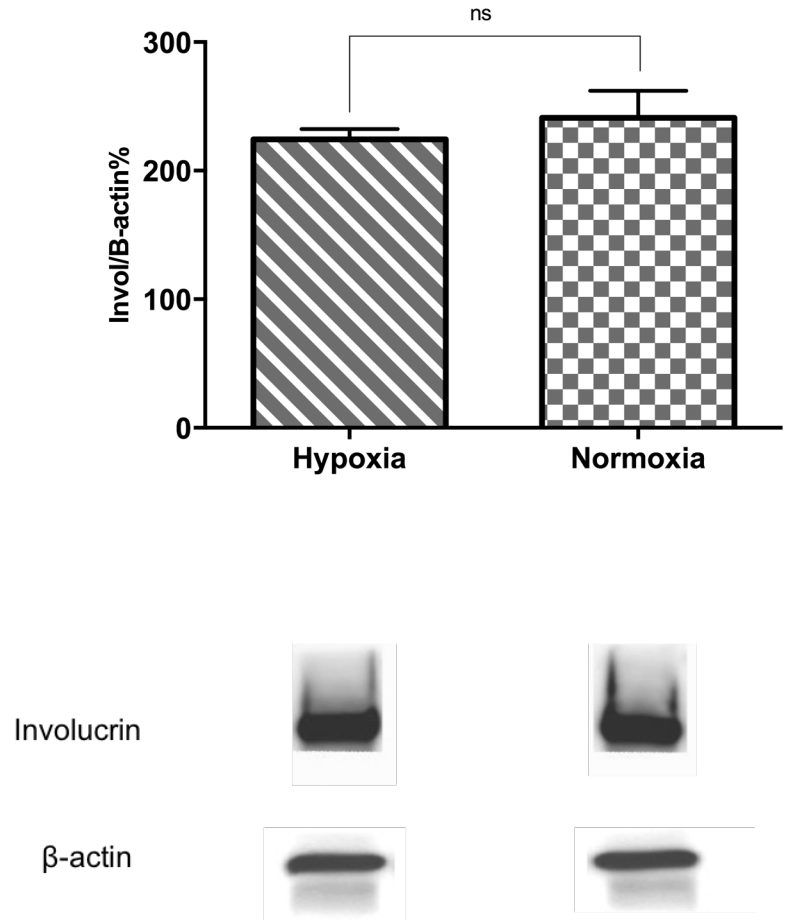

Figure 3-7 The effect of hypoxia on the Western blots analysis of involucrin. HaCaT cells were exposed to hypoxic and normoxic conditions at 1 ATA for 72 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control. The histogram represents the numerical data of involucrin normalized against β-actin (Mean ± s.d. of hypoxic: 224.33 ± 8.02% and normoxic: 241± 21.07%. n=3, ns *p*Value>0.05)

3.2.4 The effect of hypoxia on the release of IL-6 and IL-8

Expression of two pro-inflammatory cytokines (IL-6 and IL-8) was also examined using ELISA (section 2.2.6). Following cell exposure to the chronic hypoxic condition, a significant decrease in the expression of IL-6 was observed compared to the normoxic condition (0 ± 0 pg/ml and 51.4 ± 18.7 pg/ml respectively, Figure 3-7A). On the other hand, IL-8 expression was significantly reduced (491.9 ± 18.6 pg/ml and 907.8 ± 71.3 pg/ml respectively, Figure 3-7B). This demonstrated that chronic hypoxia significantly attenuates the expression of the pro-inflammatory cytokines IL-6 and IL-8 in HaCaT cells.

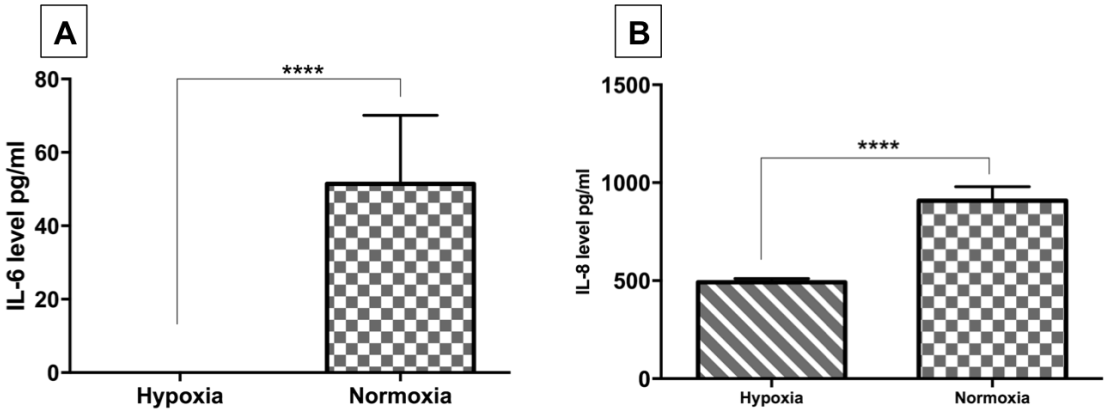

Figure 3-8 (A) IL-6 and (B) IL-8 levels in HaCaT supernant following 72 hours of treatment of the cells with chronic hypoxic condition quantified by ELISA. (The mean values ± s.d. of IL-6 in hypoxic and Normoxic conditions were 0 ± 0 pg/ml and 51.4 ± 18.7 pg/ml, respectively. The mean values ± s.d. of IL-8 in hypoxic and Normoxic conditions were 491.9 ± 18.6 pg/ml and 907.8 ± 71.3 pg/ml, respectively. n=3, ****p<0.0001)

3.3 Discussion

In this chapter, the effects of chronic hypoxia on human keratinocyte migration, proliferation and differentiation were investigated. Published studies on re-epithelialization do not provide a clear separate measurement to test proliferation and migration of keratinocytes. In this study, these two processes have been studied independently. The hypothesis behind the present study was that chronic hypoxia, as the main characteristic of ischemic wounds, could alter the keratinocyte activity and that this perhaps delays the re-epithelialization process. The present work was designed to demonstrate the mechanisms and the effects of chronic hypoxia on re-epithelialization of the skin in an *in vitro* model.

The rate of re-epithelialization is determined by several factors, including age, oxygen tension and matrix formation (Rezvani et al., 2011). Keratinocyte motility is an early event in the process of re-epithelialization. They migrate from wound margins horizontally towards the wound bed and eventually proliferate to close the gap caused by injury (Clark, 1996). Moreover, migration occurs within several hours of injury whereas proliferation begins few days after the migration step (Xia et al., 2001). It was proposed that the proliferation of keratinocytes is determined by the availability of adequate growth factors, cell attachment to the substrate and cell differentiation. In the re-epithelialization process, keratinocytes proliferate to ensure an adequate supply of the cells for successful wound closure (Pastar et al., 2014). Throughout cutaneous wound regeneration, keratinocytes at the wound edges undergo functional and phenotypic modifications. These changes are characterized by disruption of cell-cell interactions and cell-substrate adhesion (Chavez et al., 2012).

The effect of hypoxia has been previously examined in some systems, including dermal fibroblasts and arterial remodeling, and demonstrated that temporary hypoxia stimulates fibroblasts from young patients (aged 24-33 years) to migrate towards the center of a wound and to produce collagen and other various extracellular matrixes (Falanga et al., 1993; Helfman and Falanga, 1993). Other studies suggested that prolonged hypoxia (such as those found in chronic wounds in aged population) caused a detrimental effect on wound healing (Wu et al., 1999; Eaglstein and Falanga, 1997; Eaglstein, 1989; Wu et al., 1997). However, *in vivo* studies reveal that under hypoxic conditions, a significant delay in re-epithelialization of ulcerated wounds was found (Kairuz et al., 2007). In term of *in vitro* studies, chronic hypoxia is defined as exposure of the cultured cells to a hypoxic environment for more than 40 hours (Weir et al., 2011).

Interestingly, these findings clearly indicated that exposure of keratinocytes to chronic hypoxic conditions for 72 hours resulted in a significant reduction of keratinocyte migration and their motility. Furthermore, the decreased migration of keratinocytes correlated with over expression of E-cadherin, which in turn increases cell adhesion. However, future studies can be carried out investigating the influence of impacts of chronic hypoxic state on MMPs expression, which is also a key factor in determining keratinocytes movement. These results are consistent with literature studies that chronic hypoxia impairs the entire wound healing process. (Schreml et al., 2010), (Hunt and Pai, 1972), (Niinikoski, 1969), (Siddiqui et al., 1996) and (Wu et al., 1999). Also, these findings are in accordance with the fact that, while acute hypoxia promotes keratinocyte migration, chronic hypoxia exerts inhibitory effects on

wound healing processes (Wu et al., 1999; Eaglstein and Falanga, 1997; Eaglstein, 1989; Wu et al., 1997).

These findings also provided insight into the mechanism of the deleterious effect of chronic hypoxia on re-epithelialization. Soon after injury, keratinocytes at the wound margins lose their adhesion to each other and to the basal lamina, which in turn promotes their migration and accelerates wound closure. This process is achieved via dis-assembly of cell–cell and cell-matrix bindings of desmosomes (Heng, 2011). Then, the keratinocytes start to proliferate to supply the migratory edge with fresh cells (Pastar et al., 2014). The breakdown of the cell-cell junctions allows the keratinocytes to migrate. The disruption of desmosomes and hemidesmosomes required activation of PKCa which in turn converts calcium-independent to calcium-dependent desmosomes, thus disassembling of the links between the cells and the cell-matrix links (Pastar et al., 2014).

The Western blotting results demonstrated that exposure of keratinocytes to chronic hypoxic condition induces the expression of E-cadherin and thus promotes cell adhesion. These results are consistent with other studies regarding the impact of protein tight junctions on keratinocytes motility (Alt-Holland et al., 2008; Asai et al., 2016). Recent study indicated that keratinocytes can express podoplanin at the edge of the wounds and inhibition of podoplanin via transfection with podoplanin siRNA impaired keratinocyte motility. This impairment in motility was correlated with up-regulation of E-cadherin, indicating that E-cadherin is exerting an inhibitory effect on keratinocyte migration (Asai et al., 2016). In HaCaT cells, the up-regulation of E-cadherin expression via integrin α6β4 promotes cell-cell adhesion, which in turn decreases keratinocyte migration (Hintermann et al., 2005). Another study

investigated the migratory behaviour of HaCaT cells in relation to the E-cadherin expression and revealed that loss of cell adhesion enabled migration of the cells (Alt-Holland et al., 2008).

In contrast to the attenuated pro-migratory behavior of keratinocytes under chronic hypoxic condition, their proliferation increased, and the cells became hyperproliferative. These observations were confirmed by Western blot and MTT assay. Western blot analysis showed a significant up-regulation of PCNA, a proliferative marker, which was consistent with the MTT analysis, which demonstrated increased metabolic activity. These results are extremely interesting, given that chronic hypoxia provokes a highly proliferative wound edge; the wound closure is actually delayed due to the attenuated pro-migratory behavior of keratinocytes.

The phenotypes and biology of keratinocytes at the chronic wound margins is different from those of intact epidermis and acute wounds. Due to the activation and over expressions of c-Myc in the suprabasal keratinocytes from chronic wounds, they create a hyperproliferative epidermis. However, in the normal skin, only basal keratinocytes undergo active proliferations (Pastar et al., 2014).

Other studies have documented this independent proliferation behaviour from pro-migratory activity. It has been demonstrated that TGF-β inhibits keratinocytes proliferation without a significant delay in the re-epithelialization process, suggesting that TGF-β exerts pro-migratory effects on keratinocytes (Garlick and Taichman, 1994). However, due to the complex regulatory effects of hypoxia on re-epithelialization and the limited information from *in vivo* studies on hypoxia, further

investigation is needed to determine the influence of hypoxia on migration and proliferation of keratinocytes in correlation with other elements involving in the wound healing process.

Previous studies have shown that low oxygen tension in the wound site decreases the production of cytokines including IL-2 and IL-8 (Gottrup, 2004). These cytokines play a crucial role in activating monocytes and probably the endothelial cells (Rodriguez et al., 2008). However, in the wound site, these cytokines have multiple sources including monocytes and keratinocytes. Here, the influences of chronic hypoxia on the production of IL-6 and IL-8 in keratinocytes were examined using ELISA analysis. The result showed that chronic hypoxia down-regulates the expression of both IL-6 and IL-8. These results indicated that, apart from re-epithelialization, chronic hypoxia may exerts broader impacts on whole process of wound healing given that interleukins play a pivotal role as powerful chemoattractants for neutrophils to the wound site (Jiang et al., 2012). Therefore, these observations regarding the decreased level of these cytokines under chronic hypoxia could, at least partially, explain the deleterious mechanism of chronic hypoxia on the entire wound healing process.

The influence of chronic hypoxia on keratinocytes differentiation was also investigated through expression of involucrin, which is a terminal differentiation marker. The results demonstrated that chronic hypoxia did not significantly affect the expression of involucrin thus indicating the differentiation rate was not affected. This observation indicated that hyperproliferative behavior is not correlated to the increasing in cell differentiation rate, in agreement with other studies (Micallef et al., 2009 and Cho et al., 2008). Micallef et al (2009) examined the influence of

extracellular calcium on the proliferation and differentiation of cultured HaCaT cells; the resulted revealed that HaCaT cells converted to hyperproliferative cells with delayed expression of involucrin as a terminal differntiation marker (Micallef et al., 2009).

Previous studies have examined the connection between hypoxia and human keratinocyte differentiation in the context of the subsequent influence of hypoxia inducible factor HIF-1α (Cho et al., 2008 and Park et al., 2016). However, the sources and impact of HIF-1α is very controversial, and hypoxia is not the only stimulant for HIF-1α expression. Over-expression of HIF-1α is also observed in hyperproliferative keratinocytes in psoriasis (Rosenberger et al., 2007) and HIF-1α expression in LNCaP cells (prostate cancer cell line) seems to be higher under hyperoxic condition (Terraneo et al., 2014). Another study indicated that HIF-1α levels increase in *in vitro* wound models under normoxic condition. These results suggest that local hypoxia is not the sole driver for up-regulation of HIF-1α in the epidermal cells (Fitsialos et al., 2008). Apart from hypoxia, keratinocytes can modulate the expression of HIF-1α through releasing various factors including PDGF, IL-1, lL-8, TGFA and LIF (Fitsialos et al., 2008).

Taken together, these studies demonstrate that chronic hypoxia significantly attenuates the migration of keratinocytes with a substantial increase in E-cadherin expression. The role of chronic hypoxia on keratinocyte proliferation was also investigated and it was found that, under deprived oxygen tension, keratinocytes converted to hyperproliferative cells, which was correlated with up-regulation of PCNA expression and high metabolic activity. However, chronic hypoxia seems to have no effect on differentiation rate. Additionally, the data showed that chronic

hypoxia significantly reduces the production of IL-6 and IL-8, which may account for, at least partially, the broader impacts of hypoxia on the entire wound healing process.

3.4 Conclusion

In conclusion, these results indicate that low oxygen tension in the wound microenvironment exerts a substantial impact on the keratinocytes migration and proliferation. Taken together, the data suggest that keratinocyte migration and proliferation are differentially regulated by oxygen tension, but that oxygen tension *per se* does not affect differentiation. Interestingly, incubation of keratinocytes under chronic hypoxic condition resulted in delayed scratch closure. Although under prolonged hypoxia keratinocytes are modified to hyperproliferative cells, they lose their pro-migratory behaviour with marked expression of E-cadherin protein. However, chronic hypoxia could act differently in complicated models such HSE because the current model is consist of simple monolayer cells.

The results also provide insights into the broader effects of chronic hypoxia via attenuation of IL-6 and IL-8 expressions by keratinocytes. These cytokines are very well known for their stimulatory effect on the activation of other steps involving in wound healing process.

These findings have implications for future research on chronic wounds, suggesting that, the problems of chronic wounds are mostly related to the attenuation in keratinocyte migration than their proliferation profile. Therefore, it is important to perform further investigations targeting the motility of keratinocytes of chronic wounds.

Chapter IV: The role of hyperbaric oxygen on HaCaT cells

4.1 Introduction

Hyperbaric oxygen therapy (HBO) is defined as intermittent breathing of 100% oxygen at pressures higher than normal atmospheric pressure (Hampson, 1999). This can result in increasing arterial and tissue oxygen tension from 100 mmHg and 40 mmHg to 2000 mmHg and 400mmHg, respectively (Tibbles and Edelsberg, 1996). HBO is extensively used for selected problematic and infectious wounds (Grundmann et al., 2000). However, the underlying cellular and molecular mechanisms whereby HBO induces wound healing are not well understood. Oxygen is essential in wound healing due to the hypoxic nature of chronic wounds (Davidson and Mustoe, 2001 and Hunt and Pai, 1972). Indeed, *in vivo* studies demonstrated that HBO enhances wound healing and decreases wound complications including major amputation (Kessler et al., 2003 and Faglia et al., 1996). However, *in vitro* studies are of crucial importance to understand the underlying cellular and molecular changes in response to HBO.

A critical step in wound healing is re-epithelialization, dominated by migration, proliferation and differentiation of keratinocytes and restoration of the basement membrane at the wound site (Martin, 1997). The disturbed vascular oxygen supply to the wounded area is the rate-limiting factor for re-epithelialization (Kairuz et al., 2007). Results from chapter three demonstrated that chronic hypoxia significantly attenuated the migration of HaCaT cells with increased cell adhesion. Therefore, it is feasible that HBO could enhance re-epithelialization. Failure in the re-epithelialization process leads to chronic wounds. Thus, it is important to explore the cellular and molecular mechanisms to promote re-epithelialization and wound closure (Raja et al., 2007). In the re-epithelialization step, the keratinocytes migrate from wound edges to

the center, at which cell-cell contact inhibits further migration, completing re-epithelialization (Chen et al., 2016). Re-epithelialization proceeded more rapidly in response to high oxygen levels. However, the mechanisms by which HBO affects re-epithelisation, including keratinocyte proliferation and differentiation, remain unknown (Kairuz et al., 2007).

Keratinocytes are the major cells in skin epidermis constituting 90% of all cells and require a continuous oxygen supply to maintain their cellular functions (Gill and Bell, 2004). Thus, molecular oxygen is an essential element for the regeneration process in wound healing (LaVan and Hunt, 1990). It has been reported that oxygenation of ischemic wounds at 1-2 ATA enhances wound healing (Lee et al., 2006) and wound healing is primarily controlled by available oxygen (Hunt and Dunphy, 1969). The partial pressure of oxygen (pO_2) is positively correlated with angiogenesis (Hopf et al., 2005), collagen deposition (Warriner and Hopf, 2012) and epithelialization (Uhl et al., 1994). Therefore, augmenting oxygen availability to tissues via HBO would promote cutaneous wound regenerations (Suh and Hunt, 1998 and Kalliainen et al., 2003).

Currently, there are limited *in vitro* studies on the influence of HBO on re-epithelialization processes. However, re-epithelialization proceeds optimally at high oxygen tensions (Ladizinsky and Roe, 2010). Previous studies indicated that HBO enhances proliferation of fibroblasts and endothelial cells (Lipsky and Berendt, 2010). Moreover, it increases keratinocytes migration and differentiation in a human equivalent model (Dimitrijevich et al., 1999 and Tompach et al., 1997) and, induces VEGF expressions (Sheikh et al., 2000).

It was found that following short exposure to HBO treatment (1.5 hour), the subcutaneous oxygen level remains elevated for several hours (Ladizinsky and Roe, 2010). The delivery of oxygen to the central area of the wound depends on the partial pressure gradient and it is proportional to the square of the diffusional distance (Rollins et al., 2006). Thus, due to the impaired blood supply to the wound site, the pO_2 at the wound site is very low and HBO is the only possible method to deliver oxygen to the center of the wound (Faglia et al., 1996 and Silver, 1969). Increasing oxygen tension at the low perfused wound site via HBO has shown a positive wound healing outcome (Rollins et al., 2006). Therefore, HBO is used clinically in the treatment of non-healing ischemic wound. However, the cellular mechanism underlying these positive outcomes is still not fully understood (Brismar et al., 1997). Despite the very promising results obtained from clinical studies in chronic and ischemic wounds, *in vitro* studies are needed to validate the underlying mechanisms of HBO effect. This chapter examines, at a cellular level, the effects of HBO therapy on migration, proliferation and differentiation of keratinocytes, being the predominant cell type in human epidermis.

4.2 Results

4.2.1 HBO on migration and adhesion of keratinocytes

To determine the impact of HBO on keratinocytes migration and their adhesion activity, HaCaT cells were divided into two groups i.e. normoxic at 1 ATA and HBO at 3 ATA, seeded in six well plates (section 2.2.1) and scratch assays were performed on monolayers of the cells (section 2.2.10). The

normoxic group was incubated in a standard 5% CO_2 incubator and the HBO group was incubated in the HBO chamber for 2 hours (section 2.2.3) before being placed inside the 5% CO_2 incubator. Cell migration across the scratched area was photographed and analysed, and shown in Figure 4.1

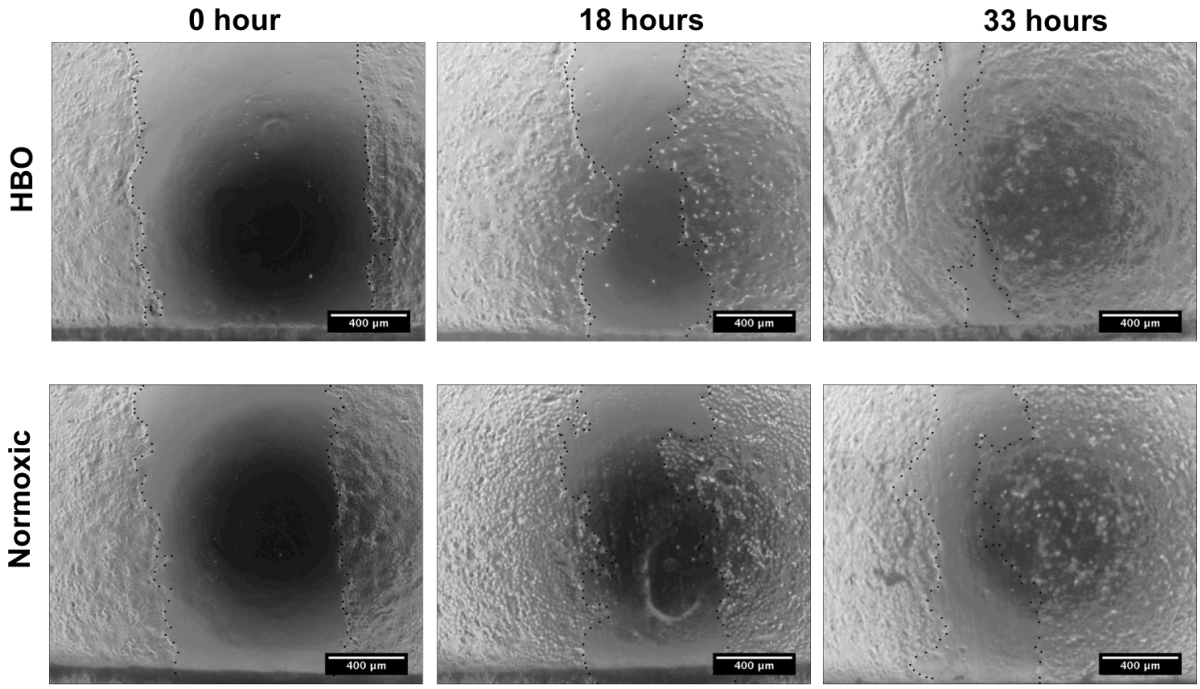

Figure 4-1 Representative images of the rate of HaCaT cell migration from the scratch assays at 0, 18, 33 hours under HBO and Normoxic conditions. The keratinocyte migration's rates were 35697± 1159µm^2/hr and 18191 ± 1347µm^2/hr under HBO and normoxic conditions, respectively.

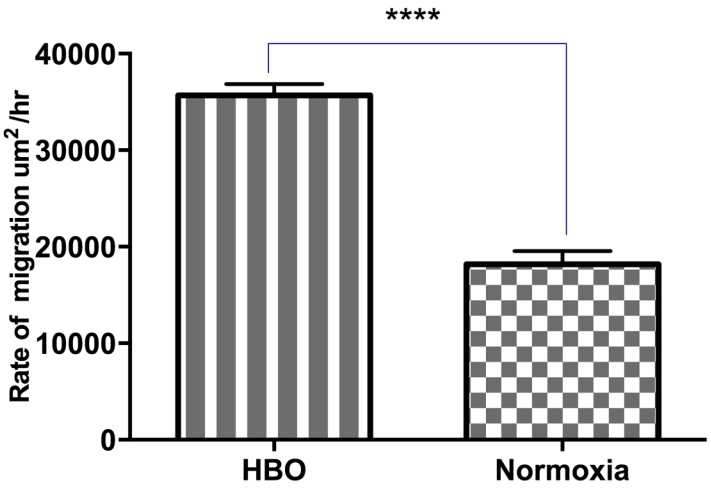

Figure 4-2 The rate of HaCaT cells migration (μm^2/hr) from scratch assay where HaCaT cells were exposed to HBO condition for 48 hour against Normoxic control. (HBO: 35697± 1159μm^2/hr and Normoxic: 18191 ± 1347μm^2/hr., n=3, ****$p \leq 0.0001$)

The effects of hyperbaric oxygenation were further investigated in terms of cell adhesion. Western blotting was performed from the cell lysates (section 2.2.8) and the expression of E-cadherin (adhesion protein) in HaCaT cells was analysed and normalized against β-actin (Figure 4-3). The mean values were 17± 7% and 52± 3% under HBO and normoxic conditions, respectively. These results indicate that HBO significantly attenuates the expression of E-cadherin in keratinocytes, thus reducing their adhesion propensity.

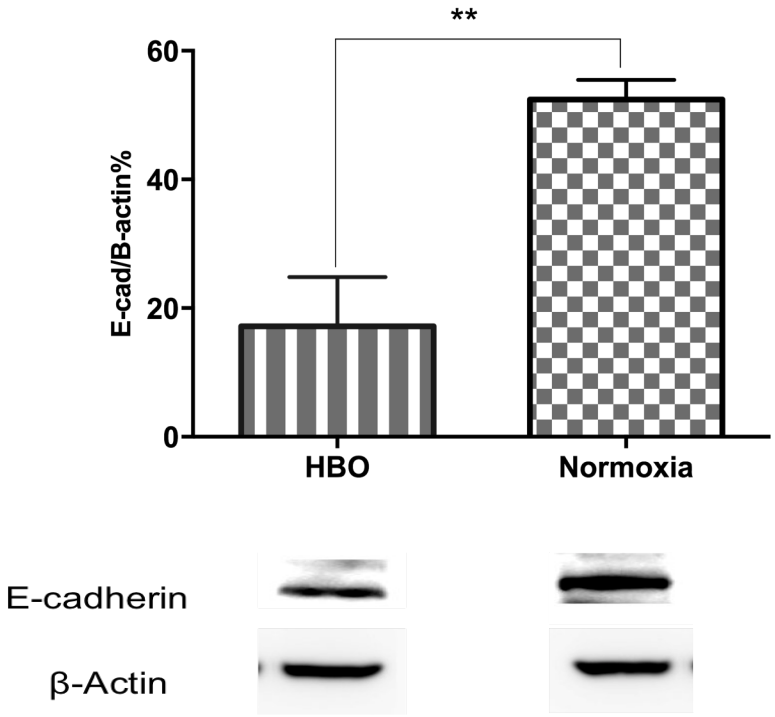

Figure 4-3 E-cadherin expression analysed by Western blotting. HaCaT cells were exposed to HBO at 3 ATA and normoxic conditions at 1 ATA for 48 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control. The histogram represents the numerical data of E-cadherin normalized against β-actin (HBO: 17± 7% and Normoxic: 52± 3%, n = 3, ***p*Value<0.01).

4.2.2 HBO on proliferation and viability of keratinocytes

To test the influence of HBO on keratinocyte proliferation and viability, MTT assay (section 2.2.7) and Western blotting (section 2.2.8) were used. MTT assay revealed that HBO significantly reduces HaCaT cells metabolic activity (Figure 4-4). To further elucidate the impact of HBO on cultured HaCaT cells at the molecular level, PCNA expression (a proliferation marker) was analysed using Western blotting. The mean PCNA expression was 6±1.4% and 77±1.5% under HBO and normoxic conditions,

respectively. The results demonstrate that exposure of keratinocytes to HBO significantly attenuated the expression of PCNA in HaCaT cells, indicating that HBO reduces HaCaT cells proliferation (Figure 4-5).

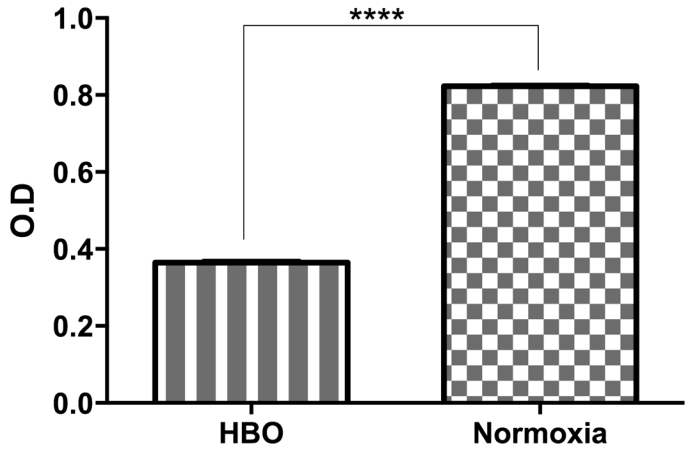

Figure 4-4 The viability of metabolically active HaCaT cells under HBO and normoxic conditions using MTT assay (n=4, ****p≤0.0001).

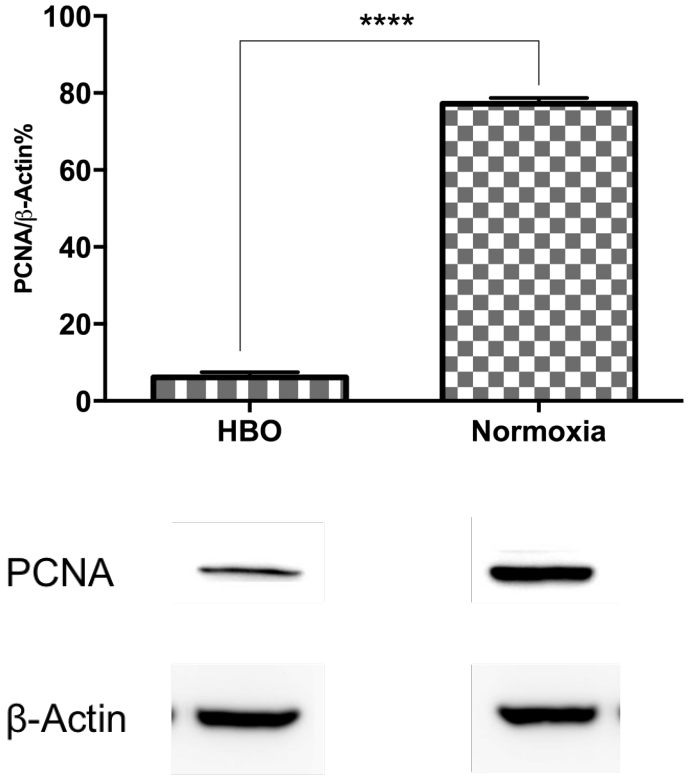

Figure 4-5 Expression of PCNA by HaCaT cells under HBO at 3 ATA and normoxic conditions at 1 ATA for 48 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control. The histogram represents the numerical data of PCNA normalized against β-actin from the quantification of the bands and presented as mean ± s.d. of three independent experiments. (HBO: 6±1.4% and Normoxic: 77±1.5%. n=3, ****pValue<0.0001)

4.2.3 The influence of HBO on differentiation of keratinocytes

HaCaT cells were exposed to normoxia and HBO, at 1ATA and 3 ATA respectively, for 48 hours. Expression of involucrin (a terminal differentiation marker) was determined using Western blotting and normalized against β-actin. Involucrin expression under normoxia and HBO conditions was 191± 16% and 222± 14%, respectively (Figure 4-6). The result indicated that HBO does not significantly affect the expression of involucrin in keratinocytes and thus, the differentiation rate. The

DMEM media containing calcium 0.2g/l that can affect the rate of differentiation and make the cells terminally differentiated. However, exposure of the HaCaT cells to HBO condition shows no effect on this process.

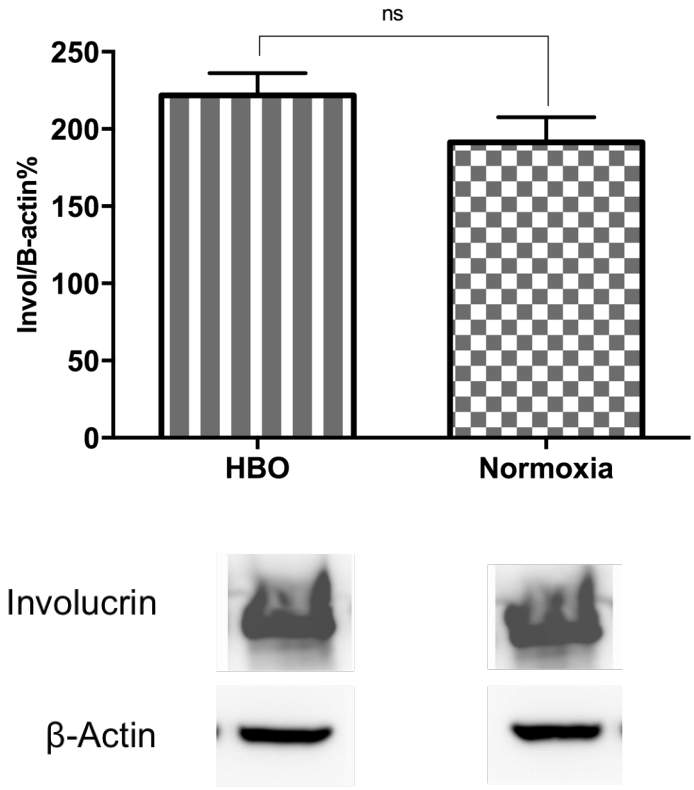

Figure 4-6 The effect of HBO on the Western blots analysis of involucrin. HaCaT cells were exposed to HBO at 3 ATA and normoxic condition at 1 ATA for 48 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control. The histogram represents the numerical data of involucrin normalized against β-actin (HBO: 222± 14% and normoxic: 191± 16%. n=3, ^{ns} *p*Value>0.05)

4.2.4 HBO on IL-6 and IL-8 secretion in keratinocytes

The influences of HBO on IL-6 and IL-8 production in keratinocytes were examined using ELISA. Following HBO exposure at 3 ATA for 48 hours, the supernatant was harvested and cytokine levels were quantified. The IL-6 concentrations were 52±2pg/ml and 57±3pg/ml in HBO and normoxic groups, respectively. in contrast, the IL-8 concentrations were 450± 40 pg/ml (HBO) and 893±171 pg/ml (normoxic). The results demonstrated that exposure of cultured keratinocytes to HBO does not change the levels of IL-6 secretion. However, HBO appeared to attenuate the production of IL-8 when compared to the normoxic group (Figure 4-7).

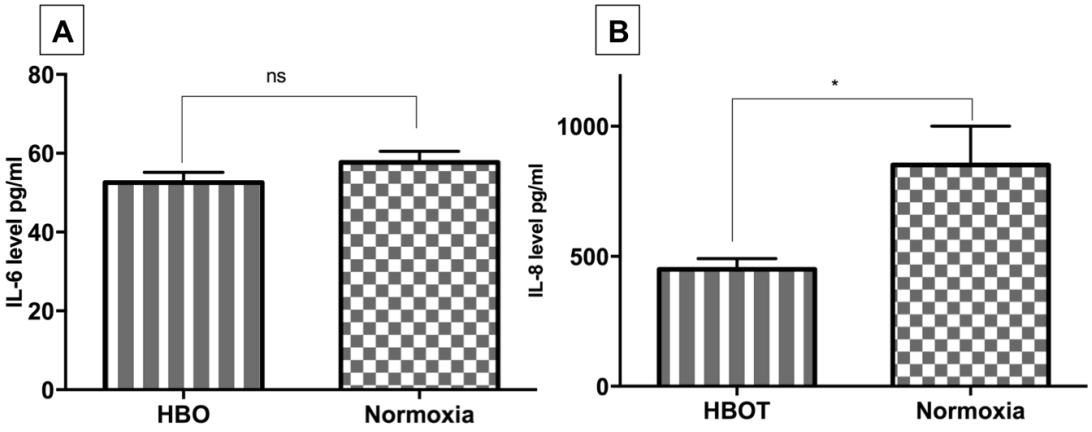

Figure 4-7 **A** is IL-6 and **B** is IL-8 levels in HaCaT supernant following 48 hours of HBO treatment quantified by ELISA. (The mean values ± s.d. of IL-6 in HBO and Normoxic conditions were 52±2 pg/ml and 57±3pg/ml, respectively. The mean values ± s.d. of IL-8 in HBO, Normoxic conditions were 450± 40 pg/ml and 893±171 pg/ml, respectively n=3, $^{ns}p>0.05$ $^{*}p<0.05$).

4.3 Discussion

The effect of HBO on re-epithelialization (namely migration, proliferation and differentiation) was evaluated *in vitro* using human keratinocytes (HaCaT cells) to understand the crucial role of oxygen at the cellular level during the wound repair process. Intracellular molecular changes, as well as extracellular changes shown in the culture supernatant, in response to HBO treatment were investigated. The *in vitro* study of cultured keratinocytes monolayer is considered as an established method for wound re-epithelialization processes (Hollander et al., 2000). Application of 95% of O_2 under 2-2.5ATA is the most frequent used method to investigate the influence of HBO on hypoxic and prolonged wounds (Oh et al., 2008).

In vivo investigations indicated the effectiveness of HBO in the treatment of ischemic wounds. Fulton (2000) demonstrated that HBO attenuates erythema and oedema, and enhanced collagen formation during healing. The effectiveness of HBO has been observed in impaired healing conditions such as in osteoradionecrosis (David et al., 2001). *Hypoxia* limits wound healing and the results of chapter 3 suggested that chronic hypoxia attenuates re-epithelialization via decreasing keratinocytes migration. Therefore, normobaric oxygen (1atm) and hyperbaric oxygen (HBO) approaches have been used clinically to oxygenate wound tissue (Patel et al., 2005). *In vivo* investigations of ischemic wounds have shown that HBO exerts positive effects on wound healing processes (Zhao et al., 1994). Clinically, it has been shown that the rate of chronic wound healing was correlated to the oxygen tension in the peri-wound tissues (Smith et al., 1996).

Apart from wound healing, HBO plays multiple roles in ischemic conditions. HBO improves cerebral metabolism (Ginsberg, 2003), reduces inflammation (Thom, 1993), attenuate apoptosis (Calvert et al., 2003), and also attenuates ischemic tolerance or ischemic preconditioning (Dong et al., 2002). However, focusing on skin re-epithelialization, these studies were designed to provide further evidence for the effectiveness of HBO in the treatment of wounds. Furthermore, better understanding of the molecular effect of oxygen on re-epithelialization may lead to more effective, specifically targeted wound treatments.

Re-epithelialization is an oxygen dependent step in the wound regeneration process. Therefore, it is logical to use HBO as a tool to deliver oxygen to the poorly-perfused wound tissues (Kuffler, 2011). Abnormalities in re-epithelialization can be divided into two categories: excessive re-epithelialization, which result in hypertrophic scars and incomplete re-epithelialization, which leads to chronic wounds (Chen et al., 2016). Therefore, attenuation of these abnormalities of re-epithelialization can promote wound healing.

Migration of keratinocytes to the wound site, among other factors, leads to coverage of the wound bed. Previous studies demonstrated that HBO enhances keratinocytes migration from dermal scaffolds (Kairuz et al., 2007). For migration to happen, the adherent cells convert to mobile cells, dissolving the desmosomal adherent and tight junctions. These junctions are necessary for tissue formation and phenotypes of the epithelial tissues (Moen et al., 2009). The influence of HBO on re-epithelialization is controversial. The possible mechanisms include: increased keratinocytes migration, increased proliferation of keratinocytes and/or decreases in deleterious factors during the wound healing process (Bilic et al., 2005). Experimental studies have shown that

HBO increases nitric oxide (NO) production at the wound site, which in turn enhances the migration of epidermal cells by converting the stationary cells to locomoting phenotypes (Boykin and Baylis, 2007).

Here, the influence of HBO on keratinocytes migration, as a critical step in re-epithelialization, was examined using the scratch assay coupled with Western blotting to quantify the adhesion molecule E-cadherin. The scratch assay showed that keratinocytes migrate significantly faster across the scratched site in response to HBO treatment when compared to the corresponding normoxic group (Figure 4-2, $p \leq 0.0001$). This finding is in agreement with previous *in vitro* and *in vivo* studies where Bilic et al (2005) demonstrated that exposure of burn wounds to HBO leads to faster epithelialization and thus wound healing. Also, animal studies revealed that daily exposure to HBO for two hours at 2ATA resulted in accelerated wound re-epithelialization of ischemic and non-ischemic wounds (Uhl et al., 1994).

Earlier studies had demonstrated that exposure of second-degree burn wounds to HBO enhances the rate of wound healing (Korn et al., 1977). HBO decreases the rate of cell apoptosis in the hypoxic microenvironment of the wound providing sufficient cells for re-epithelialization process (Niezgoda et al., 1997). Keratinocytes can survive without oxygen but they lose the capability to migrate (Bilic et al., 2005), whereas adequate oxygenation stimulates the migration and proliferation of epithelial cells (Korn et al., 1977). Others also concluded that adequate oxygen levels are essential for complete re-epithelialization and wound closure (Niezgoda et al., 1997 and Cianci et al., 2013).

To complement the gross physiological migration results, the expression of E-cadherin as an adhesion protein under HBO condition was assessed. Western blotting revealed that HBO significantly attenuates the expression of E-cadherin in keratinocytes (pValue<0.01) and subsequently minimizes cell-cell and cell-matrix adhesion, which in turn enables the cells to migrate faster, and is in agreement with a study by Sula *et al.* who also demonstrated HBO decreases E-cadherin expression (Sula et al., 2016). Down regulation of E-cadherin expression in HaCaT cells induces cell migration (Chow et al., 2016) and affects other cell types including mobilising bone marrow derived-stem/progenitor cells (SPCs) efficiently (Heyboer et al., 2014)

Following the migration, keratinocytes proliferate to supply the migratory cells with an adequate number of cells. The influence of HBO on keratinocytes proliferation is controversial. It has been shown that HBO completely arrested the growth of HaCaT and epithelial cells (Padgaonkar et al., 1993). Exposure of human keratinocytes to normobaric hyperoxic condition resulted in limited cell growth when compared with control group, while the growth of keratinocytes under 90% oxygen at 3ATA significantly inhibited cell growth (Patel et al., 2005). On the other hand, Reaven and Cox (1968) showed that keratinocytes grow optimally in an atmosphere containing 40% oxygen, and Horikoshi *et al.* (1986) also reported a higher proliferation rate for keratinocytes upon exposure to an oxygen concentration of 18%. Clearly, these conflicting data merit further investigations into the effect of HBO on keratinocytes proliferation.

The data in Figure 4-4 demonstrates that multiple exposure of keratinocytes monolayer to HBO reduces keratinocytes cellular metabolic activity, confirmed by MTT assay. Moreover, Western blot analysis of cell lysates (Figure 4-5) showed that

exposure of cells to HBO significantly attenuates the expression of PCNA, which is a proliferation marker (*p*Value<0.0001). The mean values of PCNA expressions were 6±1.4% and 77±1.5% under HBO and normoxic conditions, respectively. These results are consistent with a previous study conducted by Dimitrijevich et al. (1999) demonstrating that proliferation of human keratinocytes was significantly reduced when exposed to HBO at 3ATA .

Enhanced re-epithelialization by HBO can further be attributed to the subsequent decrease in PCNA expression, as strongly evidenced by Western blot protein analysis of HaCaT lysate and at the same time increasing in their pro-migratory behavior. PCNA analysis has been used as a marker for growth and proliferation of the cells. Its main function is to increase the activity of delta DNA polymerase during DNA replication that precedes cell division (Paulo et al., 2013). Hyperbaric oxygenation at 2.5 ATA for 90 minutes on three consecutive days reduced cell proliferation and increased cell death in normal cultured fibroblasts (Lin et al., 2008). Karasek, (1966) showed that exposure of fibroblasts to oxygen above 50% resulted in a significant reduction in proliferation.

On the other hand, the role of HBO on keratinocytes differentiation was investigated. Western blot was performed using cell lysate to analyse the level of involucrin. Results (Figure 4-6) demonstrated no significant changes (*p*>0.05) in involucrin with HBO and thus, no change in the differentiation rate. This result is consistent with a study conducted by Hollander et al., (2000) which demonstrated HBO at 3ATA did not altered keratinocytes differentiation. However, the same study revealed that HBO at lower pressure (1-2ATA) stimulate cell differentiation by up regulating cytokeratin and involucrin expressions. (Hollander et al., 2000)

HBO influences the production of a range of cytokines that are involved in wound healing (Al-Waili and Butler, 2006). For example, HBO up-regulates the expression of VEGF in the epithelial layer of wounds in rats (Haroon et al., 2000), stimulates the production of TNF-α in murine macrophages (van den Blink et al., 2002), attenuates IL-6 and enhances TNF-α production in human volunteers (Rocco et al., 2001 and Lahat et al., 1995), and induces PDGF in rabbit ear wounds (Bonomo et al., 1998). Another study demonstrated that exposure to HBO significantly reduced the levels of IL-1, IL-6 and TNF-α in patients with Crohn's disease (Weisz et al., 1997). HBO exerts a synergistic effect with FGF, TGF-B1 and PDGF in treatment of wounds (Zhao et al., 1994). HBO up-regulates the generation of FGF and TNF (Lahat et al., 1995).

The role of HBO on IL-6 and IL-8 synthesis in keratinocytes were thus also investigated. Following exposure of cells to HBO, the culture supernatants were collected and IL-6 and IL-8 secretions were quantified using ELISA. The IL-6 concentrations were 52± 2pg/ml and 57± 3pg/ml in HBO and control groups, respectively. In contrast, the IL-8 concentrations were 450± 40 pg/ml and 893±171 pg/ml. These results demonstrate that, while the level of IL-6 remains unchanged ($p>0.05$), HBO significantly attenuates the secretion of IL-8 in keratinocytes ($p<0.05$). The data is consistent with previous *in vitro* studies which demonstrated that HBO suppresses stimulus-induced pro-inflammatory cytokine production (Brenner et al., 1999). Inamoto et al. (1991) exposed mice to one hour HBO at 2.5ATA for five consecutive days before sacrificing and culturing their macrophages with LPS. Their study revealed IL-1 expression was significantly lower in macrophages of mice that were exposed to HBO when compared to the control group.

In contrast, Patel et al. (2005) studied the expression of VEGF by human keratinocytes; the result indicated that exposure to HBO induces the synthesis of VEGF. An *in vivo study* revealed that short term exposure of diabetic patients to HBO does not affect IL-8 levels in serum (Chen et al., 2007). However, current results provided further insights into the contradictory effects of HBO on cytokines synthesis confirming that HBO significantly suppresses IL-8 secretion, which is the main cytokine released by keratinocytes in response to infection (Nakamura et al., 2002). The suppression of pro-inflammatory cytokines can eventually shortening the inflammatory phase of wound healing which can be considered as another possible mechanism by which HBO improves wound healing. However, further investigations are needed to elucidate the influence of HBO on cytokines release from macrophages and neutrophils to draw conclusions on the role of HBO on the inflammation phase and overall wound healing.

4.4 Conclusion

In conclusion, a growing body of evidence suggests that hyperbaric oxygen is a useful approach to promote wound healing. However, the mechanisms by which HBO enhances wound healing are not fully understood. The current *in vitro* study was designed to investigate the role of HBO on skin re-epithelialization, as a critical step in wound healing process, using immortalized human keratinocytes (HaCaT cells). Furthermore, this model provided the opportunity to examine the cellular and molecular mechanisms through which HBO modulates cutaneous wound healing.

It was demonstrated that exposure of cultured keratinocytes to HBO stimulates keratinocyte migration with a marked reduction in E-cadherin expression indicating

that HBO decreased cell-cell and cell-matrix adhesion and protein tight junctions. In contrast to their pro-migratory activity, exposure of keratinocytes to HBO resulted in a marked decrease in the cell proliferation rate confirmed with quantitative analysis of PCNA using Western blot. This reduction in cellular proliferative activity was associated with reduced metabolic activity. Furthermore, HBO had no effect on involucrin expression and thus, the differentiation rate. Finally, though HBO did not modify the expression of IL-6, IL-8 levels were significantly down regulated by HBO.

These results illustrate the varied influences (positive and negative) of hyperbaric oxygen during key stages in wound regeneration and provide further understanding of the underlying cellular and molecular mechanisms of HBO activity during in wound re-epithelialization.

Chapter V: The influence of normobaric oxygen (NBO) and hyperbaric air (HBA) on HaCaT cells

5.1 Introduction

In wounds, vascular disruption and high oxygen consumption by metabolically active cells cause a rapid decrease in oxygen levels (Guo and Dipietro, 2010). A further decline in oxygen levels is noticed when a wound is infected (Davidson and Mustoe, 2001). This decline in oxygen leads to poor wound healing and impaired texture of the skin (Niinikoski et al., 1972b). The reduced oxygen level also disturbs the leukocyte's capability to kill bacteria, which in turn causes the wound to be more susceptible to infection. Therefore, maintaining a sufficient oxygen level in the wound site is a useful approach for improving wound healing (Davidson and Mustoe, 2001). Oxygen therapy has been proposed as an adjunct treatment for chronic wounds. Normobaric oxygen (NBO) and hyperbaric oxygen (HBO) have been used systemically to increase oxygen partial pressure in the wounded tissues. However, the exact mechanisms through which oxygen improves wound healing remain unclear and the controversial issues have been discussed in detail in Chapter 4 with regard to HBO. This chapter aims to determine the influence on wound healing of HBO's components separately i.e. oxygen as a molecule and supra-atmospheric pressure 3ATA.

The epidermis has direct contact with atmospheric oxygen. It was thought that oxygen simply diffuses from the air into the epidermis (Stücker et al., 2002), which led to investigations on the effects of topical elevated oxygen concentrations at 1ATA on surface wounds. Many studies revealed promising effects of topical oxygen (Fries et al., 2005) in the treatment of epidermal wounds.

Although the influence of HBO in the treatment of chronic wounds is well documented in clinical practice, there are several limitations in application of HBO including low availability, high cost, side effects and poor patient compliance (Han, 2016). NBO in the treatment of chronic wounds clinically has also shown promising results (Kalliainen et al., 2003). NBO is an increase in fractional inspired oxygen to almost 100 % at normobaric pressure; *in vivo* studies revealed that NBO as an adjuvant treatment attenuates infarct size following stroke and increased brain functional outcomes (Sharifipour et al., 2012). However, to date, there are very few studies that have explored the influence of NBO on the wound healing process. A clinical study revealed that NBO increases the oxygen partial pressure in the pre-wound site from 26.1 ± 11.2 mmHg to 53.4±21.7 mmHg during therapy and transcutaneous oxygen pressure (TcPO2) >40 mmHg is considered to be sufficient for wound healing (Han, 2016). NBO has several advantages over HBO in term of cost, safety and availability. Administration of NBO does not require specific equipment or specialist. In addition, NBO does not cause ear, sinus and pulmonary barotrauma, myopia, cataract, and oxygen toxicity seizures as have been reported with HBO (Sharifipour et al., 2012). NBO can potentially be used as an alternative therapy to HBO to avoid these problems.

On the other hand, hyperbaric air (HBA), which can be defined as normoxic air at a pressure higher than 1ATA (Teshigawara et al., 2010), is a key factor in oxygen therapy. Historically, in 1662 a British physician Henshaw proposed compressed air as a treatment to help respiration, to facilitate breathing, expectoration, and prevention of most afflictions of the lungs. Although at the time Henshaw's proposal did not received much attention, a few centuries later his proposal became a

plausible basis for hyperbaric medicine (Jain, 2009b). In addition to oxygen, atmospheric pressure is a fundamental factor in hyperbaric medicine. However, there are few studies exploring the physiological influences of pressure with the context of hyperbaric oxygen therapy (Dimitrijevich et al., 1999 & Tompach et al., 1997).

Elevated atmospheric pressure, either alone or within the context of hyperbaric oxygenation, can be considered as stress and cells may react differently. It has been proposed that an increase in atmospheric pressure can damage fibroblasts and endothelial cells (Tompach et al., 1997). The skin is the only organ besides the lungs that has direct exposure to atmospheric pressure, thus high atmospheric pressure can cause barotrauma lesions affecting the ears and lungs (Plafki et al., 2000). Therefore, it is compelling to explore the influence of atmospheric pressure on regeneration of cutaneous wounds and to assess the risks and benefits of pressure in oxygen therapy.

This study investigated, for the first time, the influences of normobaric oxygen and hyperbaric air on skin re-epithelialisation. The cellular and molecular changes in keratinocytes were examined in response to these conditions. It was hypothesised that oxygen is the critical molecule for enhancing re-epithelialisation in cultured human keratinocytes, and that increased pressure could alter cellular behaviour and potentially cause keratinocyte damage.

5.2 Results

5.2.1 The influence of NBO and HBA on migration and adhesion of keratinocytes

The rate of cell migration was evaluated following exposure of cultured keratinocytes (HaCaT cells) to NBO (section 2.2.4) and HBA (section 2.2.3) conditions. Scratch assays were performed (section 2.2.10) and migration of cells into the scratched area was photographed at 0, 24 and 48 hours (Figure 5-1) and analyzed using ImageJ. As shown in Figure 5-2, the rate of keratinocytes migration in HBA conditions was not significantly changed against normoxic control. On the other hand, migration rates were significantly higher in cultures grown under NBO conditions against the normoxic and HBA groups.

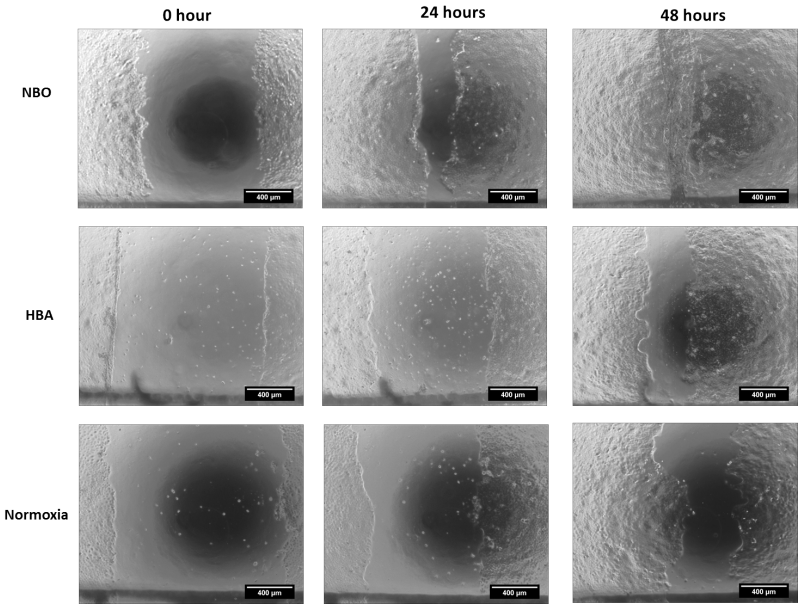

Figure 5-1 Representative images of the rate of keratinocytes cell migration from the scratch assays at 0, 24, 48 hours under NBO, HBA and normoxic control conditions. The HaCaT cells were exposed to NBO and HBO conditions for 2 hours/day in a modular chamber and oxygen vessel, respectively. Then, the cells transferred to CO_2 5% incubator.

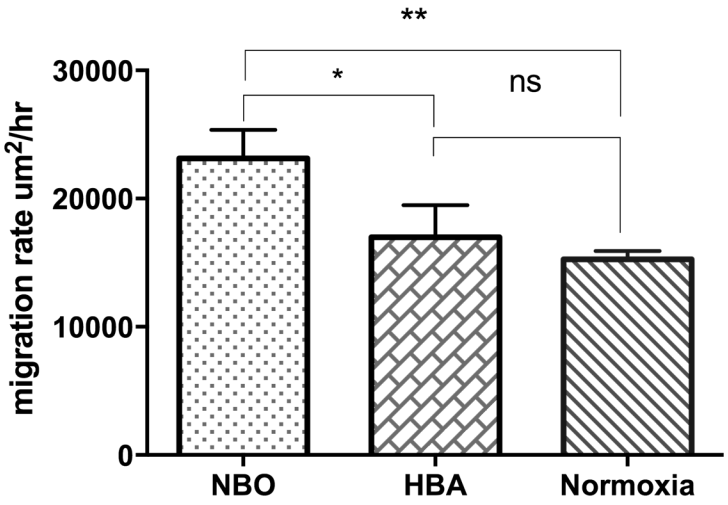

Figure 5-2 The rate of HaCaT cells migration (μm^2/hr) from scratch assay where HaCaT cells were exposed to NBO and HBA conditions for 48 hours against normoxic control. (Mean ± s.d. values NBO: 23148±2222 μm^2/hr, HBA: 16996±2483 μm^2/hr and Normoxia: 15270±649 μm^2/hr, n=3, *$p \leq 0.05$, **$p<0.01$ and $^{ns}p>0.05$)

Exposure of keratinocytes to NBO for 48 hours leads to changes in the cell adhesion profile. As shown in Figure 5-3, considerably lower expression of E-cadherin was observed in the NBO group when compared to HBA and normoxic conditions ($p<0.0001$). However, E-cadherin expression was invariant between HBA and normoxic conditions ($p > 0.05$).

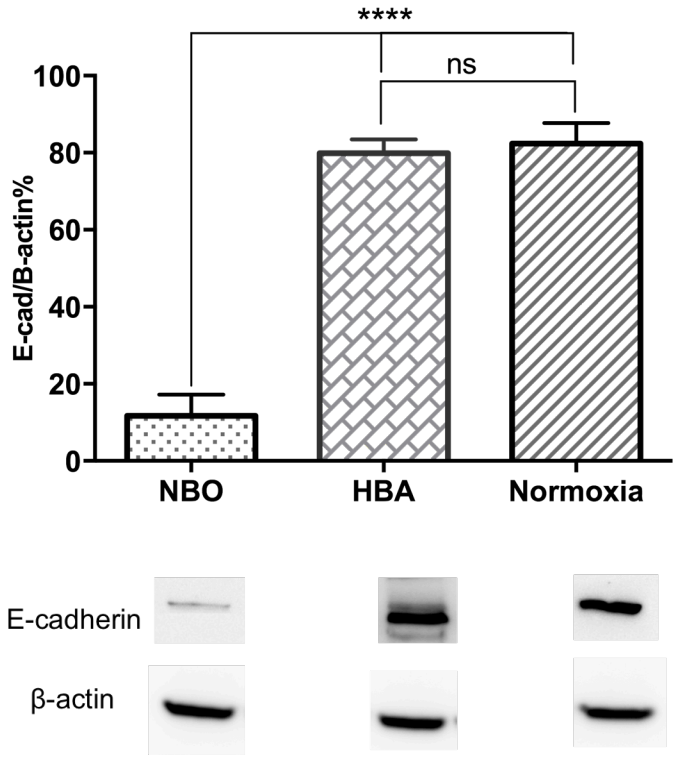

Figure 5-3 E-cadherin expression analysed by Western blotting. Cultured keratinocytes cells were exposed to NBO, HBA and normoxic conditions for 48 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control. The histogram represents the numerical data of E-cadherin normalized against β-actin (mean ± s.d. NBO: 12±5%, HBA: 80±3% and Normoxia: 82±5%, n = 3 ****pValue<0.0001 and ^{ns}pValue >0.05.)

5.2.2 The influence of NBO and HBA on proliferation of keratinocytes

Owing to the importance of proliferation of keratinocytes in skin re-epithelialisation, the influences of NBO and HBA on the rate of proliferation were examined. To determine whether NBO and HBA altered proliferation and metabolic activity of keratinocytes, MTT assay (section 2.2.7) and Western blotting (section 2.2.8) were performed where metabolic activities of cells along with PCNA expression (as a

proliferation marker) were measured. As shown in Figure 5-4, keratinocytes exposed to NBO dramatically reduced their metabolic activity when compared to the normoxic control ($p \leq 0.0001$). However, no significant changes were seen between cells cultured under HBA or normoxic conditions.

It was unclear whether the different scratch closure rates in keratinocytes grown at NBO, HBA and normoxic conditions were attributed to changes only in migration rates (Figure5-2) or whether differences in proliferation partially contributed to the scratch assay results. To assess this, the levels of PCNA expressions were determined in cell lysates of each group using Western blotting. The results revealed that there is a significant reduction in PCNA expression in keratinocytes exposed to NBO compared with both HBA and normoxic conditions ($p<0.0001$). However, PCNA expression in the HBA group showed no significant changes in comparison to the normoxic group (Figure 5-5).

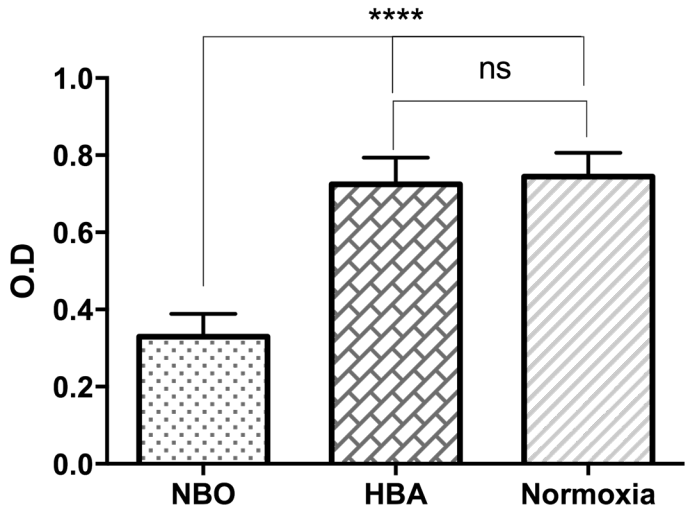

Figure 5-4 The metabolically activity of cultured keratinocytes cells under NBO, HBA and normoxic conditions using MTT assay (NBO: 0.32±0.05, HBA: 0.74±0.06 and Normoxic: 0.72±0.06., n=4, ****$p \leq 0.0001$ and $^{ns}p>0.05$).

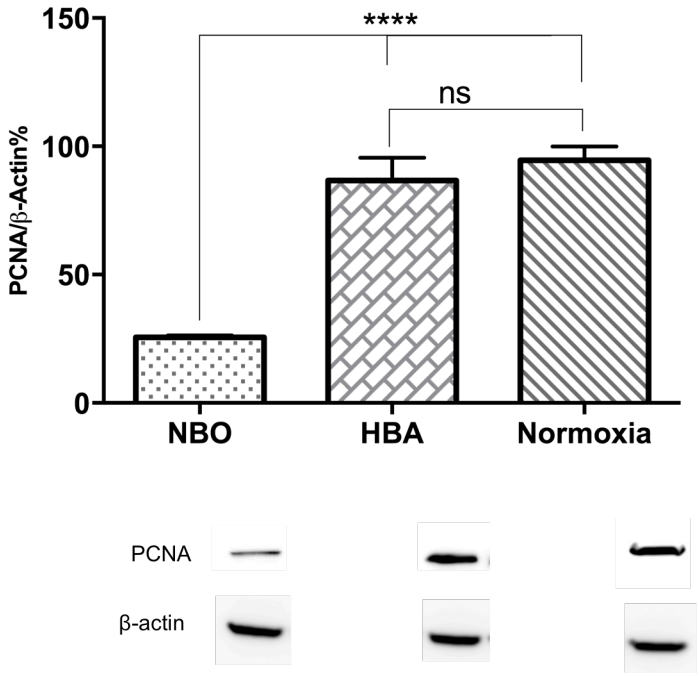

Figure 5-5 Expression of PCNA by HaCaT cells under NBO, HBA and normoxic conditions for 48 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control. The histogram represents the numerical data of PCNA normalized against β-actin from the quantification of the bands and presented as mean ± s.d. of three independent experiments. (NBO: 26±1%, HBA: 95±5%, and Normoxic: 87±9%, n=3, ****pValue<0.0001).

5.2.3 The influence of NBO and HBA on keratinocytes differentiation

Further investigations were performed to determine the influence of treatment conditions on differentiation rates of keratinocytes. Using Western blotting, involucrin, a terminal differentiation marker, was measured in cell lysates from keratinocytes exposed to different conditions. As illustrated in Figure 5-6, the mean values of involucrin expression (normalized against β-actin) were 213±40%, 229±12% and

224±39% in NBO, HBA and normoxic conditions, respectively. There was no significant difference in involucrin expressions between the three groups ($p > 0.05$).

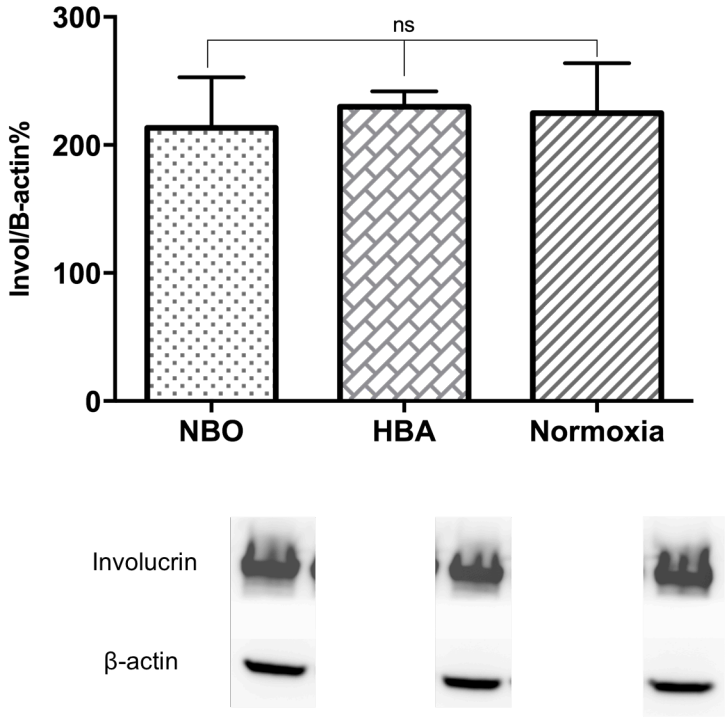

Figure 5-6 The effects of NBO and HBA on the Western blots analysis of involucrin. HaCaT cells were exposed to NBO, HBA and Normoxic conditions for 48 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control. The histogram represents the numerical data of involucrin normalized against β-actin (NBO: 213±40%, HBA: 229±12% and Normoxia: 224±39%, n=3, $^{ns}p>0.05$).

5.2.4 Cytokines IL-6 and IL-8

To investigate the influence of NBO and HBA on keratinocyte extracellular components, the synthesis and secretion of cytokines were also examined. The cells

were exposed to the different atmospheric conditions for 48 hours before the culture supernatants were collected. The levels of IL-6 and IL-8 were quantified using ELISA. The data revealed that exposure of keratinocytes to NBO caused a marked inhibition in IL-6 production against the control ($p<0.001$). However, cells treated with HBA did not affect IL-6 expression compared to normoxic controls (Figure 5-7**Error! Reference source not found.**A). On the other hand, the level of IL-8 in both NBO and HBA showed no significant difference to the normoxic control (Figure 5-7B).

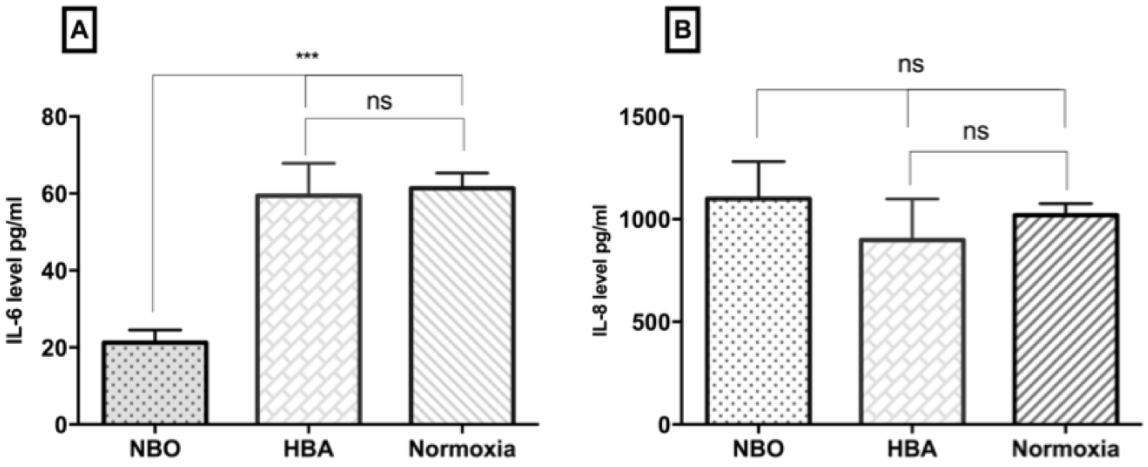

Figure 5-7 (A) IL-6 and (B) IL-8 levels in HaCaT supernant following 48 hours of NBO and HBA treatment quantified by ELISA. (The mean values ± s.d. of IL-6 in NBO, HBA and Normoxic conditions were 21±3 pg/ml, 59±8 pg/ml and 61±4 pg/ml, respectively. The mean values ± s.d. of IL-8 in NBO, HBA and Normoxic conditions were 1099±181 pg/ml, 899±200 pg/ml and 1020±56 pg/ml, respectively. , n=3, ***$p<0.001$, ns $p>0.05$)

5.3 Discussion

To the best of found knowledge, this is the first *in vitro* study investigating the effects of NBO and HBA on keratinocytes migration, proliferation and differentiation. Due to the serious side effects of HBO, researchers are exploring new and alternative

therapeutic modalities. Recently, there has been growing interest in NBO as a treatment modality for wounds. Promising data showed that NBO is effective in the treatment of thermal and chemical burns (Sharifipour et al., 2011). This study focused on the efficacy of NBO in skin re-epithelialisation. Moreover, the influence of elevated atmospheric pressure on cultured keratinocytes was also investigated at both cellular and molecular levels.

It is widely accepted that oxygen promotes wound healing. Following injury, the disrupted vasculature and metabolic activity of cells creates a hypoxic microenvironment in the wound site. Under prolonged hypoxic conditions, almost all differentiated functioning cells would die (Araki et al., 2014). Hence, oxygen has been administered therapeutically to increase the oxygen tension in wounded tissues (Horikoshi et al., 1986). Systemic administration of NBO significantly elevates oxygen partial pressure in all body tissues (Araki et al., 2014). Unlike the internal organs, the epidermis receives much of its oxygen supply directly from the atmosphere (Ngo et al., 2007). Topical oxygenation of the skin with 100% oxygen has shown a significant increase in epithelial healing (Said et al., 2005). However, the influence of HBA on skin re-epithelialisation has not been studied and has been addressed in this chapter.

The migration of keratinocytes from wound edges to the wound center is an essential step in the re-epithelialisation process (Ross et al., 2011) and it was demonstrated that oxygen levels control the rate of re-epithelialisation (Hunt and Pai, 1972). An *in vivo* study conducted by Löndahl *et al*.(2010) showed that HBO significantly increases the rate of diabetic wound healing when compared to hyperbaric air (Löndahl et al., 2010). However, another study revealed that there was no significant

difference in the rate of wound healing of ischemic Wagner grade 1 and 2 ulcers following exposure of patients to HBO against HBA (Abidia et al., 2003).

In the current study, migration of keratinocytes under NBO and HBA was investigated. Two distinct assays i.e. scratch assay and Western blotting were conducted to investigate cells migration to the scratched area and their adhesion behaviour. Data showed that keratinocytes migrate faster in response to NBO but no significant change in migration was found for keratinocytes exposed to HBA. These results were further confirmed and correlated well with cell adhesion studies, where the expression of E-cadherin (an adhesion protein) was quantified and showed a significant reduction in E-cadherin under NBO conditions but not with the HBA group, compared with the normoxic group. The result show that increasing cell migration to the scratched area correlated with the reduction in cell-cell and cell-matrix adhesion, which in turn increase cellular mobility behaviour and that these cellular changes were correlated to the oxygen level rather than elevated air pressure.

These results are consistent with other wound healing studies. It was demonstrated that plating efficiency (adhesion and cell proliferation) of cultured keratinocytes was enhanced under low oxygen tension suggesting that a better harvest of human keratinocytes is produced when cells are seeded under low oxygen when compared to hyperoxic condition (Horikoshi et al., 1986) A study conducted by Ross *et al.* (2011) revealed that increased oxygen tension in cell culture media has greater effects on keratinocytes migration rather than their proliferation (Ross et al., 2011).

In addition to migration, keratinocyte proliferation is also a critical step in the re-epithelialisation process. An *in vitro* study demonstrated that proliferation of

fibroblasts and keratinocytes was dramatically decreased when the cells were exposed to pressure above 2 ATA (Dimitrijevich et al., 1999). However, only a few studies have reported the biological effects caused by high atmospheric pressure. Results of one study revealed that increasing pressure from 40 to 120 mmHg promotes DNA synthesis and proliferation in cultured rat vascular smooth muscle cells (Hishikawa et al., 1994). Long term exposure of cells to hyperoxic conditions has an inhibitory influence on cell growth (Ngo et al., 2007). Moreover, the result of the studies here revealed that exposing cultured keratinocytes to intermittent NBO dramatically down-regulates the expression of PCNA, indicating that keratinocytes proliferation was significantly inhibited. However, no significant changes in PCNA levels were found in keratinocytes exposed to HBA when compared to normoxic condition. Using an MTT assay to measure metabolic activity of cells (Kupcsik, 2011), gave results consistent with the above; the keratinocytes under NBO conditions had reduced cellular metabolic activity compared to HBA and normoxic conditions, between which were themselves invariant.

These results are in accord with previous studies investigating the relationship between keratinocyte proliferation and different oxygen concentrations, which demonstrated that oxygen concentrations between 20% and 40% cause higher levels of keratinocyte proliferation. However, oxygen concentrations of more than 50% or below 10% resulted in a marked reduction in proliferation (Karasek, 1966). Similarly, a study conducted by Reaven *et al*. (1968) revealed that 40% oxygen is the optimum condition for keratinocytes to proliferate (Reaven and Cox, 1968). In contrast, optimum keratinocyte growth and proliferation was observed under low oxygen tension (18%) (Horikoshi et al., 1986). These findings, that the optimum oxygen

tension for proliferation lies between ~18 and 40%, are consistent with the results presented here and the results presented in chapter 3.

On the other hand, increasing the pressure in the context of HBO from 1 to 2.5 ATA did not produce a significant increase in keratinocyte proliferation rates (Dimitrijevich et al., 1999) illustrating that the rate of keratinocytes proliferation is independent of high pressure. Another study investigated the proliferation of fibroblast and endothelial cell under HBO with various pressure ranging from 2.4 to 4 ATA; the results showed no significant differences in the cell proliferation rates with pressure (Tompach et al., 1997).

Apart from migration and proliferation, differentiation of keratinocytes was also examined under NBO and HBA conditions by quantifying the expression of involucrin as the terminal differentiation marker. Data revealed that there were no significant differences under NBO and HBA when compared to the normoxic control. These results indicated that the clear changes in migration and proliferation of keratinocytes under NBO conditions were not partially attributable to keratinocyte differentiation. Previous studies demonstrated that exposure of keratinocytes to long term HBO increased differentiation rates, evidenced by increased expression of K1/10/11 and K14 (Kairuz et al., 2007). It has been demonstrated that exposure of cultured keratinocytes to low oxygen levels reduces their differentiation and stratification (Ngo et al., 2007). However, in the current study it was observed that a high oxygen concentration and supra atmospheric pressure does not affect the differentiation of keratinocytes.

Cytokines play a crucial role in the wound healing process (Gharaee-Kermani and Phan, 2001). Keratinocytes are considered a rich source of certain types of cytokines including IL-6 and IL-8 (Kondo et al., 1993). An *in vivo* study has been conducted investigating the influence of NBO, HBA and HBO on IFNγ synthesis in healthy volunteers. HBA inhibited IFNγ secretion in lymphocytes while NBO had no significant effect on IFNγ synthesis (Granowitz et al., 2002). In the results here, the influences of NBO and HBA on the production of IL-6 and IL-8 in cultured keratinocytes are reported. Data revealed that NBO reduces IL-6 synthesis significantly when compared to the normoxic control. However, no significant change was found in IL-6 levels between HBA and normoxic condition. Data also demonstrated that NBO and HBA did not affect the levels of IL-8 synthesis.

Some studies have found that elevated oxygen levels stimulate cytokines transcription and translation (Benson et al., 2003). However, these effects were not evident in the current study in term of IL-6 and IL-8 synthesis. Exposure of blood-derived monocyte-macrophages to HBO, HBA and NBO caused a significant increase in cytokines production in the HBO group but not in the NBO and HBA groups (Benson et al., 2003).

5.4 Conclusions

Numerous approaches have been used to avoid the possible side effects of hyperbaric oxygenation and to offer new therapeutic modalities for the treatment of chronic wounds. *In vitro* investigations of HBA and NBO on the wound healing process and determining their possible mechanisms of action can add new dimensions into these efforts. As skin re-epithelialisation is a crucial step in wound

healing, the current study investigated the influence of NBO and HBA on this process at both cellular and molecular levels.

Exposure of cultured keratinocytes to intermittent NBO stimulates the cells to migrate faster which in turn leads to a significant increase in scratch closure rate. This phenomenon was correlated to a marked decline in E-cadherin expression. However, the same phenomena was not found in the cells exposed to HBA; changes in the rate of keratinocyte migration and the expression of E-cadherin were not significant in cells grown under HBA conditions when compared to the normoxic control.

On the other hand, the *in vitro* proliferation study showed that exposure of keratinocytes to NBO, not HBA, resulted in a marked reduction in PCNA expression and cellular metablic activity. A similar phenomenon was also observed in the expression of IL-6 where a marked reduction in the production of IL-6 in the NBO atmosphere was seen. However, IL-8 and involucrin expression were not affected in either NBO or HBA groups. Together, these data indicate that oxygen affects the production of pro-inflammatory cytokines differently and further investigation is required to explain the role of oxygen on the production of individual cytokines.

The above studies suggest that pure oxygen, not the pressure, is responsible for the beneficial effects of HBO in re-epithelialisation of wounds. These data can provide new dimensions to the long-debated question of whether increased pressure during oxygen therapy has any beneficial effects in term of wound healing and whether these benefits merit the potential barotraumatic effects. The data above shows that elevating pressure has no additional benefits over the enriched oxygen environment. However, due to the very complicated nature of the wound healing, further

investigations are needed to explore the role of NBO and HBA on other steps involving wound healing process.

Chapter VI: The influence of inflammation on HaCaT cells

6.1 Introduction

Wounds become infected as a result of dynamic interactions between the host and pathogens, and can lead to high morbidity and mortality (Wilson, 2004). The microorganisms invade wounded tissues causing cellular injuries and immunological responses (Cutting and White, 2005). Cellular inflammatory responses, following infection, attract neutrophils and macrophages to the wounded area. These cells compete for nutrients and oxygen with other cell types (i.e. keratinocytes and fibroblasts) and also release enzymes and free radicals that cause a cycle of microbial proliferation and further tissue damage (Krasner et al., 2012). Bjarnsholt *et al*. (2008) proposed that delayed wound healing is, partially, caused by inadequate eradication of infecting pathogens (Bjarnsholt et al., 2008). It has been demonstrated that treatment of dorsal skin incision in rats with LPS (the main component of Gram-negative bacteria outer membranes) cause a significant delay in wound healing, reducing the levels of fibroblast growth factors and inducing TNF-α production (Kawaguchi et al., 1995).

Skin homeostasis is controlled by a continuous “cross-talk” amongst keratinocytes, fibroblasts and immune cells. This cross-talk is coordinated by specific cytokines (Nestle et al., 2009). One of the main targets for these cytokines is the epidermal keratinocytes, which then in turn regulates their cellular activities including migration, differentiation, as well as production of cytokines, chemokines and antimicrobial peptides (Rabeony et al., 2014).

Several cytokines including interleukins, lymphokines, and signaling molecules such as TNF-α and interferons play a role in wound healing. Accumulating evidence

suggests that cytokines play a key role in wound re-epithelisation, remodelling and angiogenesis (Werner and Grose, 2003). IL-6 also plays a crucial role in the wound healing process, and stimulation of various cells including macrophages, T-cells, fibroblasts, keratinocytes, and endothelial cells, can generate IL-6 (Lin, 2003). On the other hand, high levels of IL-8 expression was associated with impaired wound healing, decreasing keratinocytes proliferation and collagen production (Iocono et al., 2000).

Successful wound healing depends on various cytokines, chemokines and growth factors that regulate the complex molecular and cellular processes that occur. Cytokines, including IL-1, IL-6 and TNF-α, are up-regulated during the inflammatory phase of wound healing (Singer and Clark, 1999). High levels of TNF-α and IL-1 have been detected in chronic wounds (Tarnuzzer and Schultz, 1996 & Wallace and Stacey, 1998). Infection is common in chronic wounds and this further prolongs the inflammatory phase. Therefore, it has been hypothesized that in chronic wounds, chronic inflammation causes inflammatory cells to secrete TNF-α and IL-1b that synergistically inhibit cell migration and collagen deposition (Mast and Schultz, 1996). The epithelium is a strong barrier to prevent infection. However, efficient wound healing is needed to minimise risks of infection. *In vitro* studies demonstrated that bacterial cells inhibit migration of fibroblasts. However, LPS depleted bacterium (*S. marcescens)* with polymyxin-B agarose attenuated such effects of bacterial cells on migration of fibroblasts (Brothers et al., 2015).

Cross-talk between keratinocytes and monocytes is a crucial step in wound repair processes. *In vitro* co-cultivation of HaCaT keratinocytes with THP-1 (a monocyte-derived cell line) is considered an established method to study this phenomenon and

is increasingly being used to study host responses to infection (Zhang et al., 2011 & Berin et al., 1999). One benefit of co-culture compared to monoculture is its capacity to better understand the *in vivo* biology of cytokines, growth factors, and their impact on wound repair process. In the cross-talk culture model, two host cells (one being an immune cell) are co-cultured together and are challenged with a stimulant such as LPS to study the pathogenesis of infection and elucidate host responses towards infection.

These studies were designed to evaluate whether bacteria that commonly infect wounded tissues under hypoxic condition and cause wound complications, are able to alter re-epithelialization process in wound healing. It is hypothesised that co-culture of keratinocytes with LPS and monocytes will modulate the migration and proliferation of keratinocytes via alteration of cytokines expressions.

6.2 Results

6.2.1 Keratinocytes migration under hypoxic- inflammatory condition

Cell migration assays were performed (Section 2.2.10) to determine whether migration of keratinocytes altered upon treatment with various models of inflammatory conditions. Keratinocytes were grown to confluency, then scratched and co-cultured with either THP-1 monocytes or LPS 100µg/ml. The monocytes were previously treated with LPS (4µg/ml). The co-culture model (THP-1 model: HaCaT+THP-1) and the LPS stimulated model (HLPS: HaCaT+LPS) were exposed to hypoxic condition (denoted “H” for hypoxic below, as HLPS or HTHP-1) in a

modular chamber (section 2.2.4). Photographs of scratched areas were taken for 58 hours as shown in Figure 6-1. Comparison of each group revealed a different migratory response of these cells when grown in hypoxic vs normoxic control conditions (Figure 6-2). Keratinocytes migrated faster under normoxic condition when compared to hypoxic and HLPS models ($p \leq 0.05$), However, these phenomenon was not evident in the co-culture model of keratinocytes with THP-1 (HTHP-1) in which a non-significant difference was found when compared with the normoxic model ($p > 0.05$).

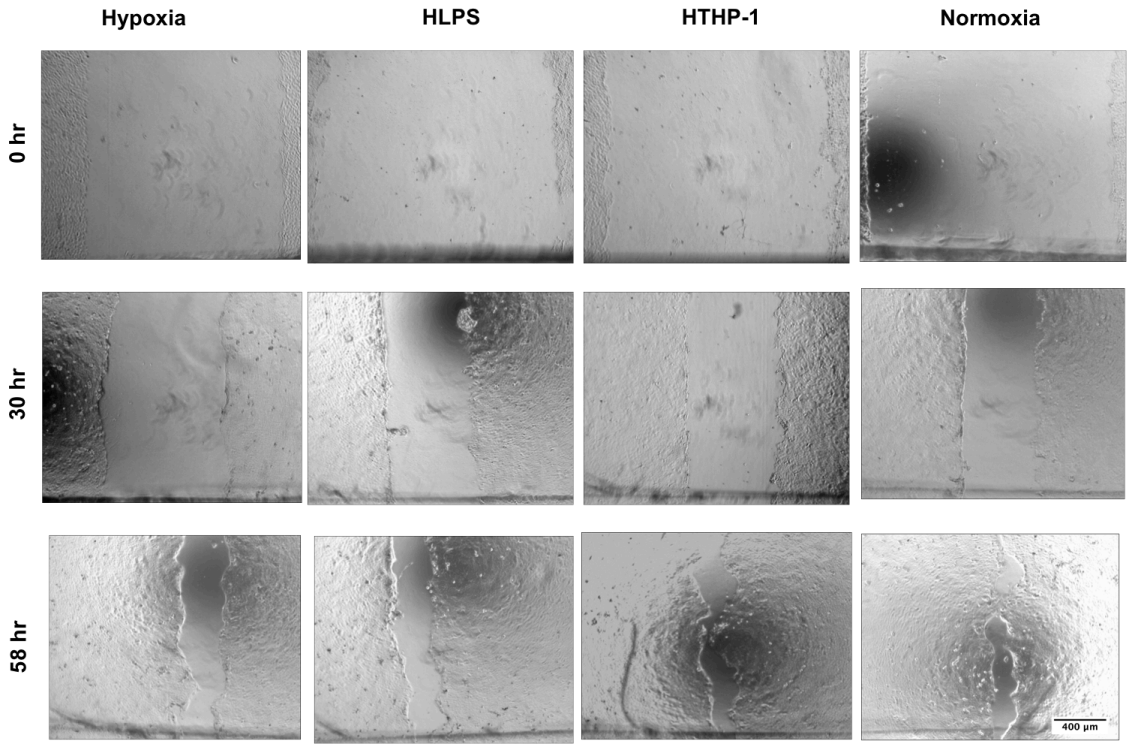

Figure 6-1 Representative images of the rate of HaCaT cell migration from scratch assays at 0, 30, 58 hours under hypoxia, HLPS, HTHP-1 and normoxic control conditions. The cells were exposed to chronic hypoxic condition for 58hrs under LPS stimulated (HLPS) and non-stimulated condition (hypoxia).

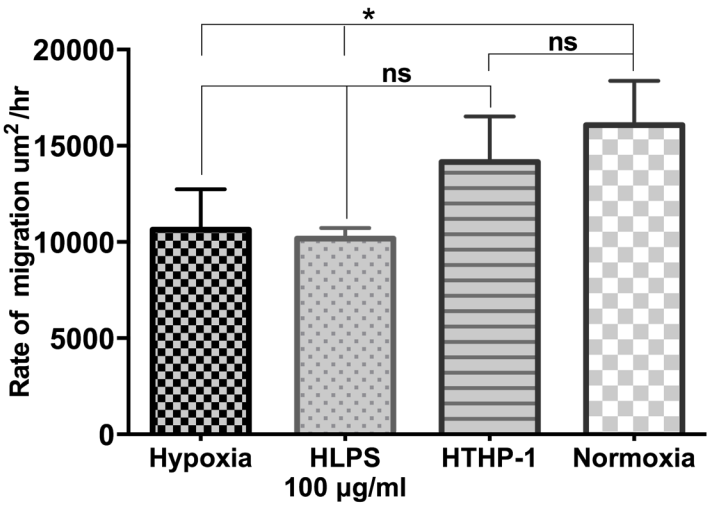

Figure 6-2 The rate of HaCaT cells migration (µm^2/hr) from scratch assays where HaCaT cells were exposed to hypoxia as controls, HLPS and HTHP-1, or normoxic control conditions for 72 hour. (Hypoxia: 10641±2105 µm^2/hr, HLPS: 10168±551 µm^2/hr, HTHP-1: 14152±2365 µm^2/hr and Normoxia: 16083±2284 µm^2/hr. n=3, $^{ns}p>0.05$ *$p \leq 0.05$).

Further investigations were conducted to evaluate the influence of different inflammatory models on keratinocyte migration. Here, the keratinocytes were treated with three different conditions under normoxia (denoted with “N” as a prefix for normoxia): 1) with IL-6 50pg/ml, 2) with IL-8 20ng/ml and 3) co-cultured with THP-1 cells previously stimulated with 4µg/ml LPS (NTHP-1). Images of the scratched areas were taken over 60 hours (Figure 6-3). Data revealed that treatment with IL-6 leads to a significant reduction in keratinocytes migration compared to IL-8 ($p < 0.05$) and normoxic control groups ($p<0.05$). However, no significant difference was found between the IL-6 group compared to the co-cultured condition ($p>0.05$). Moreover, the migration of keratinocytes in both IL-8 and NTHP-1 groups showed no significant change compared to the normoxic control ($p>0.05$) (Figure 6-4).

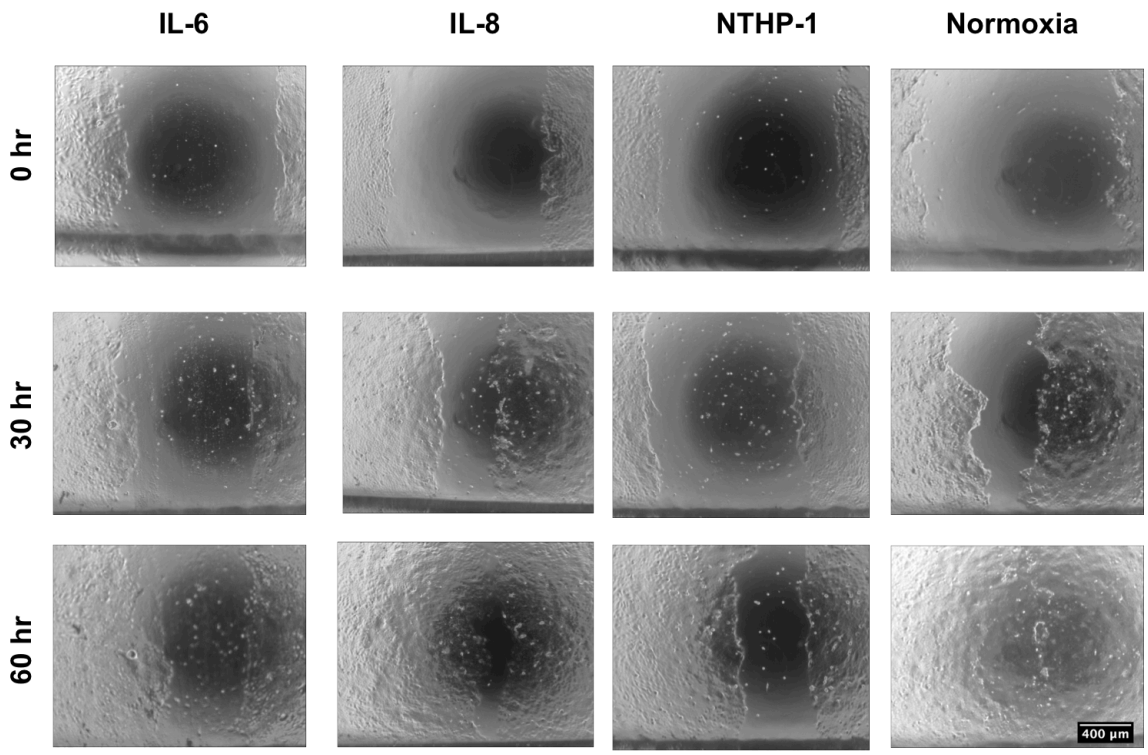

Figure 6-3 Representative images of the rate of HaCaT cell migration from the scratch assays at 0, 30, 60 hours. The HaCaT cells were exposed to with IL-6 50pg/ml, IL-8 20ng/ml and co-cultured with THP-1 cells previously stimulated with 4µg/ml LPS (NTHP-1).

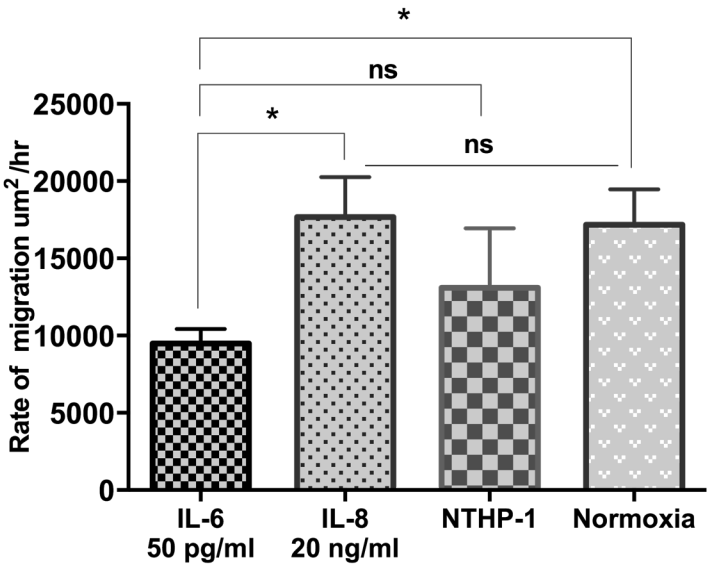

Figure 6-4 The rate of HaCaT cells migration (μm^2/hr) from scratch assays. HaCaT cells exposed to normoxic condition in a CO_2 incubator for 60 hours, the cells were treated with IL-6 (50pg/ml), IL-8 (20ng/ml) and NTHP-1 stimulated with LPS (4µg/ml).(IL-6: 9495±935 μm^2/hr, IL-8: 17674±2581 μm^2/hr, THP-1:13100±3844 μm^2/hr and Normoxia: 17176±2288 μm^2/hr, n=3, $^{ns}p>0.05$ $*p \leq 0.05$)

Cellular adhesion (section 2.2.8) was also examined under hypoxic, HLPS, IL-6 and normoxic conditions and the results indicated that both hypoxic conditions i.e. hypoxic and hypoxic stimulated with LPS, significantly increased the expression of E-cadherin in keratinocytes ($p<0.01$ and $p<0.001$ respectively) compared with the normoxic controls, thus elevating their adhesion propensity (Figure 6-5). However, there is no significant difference between the hypoxic conditions (controls or LPS stimulated). When cells were treated with IL-6 under normoxic condition, the expression of E-cadherin was significantly higher when compared with normoxic control ($p<0.01$, Figure 6-6).

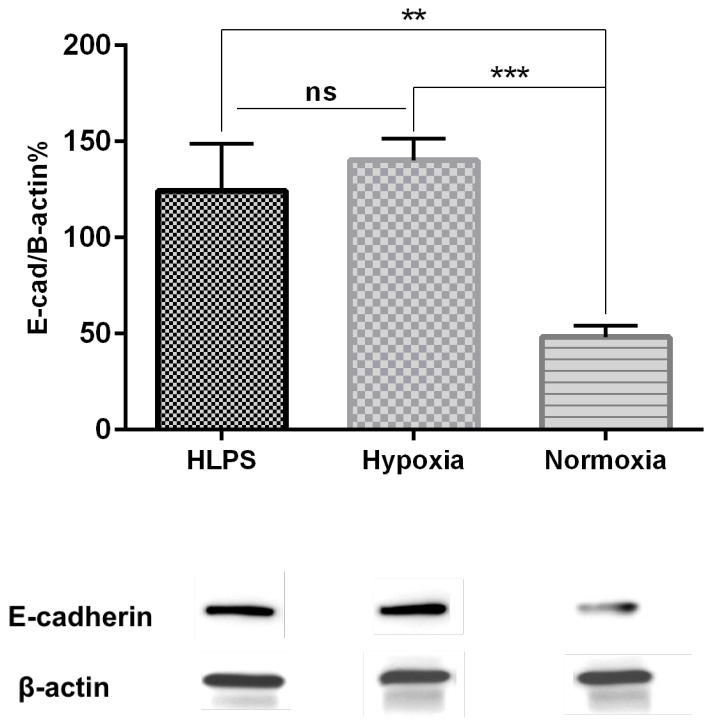

Figure 6-5 E-cadherin expression in HaCaT cells treated with LPS 100μg/ml and exposed to hypoxic condition at a modular chamber for 72 hours and analysed by Western blotting. The histogram represents the numerical data of E-cadherin normalized against β-actin as the loading control (HLPS: 124±24%, Hypoxia (control): 140±11% and Normoxia (control): 48±6%, n=3, $^{ns}p>0.05$ ** *p*Value<0.01.)

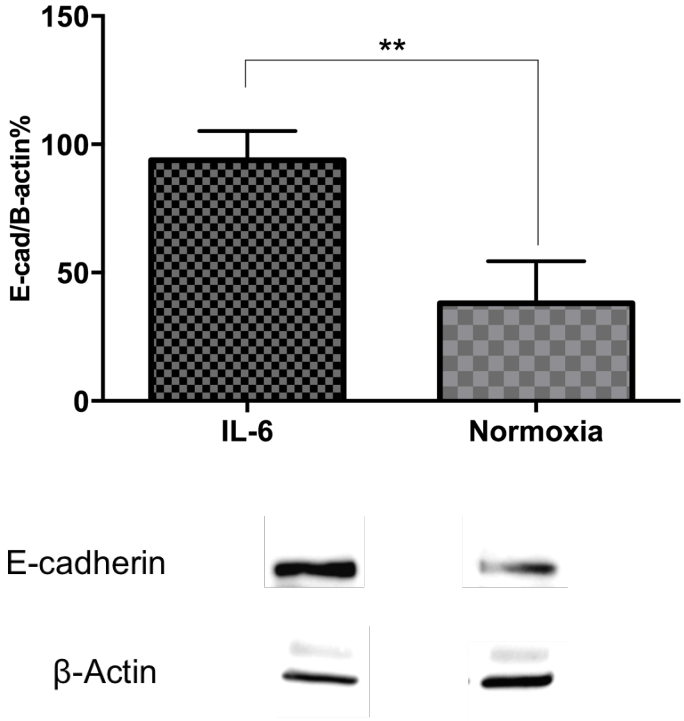

Figure 6-6 E-cadherin expression in HaCaT cells treated with IL-6 (50pg/ml) under normoxic conditions at 1 ATA for 60 hours analysed by Western blotting. The histogram represents the numerical data of E-cadherin normalized against β-actin as the loading control (IL-6: 94±11% and Normoxia: 38±16%, n = 3, ***p*Value<0.01)

6.2.2 Keratinocytes proliferation under hypoxic- inflammatory conditions

To evaluate the influence of inflammatory models on keratinocytes proliferation and metabolic activity, MTT and Western blotting were used. The MTT assay revealed that the keratinocytes cultured under hypoxic and HLPS conditions (hypoxic with LPS 100μg/ml) show significantly higher metabolic activity compared to the normoxic control ($p<0.01$ and $p<0.001$ respectively) (Figure 6-7). This is consistent with Western blotting results where a significantly higher expression of PCNA as a

proliferation marker were found ($p<0.0001$). No significant different was found between hypoxic and the HLPS conditions ($p>0.05$) (Figure 6-8).

On the other hand, when HaCaT cells were treated with IL-6 50pg/mL under normoxic condition, no significant difference was found in term of PCNA expression and cellular metabolic activity compared to normoxic control (Figure 6-9 and Figure 6-10 respectively).

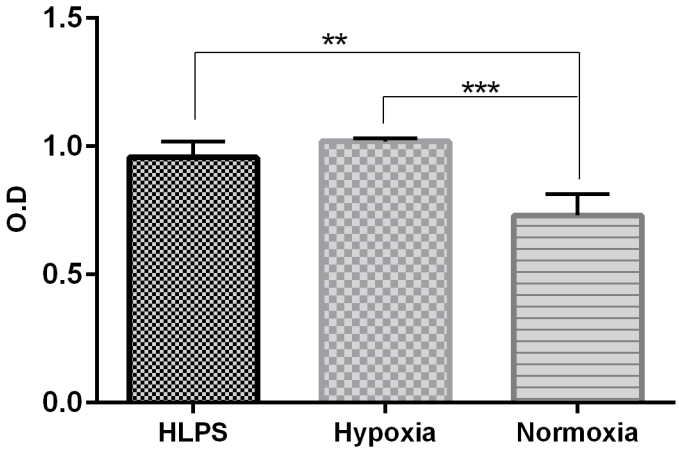

Figure 6-7 The viability of metabolically active HaCaT cells under hypoxic, HLPS and normoxic conditions using MTT assay (Mean ± s.d., n=3, **p≤0.01 ***p≤0.001,).

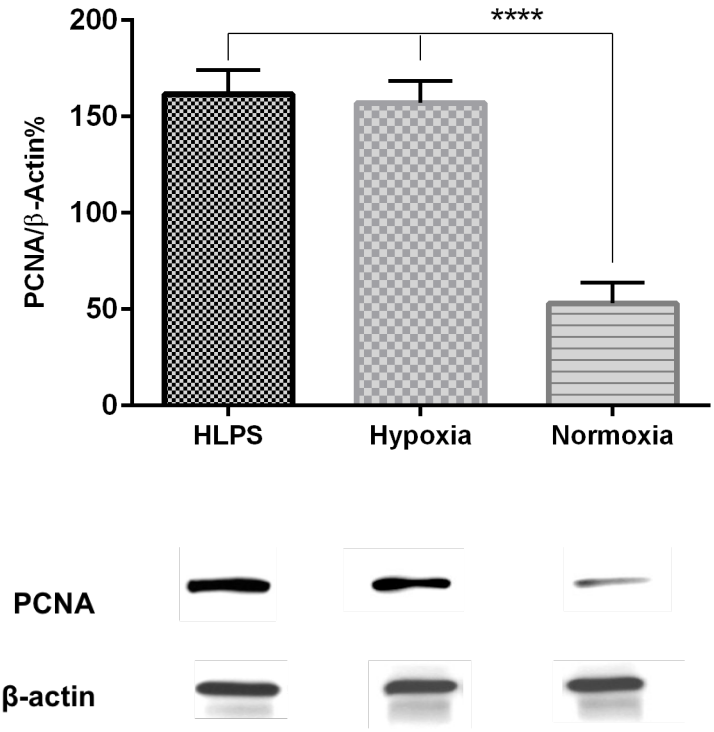

Figure 6-8 PCNA by HaCaT cells treated with LPS 100µg/ml under hypoxic for 72 hours analysed by Western blotting. The histogram represents the numerical data of PCNA normalized against β-actin. (HLPS: 162±13%, Hypoxia: 157±12% and Normoxia: 53±11%, n=3, ****p>0.0001)

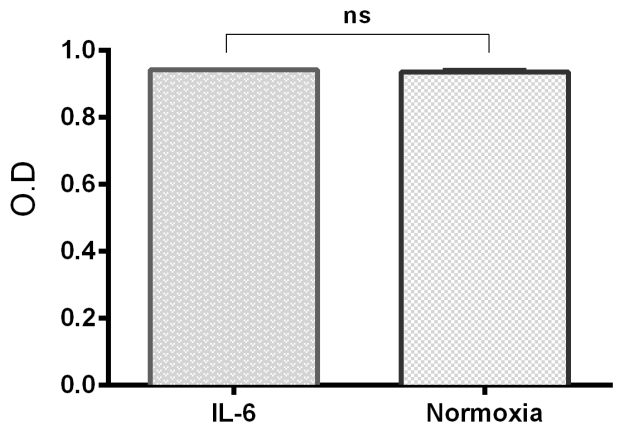

Figure 6-9 The viability of metabolically active HaCaT cells treated IL-6 50pg/ml under normoxic conditions using MTT assay (n=3, ^{ns}p>0.05).

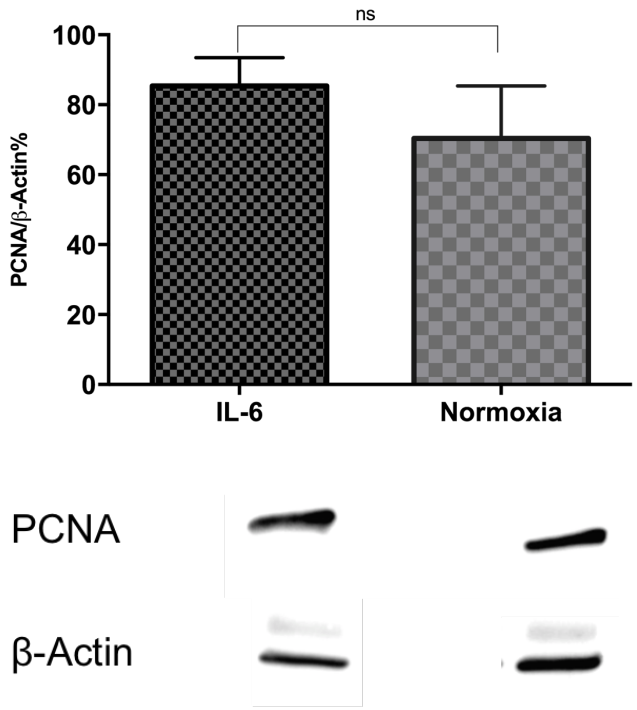

Figure 6-10 PCNA expression in HaCaT cells treated with IL-6 50pg/ml under normoxic conditions at 1 ATA for 60 hours analysed by Western blotting. The histogram represents the numerical data of PCNA normalized against β-actin (IL-6: 85±8% and 70±15%. n=3, $^{ns}p>0.05$).

6.2.3 Keratinocytes differentiation under inflammatory conditions

Keratinocyte differentiation was measured by involucrin expression (section 2.2.8). The cells were either cultured for 72 hours under hypoxic condition after being stimulation with LPS (100μg/ml) or cultured for 60 hours under normoxic condition after being stimulation with IL-6 50pg/ml. The results of Western blotting assays demonstrated that there is no significant difference in the involucrin expression among all the 3 groups when compared with the normoxic control (Figure 6-11 and Figure 6-12).

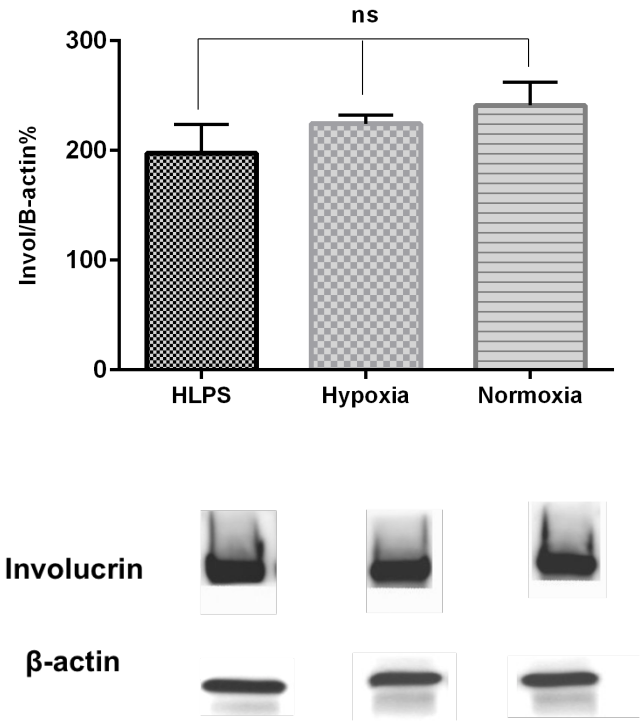

Figure 6-11 Involucrin expression in HaCaT cells treated with LPS 100µg/ml and then exposed to hypoxic condition for 72 hours. The histogram represents the numerical data of involucrin normalized against β-actin from the quantification of the bands and presented as mean ± s.d. of three independent experiments. (Hypoxia : 224±85%, HLPS: 198±26% and Normoxia : 241±21%. N=3, $^{ns}p>0.05$)

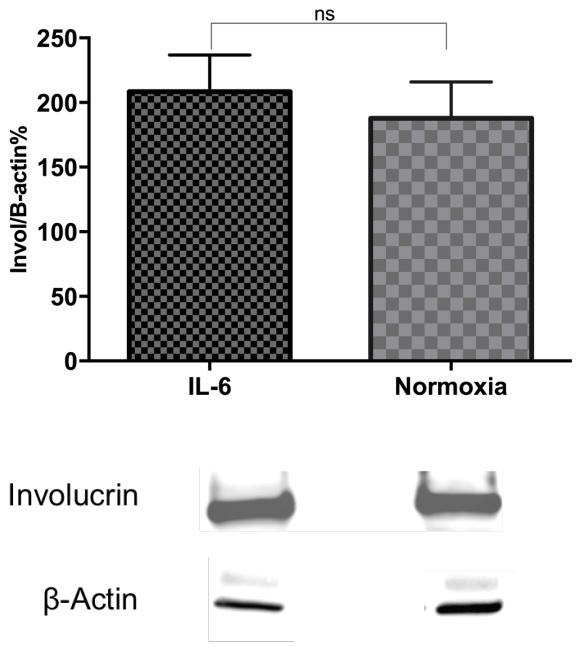

Figure 6-12 Involucrin in HaCaT cells treated with IL-6 50pg/ml under normoxic condition at 1 ATA for 60 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control. (IL-6: 209±28% and Normoxia (control): 188±28%. n=3, $^{ns}p>0.05$)

6.2.4 Cytokines and LPS stimulation

The levels of IL-6, IL-8 and TNF-α expression were examined in HaCaT and THP-1 cells using ELISA (section 2.2.6). The HaCaT cells were stimulated with LPS (100µg/ml) under both hypoxic and normoxic conditions and the NTHP-1 cells (as a positive control) were stimulated with LPS (4µg/ml) under normoxic condition for 48 hours. The results indicated that, upon stimulation with LPS, both cell lines released significant amounts of IL-6 (Figure 6-13 & Figure 6-15) and IL-8 (Figure 6-14 & Figure 6-15) under hypoxic (HaCaT) and normoxic conditions (both for HaCaT and NTHP-1). However, HaCaT released a non-significant amount of TNF-α under

maximum stimulation with LPS (200µg/ml) when compared with the normoxic group (Figure 6-16). This is in contrast to the THP-1 cell line in which a significant amount of TNF-α was been detected when the cells were stimulated with LPS 4µg/ml (Figure 6-16). As a further control, a spike recovery investigation was performed in order to assess any possible interaction of the HaCaT cell culture medium (DMEM) with TNF-α. As shown in (**Error! Reference source not found.**), the result indicated that the medium exerts no effect on TNF-α quantification.

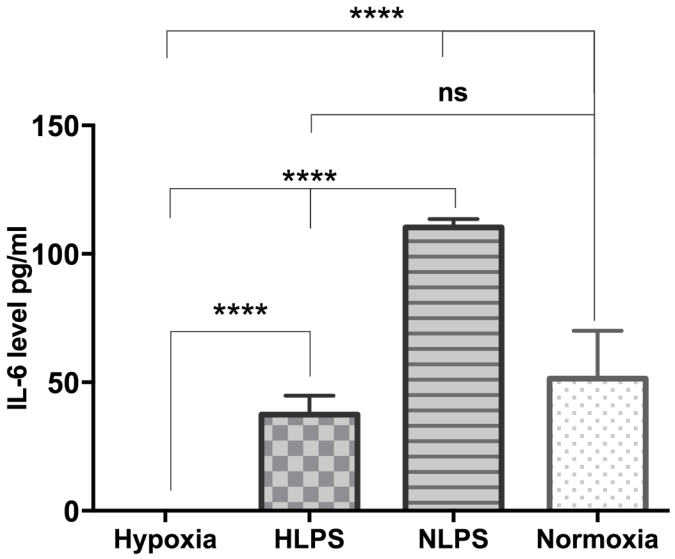

Figure 6-13 IL-6 levels in HaCaT supernant following 48 hours of LPS treatment under hypoxic and normoxic conditions quantified by ELISA. (Hypoxia: 0±0 pg/ml, Normoxia: 51±19 pg/ml, HLPS (hypoxic LPS stimulation): 37±7 pg/ml and NLPS (normoxic LPS stimulation): 110±3 pg/ml. n=3, $^{ns}p>0.05$ ****pValue<0.0001.)

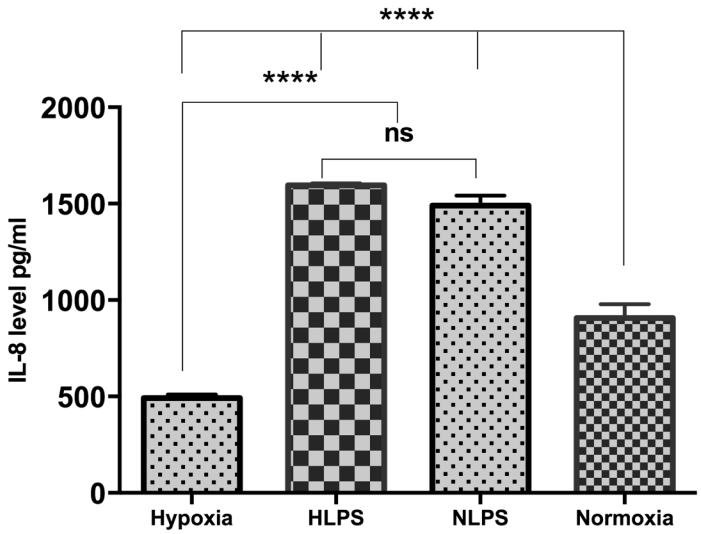

Figure 6-14 IL-8 levels in HaCaT supernant following 48 hours of LPS treatment under hypoxic and normoxic conditions quantified by ELISA. (Hypoxia: 492±19 pg/ml Normoxia: 908±71 pg/ml, HLPS: 1596±10 pg/ml and NLPS: 1491±53 pg/ml. n=3, $^{ns}p>0.05$ ****pValue<0.0001.)

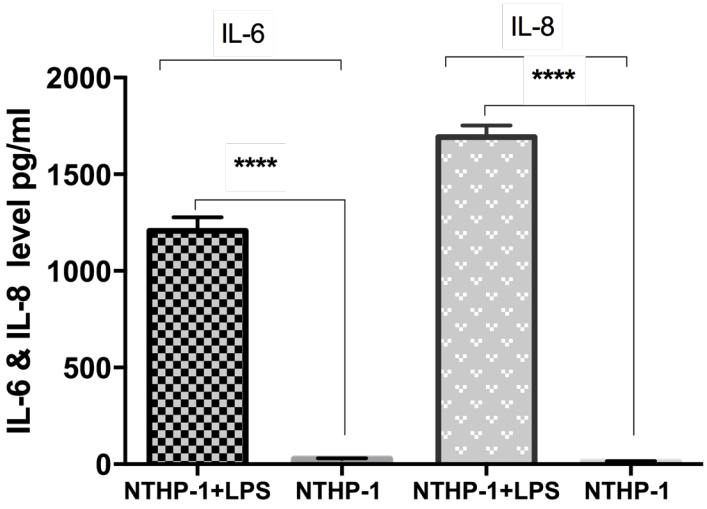

Figure 6-15 IL-6 and IL-8 levels in THP-1 cell supernant following 48 hours of treatment with LPS 4µg/ml under normoxic conditions, quantified by ELISA. (IL-6: NTHP-1+LPS: 1207±71pg/ml and NTHP-1: 28±2pg/ml. IL-8: NTHP-1+LPS: 1692±61pg/ml and NTHP-1: 11±4pg/ml, n =3, ****p<0.0001).

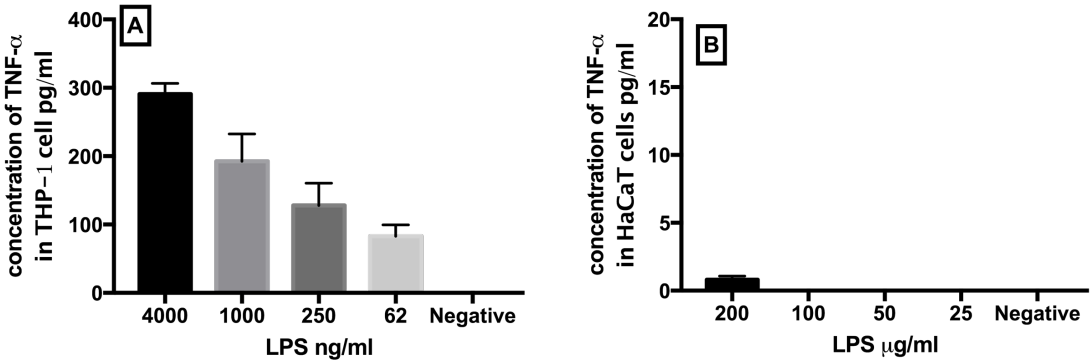

Figure 6-16 The cellular levels of TNF-α (pg/ml) after 48 hr stimulation with a range of LPS concentrations. **A**: in THP-1 cells (LPS400ng/ml: 291±16pg/ml, LPS1000pn/ml: 193±40pg/ml, LPS250ng/ml: 128±33pg/ml, LPS62ng/ml: 83±17pg/ml and Negative:0±0pg/ml **B**: in HaCaT cells (LPS200ng/ml: 0.8±0.3pg/ml, LPS100ng/ml: 0±0pg/ml, LPS50ng/ml: 0±0pg/ml, LPS25ng/ml: 0±0pg/ml, and Negative: 0±0pg/ml).

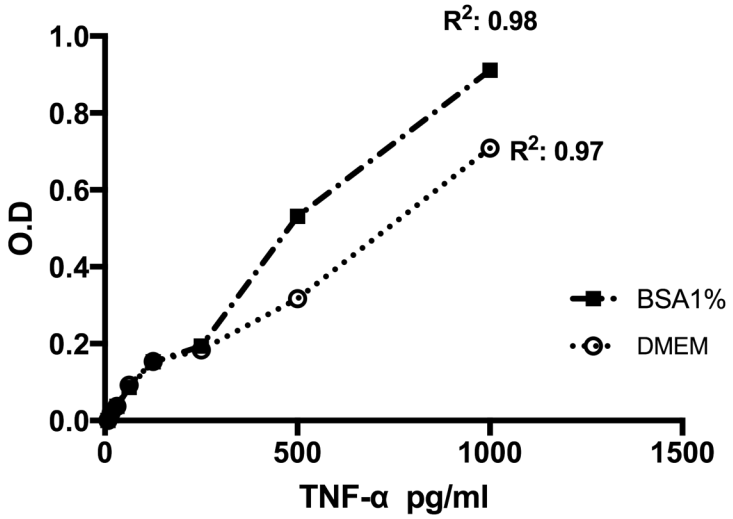

Figure 6-17 spike recovery, the standard curves of recombinant TNF-α using 1% BAS and HaCaT culture medium (DMEM) as the assay diluents.

6.2.5 Keratinocytes adhesion

As described in section 2.2.1.3, HaCaT cells are adherent and the subculturing process requires the cells to become detached and floating in the media as a cell suspension. This can be achieved via trypsinisation, which breaks down the cell-cell and cell-matrix peptide bonds and in turn changes the cell morphology to small spheres floating in the media. An unexpected phenomenon was observed following trypsinisation of HaCaT cells pre-treated with IL-6 and IL-8. In the case of IL-8, the trypsinated cells were detached but floated as a sheet of cells. On the other hand, the cells treated with IL-6 became more adherent and remained attached to the culture plate (Figure 6-18). A substantial increase in the cellular adhesion and

desmosomal junctions was evident in the keratinocytes treated with IL-6 as shown in Figure 6-19.

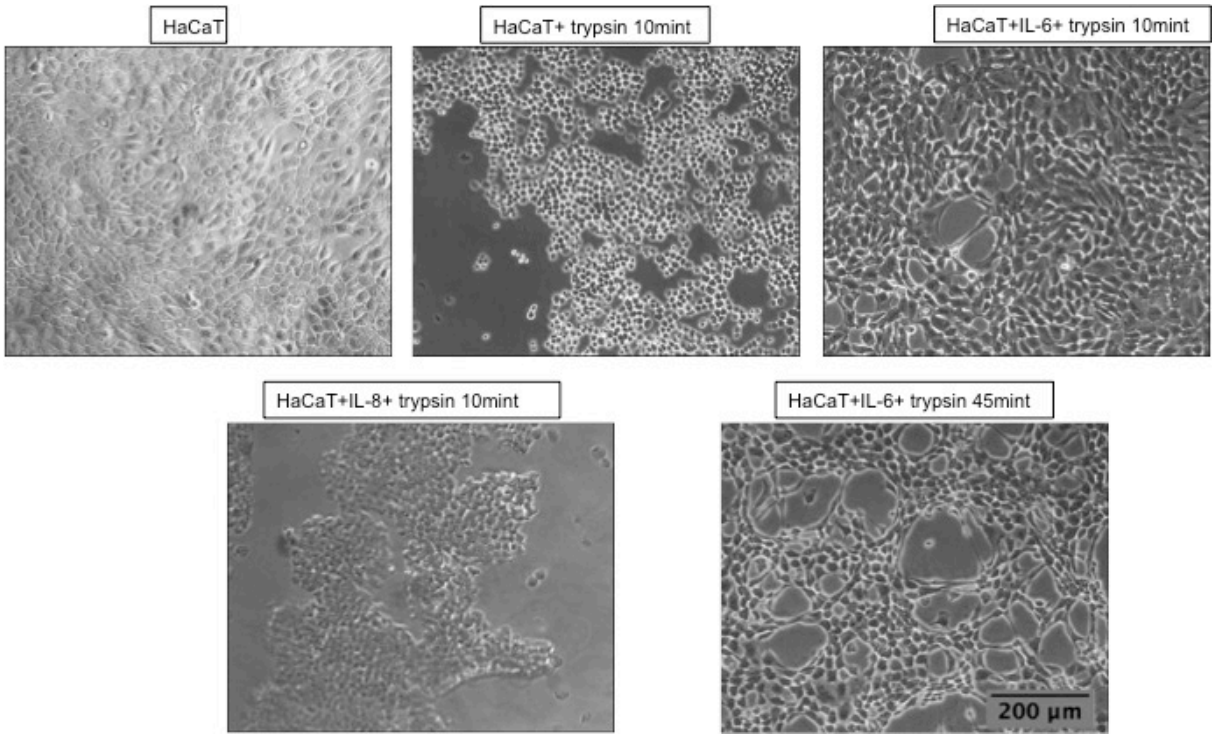

Figure 6-18 Representative images of HaCaT cells morphology following trypsinisation under different conditions i.e. IL-6 50pg/ml, IL-8 20ng/ml and THP-1.

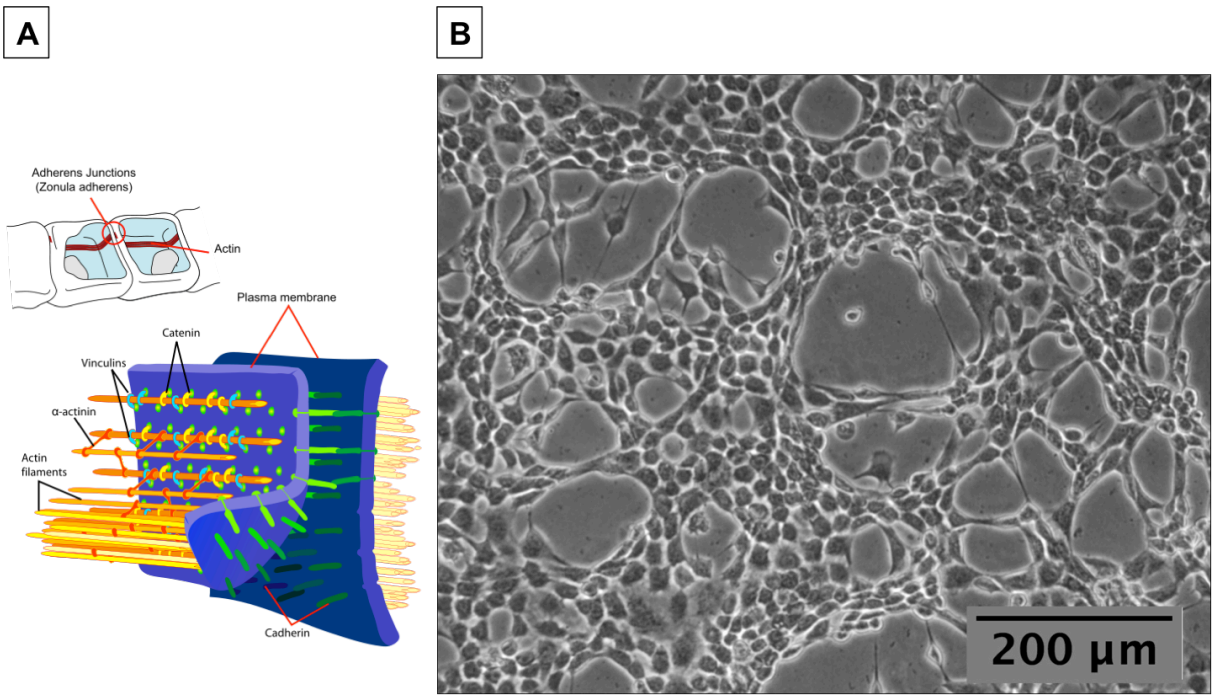

Figure 6-19 A) Diagrammatical illustration of intra-cellular desmosomes junction (adapted from LadyofHats.com with permission). B) Representative image of morphological changes in culture HaCaT cells treated with IL-6 50pg/ml. Increased adhesion due to increased intracellular junctions (desmosomes) is evident in the cells following trypsinisation.

6.3 Discussion

When skin is damaged, microorganisms can cross the skin to the underlying tissues. Replication of microorganisms within a wound causes host injury and delayed wound healing (Edwards and Harding, 2004). Both bacteria and endotoxins can lead to an elongated inflammatory phase via prolonged elevation of pro-inflammatory cytokines such as IL-1 and TNF-α, which in turn cause chronic wounds (Edwards and Harding, 2004). Diabetic ulcers frequently do not heal because of the combination of hypoxia and infection (Faglia et al., 1996).

LPS is the main biologically active glycosylated phospholipid present on the outer membrane of Gram-negative bacteria (Brothers et al., 2015). LPS and cell injuries both stimulate cytokine production via TLR4, suggesting the important role of TLR4 in wound healing process (Chen et al., 2013). Upon injury, keratinocytes are activated and release a number of cytokines that promote skin re-regeneration processes through re-epithelialisation (Kim et al., 2013) Therefore, in this study the influence of LPS on skin re-epithelialisation was examined using a co-culture model of keratinocytes with monocytes. LPS is the main exogenous ligand of TLR4 (Kostarnoy et al., 2013). LPS up regulates the production of IL-6, IL-1B, TGF- β1 and TNF-α affecting the inflammatory process and enhancing the wound healing of injured skin (Kostarnoy et al., 2013). In the current study, the influences of LPS stimulation and cytokines, in particular IL-6, on the re-epithelialisation process was investigated in term of keratinocytes migration, proliferation and differentiation.

The co-culture model using HaCaT and THP-1 cells is a promising technique to elucidate the influence of “cross-talk” on cellular interaction between the metabolically competent keratinocyte cell line (HaCaT) and the monocyte cell line (THP-1). In this model there is direct contact between keratinocytes and the monocytes providing a more realistic model of actual wounds (Hennen et al., 2011). IL-6 has a substantial role in the wound healing process. During the inflammatory phase of wound healing, the neutrophils and macrophages infiltrate to the wound site (Lin, 2003). Administration of IL-6 to mice with injured skin lead to accelerated infiltration of leukocytes to the wounded area (Saba et al., 1996). IL-6 up-regulates IL-1 expression in leukocytes which in turn stimulates the production of adhesive molecules and leukocytes adhesion (Lin, 2003). IL-6 could directly contribute to the

epidermal hyperplasia seen in psoriatic epithelium (Ishigame et al., 2009). Another study investigated the influence of IL-6 on epidermal human keratinocytes in serum free medium and demonstrated that IL-6 at 2.5-50ng/ml promotes keratinocytes proliferation where the keratinocytes formed highly stratified “colonies” with a narrower proliferative/migratory rim. (Hernández-Quintero et al., 2006).

The experiments in which exogenous LPS was added to the cells in this study were designed to evaluate the effect of endotoxins on monocytes and macrophages and their responses to immunological challenge. In hypoxic wounds, the leukocytes’ ability to fight bacteria via oxidative phosphorylation is severely impeded exposing the wounded tissues to infection (Kuffler, 2011).

Cytokines and growth factors play a crucial role in tissue repair processes and they are potential candidates as therapeutic modalities in the treatment of chronic wounds (Greenhalgh, 1996). Chronic wounds are characterized, in part, by deficiency in cytokines and growth factors originated in macrophages, keratinocytes and fibroblasts (Greenhalgh, 1996). Therefore, the potential applications of cytokines and growth factors as treatment modalities for chronic wounds are logical targets for scientific investigations (Blumenfeld et al., 2003)

Keratinocytes are a rich source of cytokines including IL-8 but the influence of IL-8 in wound healing and its autocrine effects on keratinocytes has not been studied extensively (Jiang et al., 2012). IL-8 can be considered as a chemotactic factor for keratinocytes since experimental wounds have shown a significant delay in re-epithelialisation and angiogenesis in IL-8R knockout mice (Devalaraja et al., 2000). An *in vivo* study indicated that topical application of IL-8 to experimental animal

wounds resulted in a significant increase in the re-epithelialisation and the overall wound repair process. (Blumenfeld et al., 2003)

However, the data presented in this study indicated that LPS challenge, cross talk of keratinocytes with monocytes and cytokines stimulation have variable effects on keratinocytes migration. Firstly, hypoxic conditions (hypoxic and HLPS) significantly attenuated the migration of keratinocytes when compared with normoxic control. However, the data demonstrated that there is no significant difference between hypoxic co-cultured (HaCaT+THP-1) conditions and the normoxic control in term of keratinocyte migration suggesting that monocytes co-cultured with keratinocytes can compensate for the influence of hypoxia on keratinocytes promigratory propensity. Secondly, stimulation of keratinocytes with IL-6 (50pg/ml) significantly reduced the ability of keratinocytes to migrate when compared with normoxic control and IL-8 groups. However, this phenomenon was not evident in the co-cultured model. The data also demonstrated that treatment of keratinocytes with IL-8 does not affect the migration of keratinocytes when compared with normoxic model.

These results suggest that, apart from IL-8, a mixture of cytokines produced via stimulation of THP-1 can neutralise the effect of IL-6, at least on keratinocytes migration. The keratinocytes adhesion was differently affected when the cells were challenged with LPS or cytokines under hypoxic and normoxic conditions. E-cadherin expression was significantly increased in keratinocytes treated with LPS and IL-6 under hypoxic and normoxic conditions, respectively. The cell adhesion effects can partially explain the reduction in pro-migratory activity of keratinocytes under these challenges. However, further investigation is needed to elucidate other molecular bases for this reduction in pro-migratory behaviour of keratinocytes.

On the other hand, the keratinocytes treated with IL-6 showed a substantial increase in cellular adhesion behaviour suggesting a marked increase in the number of desmosomal junctions. A greater number of desmosomal junctions leads to increased elasticity behaviour of keratinocytes (Fung et al., 2011).

A previous study demonstrated that LPS inhibits keratinocyte migration through TLR4 and TLR2. This effect was reversed via neutralizing antibodies to TLRs (Loryman and Mansbridge, 2008). In contrast, animal studies have shown that IL-1b and IL-6 levels were significantly reduced in TLR4 deficient mice, which in turn caused a significant delay in the wound healing process. (Chen et al., 2013)

Apart from migration, keratinocytes proliferation plays a pivotal role in re-epithelialisation of the skin. Previous studies demonstrated that cytokines affect keratinocytes proliferation rates; TGF-a1 stimulates epithelial cells migration and proliferation (Hebda, 1988) and promoted re-epithelisation of skin in rat (Mustoe et al., 1987). Over expression of IL-6 in rat's skin results in epidermal hyper proliferation (Sawamura et al., 1998).

The results presented here revealed that stimulation of keratinocytes with LPS under hypoxic condition did not change their proliferative profile when compared with the non-stimulated hypoxic model. However, hypoxic models (stimulated and non-stimulated) attenuate the rate of keratinocytes proliferation and cellular metabolic activity when compared with normoxic control. These data were further compared using MTT and Western blotting. Although IL-6 attenuates the migration rate of keratinocytes and induced their adhesion and elasticity propensity, further data suggested that IL-6 did not affect keratinocytes proliferation (through PCNA

expression) and metabolic activity (MTT assay). This is in contrast to Hernández-Quintero *et al.* (2006) who demonstrated that IL-6 promotes keratinocytes proliferation forming stratified colonies with a narrowed migratory rim.

Differentiation of keratinocytes was also investigated in this study. Using Western blotting, the cellular expression of involucrin as a terminal differentiation marker was quantified under various conditions. Previous studies demonstrated that pro-inflammatory cytokines are involving in keratinocytes differentiation; IL-22 reduced the expression of numerous keratinocyte differentiation markers including involucrin and cytokeratin (Wolk et al., 2006 & Boniface et al., 2005). Injection of cytokines into the ears of mice was associated with decreased differentiation and increased keratinocyte proliferation illustrated by Ki-67 staining (Nestle et al., 2009). However, data from the current study clearly demonstrated that exposure of keratinocytes to hypoxic condition or treating the cells with an endotoxin (HLPS and NLPS) and IL-6 did not affect the differentiation rate of keratinocyte. These results suggesting that the overall changes in migration and proliferation rates of keratinocytes along with their metabolic activity and adhesion behavior are not correlated with the modification in the keratinocytes differentiation.

Cytokines are also active components in the wound healing process and stimulate the migration of multiple cell types, such as monocytes, to the wounded area, regulating the re-epithelialization, tissue remodeling, and angiogenesis processes (Barrientos et al., 2008). IL-6 deficient mice showed a delay in wound healing, while exogenous administration of IL-6 reversed this effect suggesting that IL-6 plays a crucial role in wound healing process (Lin, 2003). Various cells including macrophages, T-cells, fibroblasts, keratinocytes, and endothelial cells, upon

stimulation, can generate IL-6 (Lin, 2003). In contrast, high levels of IL-8 expression at the wound site were associated with impaired wound healing, decreasing keratinocytes proliferation and collagen production (Iocono et al., 2000). However, opposite results were found in another *in vitro* study where IL-8 induces human keratinocytes proliferation (Rennekampff et al., 2000). Furthermore, topical application of IL-8 on human skin grafts was shown to stimulate human keratinocytes proliferation and thus the re-epithelialisation process (Rennekampff et al., 2000).

Fibroblasts are a rich source of IL-8; this cytokine exerts an autocrine effect on cultured human fibroblast cells through increasing in the percentage of cells lacking focal adhesions and subsequently enhancing their migration profile. IL-8 has been shown to be a potent chemoattractant for neutrophils, leukocytes and keratinocytes (Dunlevy and Couchman, 1995).

Several reports show that targeting cytokines, such as TNF-α or IL-1, is a potential therapeutic strategy for skin disorders (Kupetsky et al., 2013). Establishment of an *in vitro* model to study the role of these cytokines can be of great importance to clarify the role of these cytokines in cutaneous wound healing. Therefore, here, the cytokine profile of keratinocytes was examined using ELISA. The HaCaT cells were stimulated with LPS and THP-1 used as positive control as a well-established cell line with a high cytokines profile. The results demonstrated that, upon LPS stimulation, keratinocytes release a significant amount of IL-6 and IL-8. However, no significant difference in the levels of TNF-α release was found when the HaCaT cells were exposed to LPS (200μg/ml) compared to the normoxic control. To validate the experiment, the THP-1 cell line was used as a positive control and a spike recovery

assay conducted, indicating that the protocol was appropriate and that there was no interaction between the culture media and TNF-α. In contrast to the TNF-α results presented here, a study conducted by Ibisch *et al*. (2007) demonstrated that LPS (40μg/ml) caused maximum secretion of TNF-α in canine keratinocytes (Ibisch et al., 2007), However, there may be differences in the responses from human and canine keratinocytes towards an LPS challenge.

The results of ELISA on IL-6 and IL-8 levels in keratinocytes is very important given that successful wound healing depends on various cytokines, chemokines and growth factors that regulate the complex molecular and cellular processes involving in wound healing. Cytokines including IL-1, IL-6 and TNF-α are up-regulated during the inflammatory phase of wound healing (Singer and Clark, 1999). Keratinocytes are a rich source of IL-1 that exerts paracrine and autocrine effects inducing the migration and proliferation of keratinocytes (Raja et al., 2007). IL-6 is produced by neutrophils, monocytes and keratinocytes and its level is increased in the wound site (Sogabe et al., 2006). On the other hand, it is found that TNF-α, at low levels, promotes wound healing via increasing macrophage-produced growth factors. However, exposure of a wound to continuously elevated levels of TNF-α and IL-1 has a detrimental effect on wound re-epithelialisation (So et al., 1992 & Mast and Schultz, 1996).

High levels of TNF-α and IL-1 have been detected in chronic wounds (Tarnuzzer and Schultz, 1996 & Wallace and Stacey, 1998). In addition, infection that is common in chronic wounds further contributes to prolonged inflammation. Therefore, It has been hypothesized that in chronic wounds, chronic inflammation causes inflammatory cells

to secrete TNF-α and IL-1b that synergistically inhibit cell migration and collagen deposition (Mast and Schultz, 1996).

Therefore, the results of the study presented here, in term of cytokines effects on re-epithelialisation, offer insights into the potential role of cytokines as therapeutic candidates for wound healing. IL-6 decreases keratinocytes migration but increases the cellular elasticity adhesion propensity, which can be valuable in specific stages of wound healing where tensile strength of the skin is required. Another interesting outcome of this study is that IL-8 had no effects on the migration of keratinocytes and that human keratinocytes cannot generate TNF-α under maximum LPS challenge. As previously discussed, these two cytokines have been found in chronic wounds and continuous exposure of wounds to these two cytokines can impair wound healing process.

6.4 Conclusion

Co-culture of keratinocytes with stimulated monocytes under hypoxic condition could improve their pro-migratory propensity. However, it did not accelerate the rate of HaCaT cell migration in comparison to the normoxic model. One possible mechanism of this effect could be due to the secretion of cytokines and growth factors from the stimulated monocytes with LPS. Moreover, the stimulation of keratinocytes with LPS resulted in a significant release of IL-6 and IL-8. The LPS stimulation of keratinocytes did not change the rate of migration and proliferation of keratinocytes compared to non-stimulated conditions. The same result was found for IL-8 stimulation. However, treatment of keratinocytes with IL-6 significantly attenuates the migration of keratinocytes without any significant influence on proliferation and differentiation

rates. On the other hand, IL-6, not LPS or IL-8, increased cell adhesion and elasticity of keratinocytes that might contribute to improving skin integrity and tensile strength.

Chapter VII: General Discussion

7.1 General discussion

Skin is the largest organ of the human body. It is the body's first line of defence, but has multiple functions, including protection, sensation, aesthetics and communication, water resistance, controls absorption, thermoregulation, control of evaporation, storage and synthesis. Damage to this external covering by injury or illness can lead to acute loss of physiological balance, disability or even death (Clark et al., 2007).

The word "wound" has been defined as a disruption of normal anatomical structure and, more importantly, functions. Wound healing is a complex, protracted and dynamic process that results in the re-establishment of anatomical continuity and functional integrity (Diegelmann and Evans, 2004). The wound healing process involves a variety of cells, such as epidermal cells and fibroblasts and is a collaborative activity involving cell migration, proliferation, differentiation and apoptosis (Staiano-Coico et al., 2000), but the intricate details are not well understood. Nonetheless, wound healing generally consists of four overlapping phases, i.e. haemostasis, inflammation, granulation and remodelling (Thackham et al., 2008). Chronic wounds are those that do not heal in an orderly sequence and/or over the expected time frame compared to normal wounds. In general, any wound that fails to heal within three months is termed a chronic wound (Mustoe, 2004 & Schultz et al., 2003).

Restoration of microcirculation and nutrient supply to the tissue are the critical steps in wound healing (Thackham et al., 2008). The most essential nutrient is oxygen, which is critical for the production of granulation tissue and resistance to infection.

Hypoxia is the most common feature of chronic wounds. Impaired microcirculation and high oxygen consumption by metabolically active cells lead to a hypoxic microenvironment in the wounded tissues (Davidson and Mustoe, 2001). The oxygen tension in this hypoxic microenvironment ranges from 5-20 mmHg compared with typically 30-50 mmHg in healthy tissue (Sheffield, 1998). Hypoxia affects various aspects of the wound healing process, including angiogenesis, collagen synthesis and deposition, and re-epithelialisation .

A critical step in wound healing is re-epithelialization, dominated by migration, proliferation and differentiation of keratinocytes and restoration of the basement membrane at the wound site (Martin, 1997). The disturbed vascular oxygen supply to the wounded area is the rate-limiting factor for re-epithelialization (Kairuz et al., 2007). Keratinocytes are the major cells in skin epidermis constituting 90% of all cells and require a continuous oxygen supply to maintain their cellular functions (Gill and Bell, 2004). Thus, molecular oxygen is an essential element for the regeneration process in wound healing (LaVan and Hunt, 1990). It has been reported that oxygenation of ischemic wounds at 1-2 ATA enhances wound healing (Lee et al., 2006) and wound healing is primarily controlled by available oxygen (Hunt and Dunphy, 1969). The partial pressure of oxygen (pO_2) is positively correlated with angiogenesis (Hopf et al., 2005), collagen deposition (Warriner and Hopf, 2012) and epithelization (Uhl et al., 1994). Therefore, augmenting oxygen availability to tissues via HBO would promote cutaneous wound regeneration (Suh and Hunt, 1998 and Kalliainen et al., 2003).

In this thesis, processes in re-epithelialisation of the skin were investigated using cultured human immortalized keratinocytes (HaCaT). Various techniques (scratch

assay, Western blotting, ELISA and MTT) were used to investigate the migration, proliferation and differentiation of keratinocytes. Moreover, various models of cultured cells were created including hypoxic, normoxic and inflammatory models and were exposed to different treatments strategies.

The effect of acute and chronic hypoxia were previously examined in *in vitro* and *in vivo* models, including dermal fibroblasts and arterial remodeling, and demonstrated that temporary hypoxia stimulates fibroblasts from young patients (aged 24-33 years) to migrate towards the center of a wound and to produce collagen and other various extracellular matrixes (Falanga et al., 1993; Helfman and Falanga, 1993). Other studies suggested that prolonged hypoxia caused a detrimental effect on wound healing (Wu et al., 1999; Eaglstein and Falanga, 1997; Eaglstein, 1989; Wu et al., 1997). However, *in vivo* studies revealed that under hypoxic conditions, a significant delay in re-epithelialization of ulcerated wounds was found (Kairuz et al., 2007).

The role of chronic hypoxia, being the most common feature in chronic wounds, on migration, proliferation and differentiations of HaCaT cells were investigated in Chapter Three. A chronic hypoxic model was achieved via exposure of the human keratinocytes to a hypoxic environment for 72 hours in a modular chamber. The results clearly indicated that chronic hypoxia attenuated keratinocytes migration ($p<0.05$) as stated in Figure 3.1, of the rate keratinocyte's migration under hypoxic and normoxic conditions were 10641 ± 2105 μm2/hr and 16083 ± 2284 μm2/hr, subsequently. On the other hand, increased HaCaT cell proliferation ($p<0.001$) (Figure 3.5), metabolic activity ($p<0.001$) was observed. As shown in Figure 3.4, the PCNA expression under hypoxic and normoxic were 157 ± 11% and 53 ± 10% PCNA/ β-actin%, respectively.

and cell adhesion (hypoxic: 140.33±11.02% and normoxic 48.33±5.77% E-cadherin/β-actin%. $p<0.001$) were observed when the cells exposed to hypoxic condition. However, no effect on differentiation rates was found as shown in figure 3.6. Cells exposed to chronic hypoxic conditions attenuated both IL-6 and IL-8 expressions ($p<0.0001$). The data demonstrated that chronic hypoxia significantly delays re-epithelialisation. Interestingly, data suggested a strong relationship between hypoxia and retardation of cell migration rather than cell proliferation. The attenuation of keratinocytes motility was also correlated with higher expression of E-cadherin as the adhesion molecule. On the other hand, hyperproliferative keratinocytes and increased cellular metabolic activity under chronic hypoxic condition suggested epidermal hyperplasia is a possible consequence of chronic hypoxic wounds. This phenomenon (epidermal hyperproliferation along with impaired wound healing) has been observed in other wound studies (Pastar et al., 2014 & Florin et al., 2006). This epidermal hyperplasia can actively contribute in wound healing when it contains pro-migratory cells (Braiman-Wiksman et al., 2007). However, the results presented here demonstrated that the keratinocytes decreased their pro-migratory activity under chronic hypoxic condition.

As explained in Chapter One, oxygenation of wounds has been considered as a potential wound therapeutical modality. Currently, there are limited *in vitro* studies on the influence of hyperbaric oxygen therapy (HBO) on re-epithelialization processes. However, re-epithelialization is shown to be optimal at high oxygen tensions (Ladizinsky and Roe, 2010). Previous studies indicated that HBO enhanced proliferation of fibroblasts and endothelial cells (Lipsky and Berendt, 2010). Moreover, it increased keratinocytes migration and differentiation in a human

equivalent model (Dimitrijevich et al., 1999 and Tompach et al., 1997) and, induces VEGF expressions (Sheikh et al., 2000). Chapter Four was devoted to investigate the influence of hyperbaric oxygenation as a treatment modality on wound healing process in term of re-epithelialisation. In contrast to the results obtained from hypoxic model (in Chapter Three), hyperbaric oxygenation stimulated scratch wound closure process via acceleration of keratinocytes migration ($p<0.0001$); the keratinocyte migration's rates were 35697± 1159μm2/hr and 18191 ± 1347μm2/hr under HBO and normoxic conditions, respectively (Figure 4-2) and attenuating cell-cell and cell-matrix adhesion ($p<0.01$), the mean values were 17± 7% and 52± 3% under HBO and normoxic conditions, respectively as shown in figure 4.3. The mean PCNA expression was 6±1.4% and 77±1.5% under HBO and normoxic conditions, respectively. The results demonstrate that exposure of keratinocytes to HBO significantly attenuated the expression of PCNA in HaCaT cells, indicating that HBO reduces HaCaT cells proliferation (Figure 4-5). Moreover, the cellular expression of IL-8, but not IL-6, was decreased significantly ($p<0.05$). However, involucrin expression, as a terminal differentiation marker, was not changed throughout the course of the HBO treatment (Figure 4.6).

These findings are in agreement with other studies demonstrating that exposure of burn wounds to HBO leads to faster epithelialization and thus wound healing (Bilic et al., 2005) . Also, animal studies revealed that daily exposure to HBO for two hours at 2ATA resulted in accelerated wound re-epithelialization of ischemic and non-ischemic wounds (Uhl et al., 1994). Other studies showed that HBO increased nitric oxide (NO) production at the wound site, which in turn enhanced the migration of

epidermal cells by converting the stationary cells to locomoting phenotypes (Boykin and Baylis, 2007).

The results of the HBO investigations are significant in at least two major aspects: Firstly, they revealed that HBO can efficiently reverse all cellular and molecular changes (in keratinocytes) resulting from chronic hypoxic conditions. Secondly, it further confirmed that delayed re-epithelialisation is more related to impaired pro-migratory activity of the cells rather than their proliferation and differentiation rate. Therefore, investigating keratinocyte's locomotion is an important target in wound healing research.

Clinically, HBO has its limitations including facility accessibility, high cost, side effects and poor patient compliance (Han, 2016). Some of the side effects reported were serious problems including ear, sinus and pulmonary barotrauma, myopia, cataract, and oxygen toxicity seizures (Sharifipour et al., 2012). Therefore, it is rational to explore other strategies to minimise these serious side effects. Normobaric oxygen (NBO) has been proposed as an alternative therapy to HBO. NBO is the increased in fractional inspired oxygen to almost 100 % at normobaric pressure. *In vivo* studies revealed that NBO as an adjuvant treatment attenuated infarct size following stroke and increased brain functional outcomes (Sharifipour et al., 2012), Clinically, NBO in the treatment of chronic wounds has also shown promising results (Kalliainen et al., 2003). However, there is limited *in vitro* data regarding the efficiency of NBO in cutaneous wound healing.

In chapter five, the influence of NBO and the impacts of pressure alone (hyperbaric air, HBA) on keratinocytes were studied. The later was to understand any role that air

pressure alone had on cellular and molecular processes involved in the wound healing process.

Cultured keratinocytes were exposed to either NBO (in a modular chamber) or HBA (in an oxygen combustion vessel) for 48 hours. Results demonstrated that exposure to NBO resulted in a faster migration of keratinocytes to the scratched area compared to the normoxic control group (NBO: 23148±2222 μm2/hr, HBA: 16996±2483 μm2/hr and Normoxia: 15270±649 μm2/hr, $p<0.01$) (Figure 5.2) with a significant reduction in cell adhesion expressed by E-cadherin (mean ± s.d. NBO: 12±5%, HBA: 80±3% and Normoxia: 82±5%, ($p<0.0001$). On the other hand, NBO significantly attenuated proliferation and cellular metabolic activity of the keratinocytes. The PCNA expressions in NBO, HBA and normoxic groups were 26±1%, 95±5%, and 87±9%, respectively (Figure 5.5). Moreover, NBO significant diminished IL-6 expression compared to control ($p<0.001$), whereas no effect on IL-8 expression and cell differentiation (Figure 5.6). Regarding the HBA group, no significant differences were found in cell migration, adhesion, proliferation, differentiation, metabolic activity, and IL-6 and IL-8 productions when compared to normoxic model. These indicated that pressure alone does not seem to be a rate-limiting factor in re-epithelialisation processes.

Vs. Normoxia	Migration	Adhesion	Proliferation	Differentiation	Metabolic activity	IL-6	IL-8
Hypoxia	↓	↑	↑	ns	↑	↓	↓
HBO	↑	↓	↓	ns	↓	ns	↓
NBO	↑	↓	↓	ns	↓	↓	ns
HBA	ns	ns	ns	ns	ns	ns	ns

Table 7-1 The influences of various oxygen and ambient pressure levels on keratinocytes activity. ns = no significant effect ($P<0.05$ or lower)

A summary of all the findings of different growth environments on keratinocyte behaviour is given in Table 7-1. Interestingly, NBO and HBO had similar effects on keratinocytes migration and proliferation. Furthermore, HBA showed no influences on re-epithelialisation processes. These results are extremely important given that majority of side effects of HBO are related to the high air pressure rather than oxygen level. More importantly, NBO has similar re-epithelialisation effects as HBO, therefore, it can be considered as a potential alternative to hyperbaric oxygenation. However, further investigations are needed in term of *in vivo* and *in vitro* studies to elucidate the role of NBO on different stages and various cellular responses in the very complicated process of wound healing.

Apart from hypoxia, infection is considered as a serious challenge in the wound healing process. The majority of skin wounds are colonised with microorganisms which are critical in predicting wound healing (Cutting and White, 2005). The micro-environment of the wounded tissue provides moist, warm, and nutritious conditions, which is conducive to microbial colonization and proliferation (Bowler et al., 2001). In the event of infection, a wound may fail to heal in a timely manner, and managing

non-healing wounds can be expensive. Therefore, research explaining the role of infection on wound healing is compelling. In terms of *in vitro* studies, various models have been proposed including LPS stimulation as the main component of outer membrane of Gram-negative bacteria (Kawaguchi et al., 1995) and co-culture of keratinocytes with monocytes has also been used to elucidate the cross-talk between these crucial cells in skin disorders (Hennen et al., 2011& Schreiner et al., 2007). In chapter six, the role of infection on wound healing under hypoxic and normoxic conditions was studied. Three distinct but inter-related perspectives were investigated including stimulation of keratinocytes with LPS, co-culture of keratinocytes with monocytes and stimulation of keratinocytes with cytokines (IL-6 and IL-8). Both LPS (from Gram-negative bacteria) and monocytes play a key role in wound healing, especially during the inflammation phase via regulation of various cytokines and growth factors.

Data showed that stimulation of keratinocytes with LPS under hypoxic condition had no influences on keratinocytes migration to the scratched area when compared to the non-stimulated hypoxic group. However, as shown in Figure 6.2, both hypoxic models (stimulated and non-stimulated) significantly attenuated the migration rate when compared to the normoxic model (hypoxia: 10641±2105 μm2/hr, HLPS: 10168±551 μm2/hr, HTHP-1: 14152±2365 μm2/hr and Normoxia: 16083±2284 μm2/hr. These results suggest that hypoxia is the rate-limiting factor, rather than LPS (i.e. infection). On the other hand, co-culture of keratinocytes with monocytes under chronic hypoxic condition (HTHP-1) stimulates keratinocytes migration, though such an impact was not evident under normoxic condition (NTHP-1). Moreover, stimulation of keratinocytes with IL-6, but not IL-8, under normoxic conditions significantly

reduced keratinocyte migration (IL-6: 9495±935 μm2/hr, IL-8: 17674±2581 μm2/hr, THP-1: 13100±3844 μm2/hr and Normoxia: 17176±2288 μm2/hr) (Figure 6.4) this results also associated with higher cell adhesion and desmosomal numbers. The e-cadherin expressions were: 94±11% and 38±16% under IL-6 and normoxic conditions, respectively (Figure 6.6). Additionally, it was found that keratinocytes could not produce a detectable amount of TNF-α despite maximal stimulation with LPS (Figure 6.16).

Consistent with the results obtained in chapter three, hypoxic models (LPS stimulated and non-stimulated) along with IL-6 stimulated group, express higher levels of cell adhesion and proliferation. The e-cadherin expression under HLPS, Hypoxia and normoxia were 124±24%, 140±11% and 48±6%, respectively. The PCNA profiles were 162±13%, 157±12% and 53±11% under the same conditions respectively. However, non-significant differences were found among all treatment groups in term of differentiation (Figure 6.11 and 6.12). Interestingly, the increased adhesion and sticky behaviour of keratinocytes in IL-6 treated group suggests that IL-6 may have clinical applications for certain types of wounds, where the skin has failed to regain full strength and texture after wound closure. In summary, three key findings are evident from Chapter Six: 1. LPS has no effects on re-epithelialisation of wounds 2. Co-culture of keratinocytes with monocytes enhances keratinocytes migration under hypoxic condition and 3. IL-6 delays keratinocytes migration and increases cell binding, which in turn increases epidermal strength and texture.

Most wound infections are polymicrobial (Dowd et al., 2008) which typically have synergistic effects (Kingsley, 2001). Inflammation is a normal phase in wound healing to remove the microorganisms from the wounded tissues. Both bacteria and

endotoxins (for instance LPS) can elongate the inflammation phase via releasing pro-inflammatory cytokines including IL-1 and TNF-α from various cells involving in wound healing, which in turn delays wound healing processes (Guo and Dipietro, 2010). Although, the resulted obtained in chapter six demonstrated that LPS stimulation does not affect re-epithelialisation, elongation of the inflammation phase can explain the deleterious effects of infection on the entire wound healing process.

7.2 Limitations of the study and future works

Some limitations of the studies completed in this thesis merit consideration:

- *In vivo* skin re-epithelialisation is more complicated than the *in vitro* models, given that each state of wound healing is inter-correlated with other stages controlled by various cytokines and growth factors.
- Gene expressions of immortalized human cell lines such HaCaT cells might be different to some extent when compare with normal human keratinocytes (NHKs).
- Oxygen directly diffuses to the cell culture media, which in turn increases the level of oxygen very quickly and efficiently. However, such a rapid increase in the actual wound center is impossible.
- Wound healing is a complex process with different phases and various cells involved. Hyperbaric and normobaric oxygen therapy can improve wound healing in a variety of ways and via different mechanisms including bactericidal activity, improving neovascularisation and enhancing collagen production and wound remodelling. However, many more mechanisms have been suggested through which oxygen therapy can improve wound healing.

- In the current study the influence of hypoxia, HBO, NBO, HBA and inflammation on some aspects of re-epithelialisation have been investigated. However, future research could be undertaken to determine the influences of these models on distinct phases of wound healing including haemostasis, inflammation, proliferation and remodelling. Furthermore, in term of cytokines, in particular IL-6, *in vivo* investigation on animals can be conducted to elucidate the role of IL-6 on remodelling of the skin given that IL-6 enhances cell adhesion and sticky behaviour.

7.3 Conclusion

In the present study several conditions and experiments have been used to investigate the role of oxygen, hypoxia and inflammation on migration, proliferation and differentiation of keratinocytes. It is concluded that use of elevated oxygen, under normobaric and hyperbaric conditions, stimulates re-epithelialisation process via increasing keratinocytes migration rather than enhancing their proliferation rate. Moreover, both chronic hypoxic condition and IL-6 stimulation attenuated re-epithelisation processes. LPS and IL-8 stimulations did not influence keratinocytes migration and proliferation; however, co-culture of keratinocytes with monocytes only leads to a marginal increase of re-epithelisation under hypoxic condition. Further works is required to investigate the influence of HBO, NBO, HBA and infection on other phase of wound healing using *in vitro* and *in vivo* models.

References

Abidia, A., Laden, G., Kuhan, G., Johnson, B.F., Wilkinson, A.R., Renwick, P.M., Masson, E.A., and McCollum, P.T. (2003). The role of hyperbaric oxygen therapy in ischaemic diabetic lower extremity ulcers: a double-blind randomised-controlled trial. Eur. J. Vasc. Endovasc. Surg. Off. J. Eur. Soc. Vasc. Surg. *25*, 513–518.

Allen, D.B., Maguire, J.J., Mahdavian, M., Wicke, C., Marcocci, L., Scheuenstuhl, H., Chang, M., Le, A.X., Hopf, H.W., and Hunt, T.K. (1997). Wound hypoxia and acidosis limit neutrophil bacterial killing mechanisms. Arch. Surg. Chic. Ill 1960 *132*, 991–996.

Araki, J., Kato, H., Doi, K., Kuno, S., Kinoshita, K., Mineda, K., Kanayama, K., and Yoshimura, K. (2014). Application of Normobaric Hyperoxygenation to an Ischemic Flap and a Composite Skin Graft: Plast. Reconstr. Surg. Glob. Open *2*, e152.

Asai, J., Hirakawa, S., Sakabe, J., Kishida, T., Wada, M., Nakamura, N., Takenaka, H., Mazda, O., Urano, T., Suzuki-Inoue, K., et al. (2016). Platelets Regulate the Migration of Keratinocytes via Podoplanin/CLEC-2 Signaling during Cutaneous Wound Healing in Mice. Am. J. Pathol. *186*, 101–108.

Aydın, F., Kaya, A., Savran, A., İncesu, M., Karakuzu, C., and Öztürk, A.M. (2014). Diabetic hand infections and hyperbaric oxygen therapy. Acta Orthop. Traumatol. Turc. *48*, 649–654.

Barrientos, S., Stojadinovic, O., Golinko, M.S., Brem, H., and Tomic-Canic, M. (2008). PERSPECTIVE ARTICLE: Growth factors and cytokines in wound healing: Growth factors and cytokines in wound healing. Wound Repair Regen. *16*, 585–601.

Bedogni, B., Welford, S.M., Cassarino, D.S., Nickoloff, B.J., Giaccia, A.J., and Powell, M.B. (2005). The hypoxic microenvironment of the skin contributes to Akt-mediated melanocyte transformation. Cancer Cell *8*, 443–454.

Bellavia, G., Fasanaro, P., Melchionna, R., Capogrossi, M.C., and Napolitano, M. (2014). Transcriptional control of skin reepithelialization. J. Dermatol. Sci. *73*, 3–9.

Benson, R.M., Minter, L.M., Osborne, B.A., and Granowitz, E.V. (2003). Hyperbaric oxygen inhibits stimulus-induced proinflammatory cytokine synthesis by human blood-derived monocyte-macrophages. Clin. Exp. Immunol. *134*, 57–62.

Berin, M.C., McKay, D.M., and Perdue, M.H. (1999). Immune-epithelial interactions in host defense. Am. J. Trop. Med. Hyg. *60*, 16–25.

Bilic, I., Petri, N.M., Bezic, J., Alfirevic, D., Modun, D., Capkun, V., and Bota, B. (2005). Effects of hyperbaric oxygen therapy on experimental burn wound healing in rats: a randomized controlled study. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *32*, 1–9.

Bishop, A. (2008). Role of oxygen in wound healing. J. Wound Care *17*, 399–402.

Biswas, S., Roy, S., Banerjee, J., Hussain, S.-R.A., Khanna, S., Meenakshisundaram, G., Kuppusamy, P., Friedman, A., and Sen, C.K. (2010). Hypoxia inducible microRNA 210 attenuates keratinocyte proliferation and impairs closure in a murine model of ischemic wounds. Proc. Natl. Acad. Sci. U. S. A. *107*, 6976–6981.

Bjarnsholt, T., Kirketerp-Møller, K., Jensen, P.Ø., Madsen, K.G., Phipps, R., Krogfelt, K., Høiby, N., and Givskov, M. (2008). Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *16*, 2–10.

Blanpain, C., and Fuchs, E. (2006). Epidermal Stem Cells of the Skin. Annu. Rev. Cell Dev. Biol. *22*, 339–373.

Van den Blink, B., van der Kleij, A.J., Versteeg, H.H., and Peppelenbosch, M.P. (2002). Immunomodulatory effect of oxygen and pressure. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. *132*, 193–197.

Blumenfeld, I., Ullmann, Y., Laufer, D., and Livne, E. (2003). ENHANCEMENT OF BURN HEALING BY GROWTH FACTORS AND IL□8. Ann. Burns Fire Disasters *vol. XIII*.

Boniface, K., Bernard, F.-X., Garcia, M., Gurney, A.L., Lecron, J.-C., and Morel, F. (2005). IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. J. Immunol. Baltim. Md 1950 *174*, 3695–3702.

Bonomo, S.R., Davidson, J.D., Yu, Y., Xia, Y., Lin, X., and Mustoe, T.A. (1998). Hyperbaric oxygen as a signal transducer: upregulation of platelet derived growth factor-beta receptor in the presence of HBO2 and PDGF. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *25*, 211–216.

Boukamp, P., Petrussevska, R.T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N.E. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell Biol. *106*, 761–771.

Bowler, P.G., Duerden, B.I., and Armstrong, D.G. (2001). Wound Microbiology and Associated Approaches to Wound Management. Clin. Microbiol. Rev. *14*, 244–269.

Boykin, J.V., Jr, and Baylis, C. (2007). Hyperbaric oxygen therapy mediates increased nitric oxide production associated with wound healing: a preliminary study. Adv. Skin Wound Care *20*, 382–388.

Braiman-Wiksman, L., Solomonik, I., Spira, R., and Tennenbaum, T. (2007). Novel Insights into Wound Healing Sequence of Events. Toxicol. Pathol. *35*, 767–779.

Brenner, I., Shephard, R.J., and Shek, P.N. (1999). Immune function in hyperbaric environments, diving, and decompression. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *26*, 27–39.

Brismar, K., Lind, F., and Kratz, G. (1997). Dose-dependent hyperbaric oxygen stimulation of human fibroblast proliferation. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *5*, 147–150.

Brothers, K.M., Stella, N.A., Hunt, K.M., Romanowski, E.G., Liu, X., Klarlund, J.K., and Shanks, R.M.Q. (2015). Putting on the brakes: Bacterial impediment of wound healing. Sci. Rep. *5*, 14003.

Bryant, R., and Nix, D. (2006). Acute and Chronic Wounds (London: Elsevier Health Sciences).

Calvert, J.W., Zhou, C., Nanda, A., and Zhang, J.H. (2003). Effect of hyperbaric oxygen on apoptosis in neonatal hypoxia-ischemia rat model. J. Appl. Physiol. Bethesda Md 1985 *95*, 2072–2080.

Chan, Y.S., Chen, A.C.Y., Yuan, L.J., Lin, S.S., Yang, C.Y., Lee, M.S.S., and Ueng, S.W.N. (2007). Effects of hyperbaric oxygen and platelet derived growth factor on medial collateral ligament fibroblasts. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *34*, 181–190.

Chavez, M.G., Buhr, C.A., Petrie, W.K., Wandinger-Ness, A., Kusewitt, D.F., and Hudson, L.G. (2012). Differential downregulation of e-cadherin and desmoglein by epidermal growth factor. Dermatol. Res. Pract. *2012*, 309587.

Chen, W.Y.J., and Rogers, A.A. (2007). Recent insights into the causes of chronic leg ulceration in venous diseases and implications on other types of chronic wounds. Wound Repair Regen. *15*, 434–449.

Chen, D., Hao, H., Fu, X., and Han, W. (2016). Insight into Reepithelialization: How Do Mesenchymal Stem Cells Perform? Stem Cells Int. *2016*, 1–9.

Chen, L., Guo, S., Ranzer, M.J., and DiPietro, L.A. (2013). Toll-Like Receptor 4 Has an Essential Role in Early Skin Wound Healing. J. Invest. Dermatol. *133*, 258–267.

Chen, S.-J., Yu, C.-T., Cheng, Y.-L., Yu, S.-Y., and Lo, H.-C. (2007). Effects of hyperbaric oxygen therapy on circulating interleukin-8, nitric oxide, and insulin-like growth factors in patients with type 2 diabetes mellitus. Clin. Biochem. *40*, 30–36.

Cho, Y.-S., Bae, J.-M., Chun, Y.-S., Chung, J.-H., Jeon, Y.-K., Kim, I.-S., Kim, M.-S., and Park, J.-W. (2008). HIF-1alpha controls keratinocyte proliferation by up-regulating p21(WAF1/Cip1). Biochim. Biophys. Acta *1783*, 323–333.

Chow, S.-E., Chen, C.P.-C., Hsu, C.-C., Tsai, W.-C., Wang, J.-S., and Hsu, N.-C. (2016). Quantifying cell behaviors in negative-pressure induced monolayer cell movement. Biomed. J. *39*, 50–59.

Cianci, P., Slade, J.B., Sato, R.M., and Faulkner, J. (2013). Adjunctive hyperbaric oxygen therapy in the treatment of thermal burns. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *40*, 89–108.

Clark, R.A.F., Ghosh, K., and Tonnesen, M.G. (2007). Tissue Engineering for Cutaneous Wounds. J. Invest. Dermatol. *127*, 1018–1029.

Cutting, K.F., and White, R.J. (2005). Criteria for identifying wound infection--revisited. Ostomy. Wound Manage. *51*, 28–34.

David, L.A., Sàndor, G.K., Evans, A.W., and Brown, D.H. (2001). Hyperbaric oxygen therapy and mandibular osteoradionecrosis: a retrospective study and analysis of treatment outcomes. J. Can. Dent. Assoc. *67*, 384.

Davidson, J.D., and Mustoe, T.A. (2001). Oxygen in wound healing: more than a nutrient. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *9*, 175–177.

Dealey, C. (2012). The Care of Wounds A Guide for Nurses. (Hoboken: John Wiley & Sons).

Deaton, P.R., McKellar, C.T., Culbreth, R., Veal, C.F., and Cooper, J.A. (1994). Hyperoxia stimulates interleukin-8 release from alveolar macrophages and U937 cells: attenuation by dexamethasone. Am. J. Physiol. *267*, L187–L192.

Van De Graaff, K.M. (2011). Human anatomy and physiology (Chicago: McGraw-Hill).

Devalaraja, R.M., Nanney, L.B., Du, J., Qian, Q., Yu, Y., Devalaraja, M.N., and Richmond, A. (2000). Delayed wound healing in CXCR2 knockout mice. J. Invest. Dermatol. *115*, 234–244.

Diegelmann, R.F., and Evans, M.C. (2004). Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci *9*, 283–289.

Dimitrijevich, S.D., Paranjape, S., Wilson, J.R., Gracy, R.W., and Mills, J.G. (1999). Effect of hyperbaric oxygen on human skin cells in culture and in human dermal and skin equivalents. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *7*, 53–64.

Dong, H., Xiong, L., Zhu, Z., Chen, S., Hou, L., and Sakabe, T. (2002). Preconditioning with hyperbaric oxygen and hyperoxia induces tolerance against spinal cord ischemia in rabbits. Anesthesiology *96*, 907–912.

Dowd, S.E., Sun, Y., Secor, P.R., Rhoads, D.D., Wolcott, B.M., James, G.A., and Wolcott, R.D. (2008). Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. BMC Microbiol. *8*, 43.

Dunlevy, J.R., and Couchman, J.R. (1995). Interleukin-8 induces motile behavior and loss of focal adhesions in primary fibroblasts. J. Cell Sci. *108 (Pt 1)*, 311–321.

Eaglstein, W.H. (1989). Wound healing and aging. Clin. Geriatr. Med. *5*, 183–188.

Eaglstein, W.H., and Falanga, V. (1997). Chronic wounds. Surg. Clin. North Am. *77*, 689–700.

Eckert, J.W., Abramson, S.L., Starke, J., and Brandt, M.L. (1995). The surgical implications of chronic granulomatous disease. Am. J. Surg. *169*, 320–323.

Edwards, R., and Harding, K.G. (2004). Bacteria and wound healing. Curr. Opin. Infect. Dis. *17*, 91–96.

Efrati, S., Gall, N., Bergan, J., Fishlev, G., Bass, A., Berman, S., Hamad-Abu, R., Feigenzon, M., and Weissgarten, J. (2009). Hyperbaric oxygen, oxidative stress, NO bioavailability and ulcer oxygenation in diabetic patients. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *36*, 1–12.

Ema, M., Hirota, K., Mimura, J., Abe, H., Yodoi, J., Sogawa, K., Poellinger, L., and Fujii-Kuriyama, Y. (1999). Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J. *18*, 1905–1914.

Eming, S.A., Brachvogel, B., Odorisio, T., and Koch, M. (2007). Regulation of angiogenesis: wound healing as a model. Prog. Histochem. Cytochem. *42*, 115–170.

Escobar, S.J., Slade, J.B., Hunt, T.K., and Cianci, P. (2005). Adjuvant hyperbaric oxygen therapy (HBO2)for treatment of necrotizing fasciitis reduces mortality and amputation rate. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *32*, 437–443.

Eyre, D.R., Paz, M.A., and Gallop, P.M. (1984). Cross-linking in collagen and elastin. Annu. Rev. Biochem. *53*, 717–748.

Faglia, E., Favales, F., Aldeghi, A., Calia, P., Quarantiello, A., Oriani, G., Michael, M., Campagnoli, P., and Morabito, A. (1996). Adjunctive systemic hyperbaric oxygen therapy in treatment of severe prevalently ischemic diabetic foot ulcer. A randomized study. Diabetes Care *19*, 1338–1343.

Falanga, V. (1993). Growth factors and wound healing. J Dermatol Surg Oncol *19*, 711–714.

Falanga, V., Qian, S.W., Danielpour, D., Katz, M.H., Roberts, A.B., and Sporn, M.B. (1991). Hypoxia upregulates the synthesis of TGF-beta 1 by human dermal fibroblasts. J. Invest. Dermatol. *97*, 634–637.

Falanga, V., Martin, T.A., Takagi, H., Kirsner, R.S., Helfman, T., Pardes, J., and Ochoa, M.S. (1993). Low oxygen tension increases mRNA levels of alpha 1 (I) procollagen in human dermal fibroblasts. J. Cell. Physiol. *157*, 408–412.

Fitsialos, G., Bourget, I., Augier, S., Ginouvès, A., Rezzonico, R., Odorisio, T., Cianfarani, F., Virolle, T., Pouysségur, J., Meneguzzi, G., et al. (2008). HIF1 transcription factor regulates laminin-332 expression and keratinocyte migration. J. Cell Sci. *121*, 2992–3001.

Florin, L., Knebel, J., Zigrino, P., Vonderstrass, B., Mauch, C., Schorpp-Kistner, M., Szabowski, A., and Angel, P. (2006). Delayed Wound Healing and Epidermal Hyperproliferation in Mice Lacking JunB in the Skin. J. Invest. Dermatol. *126*, 902–911.

Folkman, J. (1982). Angiogenesis: initiation and control. Ann. N. Y. Acad. Sci. *401*, 212–227.

Fries, R.B., Wallace, W.A., Roy, S., Kuppusamy, P., Bergdall, V., Gordillo, G.M., Melvin, W.S., and Sen, C.K. (2005). Dermal excisional wound healing in pigs following treatment with topically applied pure oxygen. Mutat. Res. *579*, 172–181.

Fukumura, D., Gohongi, T., Kadambi, A., Izumi, Y., Ang, J., Yun, C.O., Buerk, D.G., Huang, P.L., and Jain, R.K. (2001). Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc. Natl. Acad. Sci. U. S. A. *98*, 2604–2609.

Fulton, J.E., Jr (2000). The use of hyperbaric oxygen (HBO) to accelerate wound healing. Dermatol. Surg. Off. Publ. Am. Soc. Dermatol. Surg. Al *26*, 1170–1172.

Fung, C.K.M., Ning Xi, Ruiguo Yang, Seiffert-Sinha, K., King Wai Chiu Lai, and Sinha, A.A. (2011). Quantitative Analysis of Human Keratinocyte Cell Elasticity Using Atomic Force Microscopy (AFM). IEEE Trans. NanoBioscience *10*, 9–15.

Gajendrareddy, P.K., Sen, C.K., Horan, M.P., and Marucha, P.T. (2005). Hyperbaric oxygen therapy ameliorates stress-impaired dermal wound healing. Brain. Behav. Immun. *19*, 217–222.

Garlick, J.A., and Taichman, L.B. (1994). Fate of human keratinocytes during reepithelialization in an organotypic culture model. Lab. Investig. J. Tech. Methods Pathol. *70*, 916–924.

Garthwaite, J. (1991). Glutamate, nitric oxide and cell-cell signalling in the nervous system. Trends Neurosci. *14*, 60–67.

Gharaee-Kermani, M., and Phan, S.H. (2001). Role of cytokines and cytokine therapy in wound healing and fibrotic diseases. Curr. Pharm. Des. *7*, 1083–1103.

Gill, A.L., and Bell, C.N.A. (2004). Hyperbaric oxygen: its uses, mechanisms of action and outcomes. QJM Mon. J. Assoc. Physicians *97*, 385–395.

Ginsberg, M.D. (2003). Adventures in the pathophysiology of brain ischemia: penumbra, gene expression, neuroprotection: the 2002 Thomas Willis Lecture. Stroke J. Cereb. Circ. *34*, 214–223.

Gordillo, G.M., and Sen, C.K. (2003). Revisiting the essential role of oxygen in wound healing. Am. J. Surg. *186*, 259–263.

Gottrup, F. (2004). Oxygen in wound healing and infection. World J. Surg. *28*, 312–315.

Granowitz, E.V., Skulsky, E.J., Benson, R.M., Wright, J., Garb, J.L., Cohen, E.R., Smithline, E.C., and Brown, R.B. (2002). Exposure to increased pressure or hyperbaric oxygen suppresses interferon-gamma secretion in whole blood cultures of healthy humans. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *29*, 216–225.

Greenhalgh, D.G. (1996). The role of growth factors in wound healing. J. Trauma *41*, 159–167.

Gregg, D., de Carvalho, D.D., and Kovacic, H. (2004). Integrins and coagulation: a role for ROS/redox signaling? Antioxid. Redox Signal. *6*, 757–764.

Greif, R., Akça, O., Horn, E.P., Kurz, A., Sessler, D.I., and Outcomes Research Group (2000). Supplemental perioperative oxygen to reduce the incidence of surgical-wound infection. N. Engl. J. Med. *342*, 161–167.

Grundmann, T., Jaehne, M., and Fritze, G. (2000). [The value of hyperbaric oxygen therapy (HBO) in treatment of problem wounds in the area of plastic-reconstructive head and neck surgery]. Laryngorhinootologie. *79*, 304–310.

Guest, J.F., Ayoub, N., McIlwraith, T., Uchegbu, I., Gerrish, A., Weidlich, D., Vowden, K., and Vowden, P. (2015). Health economic burden that wounds impose on the National Health Service in the UK. BMJ Open *5*, e009283.

Guo, S., and Dipietro, L.A. (2010). Factors affecting wound healing. J. Dent. Res. *89*, 219–229.

Hampson, N.B. (1999). Hyperbaric oxygen therapy: 1999 committee report (Kensington, Md.: Undersea and Hyperbaric Medical Society).

Han, S.-K. (2016). Innovations and advances in wound healing.

Haroon, Z.A., Raleigh, J.A., Greenberg, C.S., and Dewhirst, M.W. (2000). Early wound healing exhibits cytokine surge without evidence of hypoxia. Ann. Surg. *231*, 137–147.

Hebda, P.A. (1988). Stimulatory effects of transforming growth factor-beta and epidermal growth factor on epidermal cell outgrowth from porcine skin explant cultures. J. Invest. Dermatol. *91*, 440–445.

Helfman, T., and Falanga, V. (1993). Gene expression in low oxygen tension. Am. J. Med. Sci. *306*, 37–41.

Heng, M.C.Y. (2011). Wound healing in adult skin: aiming for perfect regeneration. Int. J. Dermatol. *50*, 1058–1066.

Hennen, J., Aeby, P., Goebel, C., Schettgen, T., Oberli, A., Kalmes, M., and Blomeke, B. (2011). Cross Talk between Keratinocytes and Dendritic Cells: Impact on the Prediction of Sensitization. Toxicol. Sci. *123*, 501–510.

Hernández-Quintero, M., Kuri-Harcuch, W., González Robles, A., and Castro-Muñozledo, F. (2006). Interleukin-6 promotes human epidermal keratinocyte proliferation and keratin cytoskeleton reorganization in culture. Cell Tissue Res. *325*, 77–90.

Heyboer, M., Milovanova, T.N., Wojcik, S., Grant, W., Chin, M., Hardy, K.R., Lambert, D.S., Logue, C., and Thom, S.R. (2014). CD34+/CD45-dim stem cell mobilization by hyperbaric oxygen — Changes with oxygen dosage. Stem Cell Res. *12*, 638–645.

Hintermann, E., Yang, N., O'Sullivan, D., Higgins, J.M.G., and Quaranta, V. (2005). Integrin alpha6beta4-erbB2 complex inhibits haptotaxis by up-regulating E-cadherin cell-cell junctions in keratinocytes. J. Biol. Chem. *280*, 8004–8015.

Hishikawa, K., Nakaki, T., Marumo, T., Hayashi, M., Suzuki, H., Kato, R., and Saruta, T. (1994). Pressure promotes DNA synthesis in rat cultured vascular smooth muscle cells. J. Clin. Invest. *93*, 1975–1980.

Alt-Holland, A., Shamis, Y., Riley, K.N., DesRochers, T.M., Fusenig, N.E., Herman, I.M., and Garlick, J.A. (2008). E-cadherin suppression directs cytoskeletal rearrangement and intraepithelial tumor cell migration in 3D human skin equivalents. J. Invest. Dermatol. *128*, 2498–2507.

Hollander, D.A., Hakimi, M.Y., Hartmann, A., Wilhelm, K., and Windolf, J. (2000). The Influence of Hyperbaric Oxygenation (HBO) on Proliferation and Differentiation of Human Keratinocyte Cultures In Vitro. Cell Tissue Bank. *1*, 261–269.

Hopf, H.W., Gibson, J.J., Angeles, A.P., Constant, J.S., Feng, J.J., Rollins, M.D., Zamirul Hussain, M., and Hunt, T.K. (2005). Hyperoxia and angiogenesis. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *13*, 558–564.

Horikoshi, T., Balin, A.K., and Carter, D.M. (1986). Effect of oxygen on the growth of human epidermal keratinocytes. J. Invest. Dermatol. *86*, 424–427.

Hunt, T.K., and Aslam, R.S. (2004). Oxygen 2002: wounds. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *31*, 147–153.

Hunt, T.K., and Dunphy, J.E. (1969). Effects of increasing oxygen supply to healing wounds. Br. J. Surg. *56*, 705.

Hunt, T.K., and Pai, M.P. (1972). The effect of varying ambient oxygen tensions on wound metabolism and collagen synthesis. Surg. Gynecol. Obstet. *135*, 561–567.

Hunt, T.K., Niinikoski, J., and Zederfeldt, B. (1972). Role of oxygen in repair processes. Acta Chir. Scand. *138*, 109–110.

Hunt, T.K., Aslam, R.S., Beckert, S., Wagner, S., Ghani, Q.P., Hussain, M.Z., Roy, S., and Sen, C.K. (2007). Aerobically derived lactate stimulates revascularization and tissue repair via redox mechanisms. Antioxid. Redox Signal. *9*, 1115–1124.

Ibisch, C., Bourdeau, P., Cadiot, C., Viac, J., and Gatto, H. (2007). Upregulation of TNF-α Production by IFN-γ and LPS in Cultured Canine Keratinocytes: Application to Monosaccharides Effects. Vet. Res. Commun. *31*, 835–846.

Inamoto, Y., Okuno, F., Saito, K., Tanaka, Y., Watanabe, K., Morimoto, I., Yamashita, U., and Eto, S. (1991). Effect of hyperbaric oxygenation on macrophage function in mice. Biochem. Biophys. Res. Commun. *179*, 886–891.

Iocono, J.A., Colleran, K.R., Remick, D.G., Gillespie, B.W., Ehrlich, H.P., and Garner, W.L. (2000). Interleukin-8 levels and activity in delayed-healing human thermal wounds. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *8*, 216–225.

Ishigame, H., Kakuta, S., and Nagai, T. (2009). Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity *30*, 108–119.

Jain, K.K. (2009a). The history of hyperbaric medicine. In Textbook of Hyperbaric Medicine, K.K. Jain, ed. (Cambridge, MA: Hogrefe Publishing), pp. 3–8.

Jain, K.K. (2009b). Textbook of hyperbaric medicine (Cambridge, MA: Hogrefe).

Jamieson, D., Chance, B., Cadenas, E., and Boveris, A. (1986). The relation of free radical production to hyperoxia. Annu. Rev. Physiol. *48*, 703–719.

Jiang, W.G., Sanders, A.J., Ruge, F., and Harding, K.G. (2012). Influence of interleukin-8 (IL-8) and IL-8 receptors on the migration of human keratinocytes, the role of PLC-γ and potential clinical implications. Exp. Ther. Med. *3*, 231–236.

Jonsson, K., Jensen, J.A., Goodson, W.H., 3rd, Scheuenstuhl, H., West, J., Hopf, H.W., and Hunt, T.K. (1991). Tissue oxygenation, anemia, and perfusion in relation to wound healing in surgical patients. Ann. Surg. *214*, 605–613.

Kairuz, E., Upton, Z., Dawson, R.A., and Malda, J. (2007). Hyperbaric oxygen stimulates epidermal reconstruction in human skin equivalents. Wound Repair Regen. *15*, 266–274.

Kalliainen, L.K., Gordillo, G.M., Schlanger, R., and Sen, C.K. (2003). Topical oxygen as an adjunct to wound healing: a clinical case series. Pathophysiol. Off. J. Int. Soc. Pathophysiol. ISP *9*, 81–87.

Karasek, M.A. (1966). In vitro Culture of Human Skin Epithelial Cells**From the Department of Dermatology, Stanford University School of Medicine, Palo Alto, California. J. Invest. Dermatol. *47*, 533–540.

Kawaguchi, H., Hizuta, A., Tanaka, N., and Orita, K. (1995). Role of endotoxin in wound healing impairment. Res. Commun. Mol. Pathol. Pharmacol. *89*, 317–327.

Kessler, L., Bilbault, P., Ortéga, F., Grasso, C., Passemard, R., Stephan, D., Pinget, M., and Schneider, F. (2003). Hyperbaric oxygenation accelerates the healing rate of nonischemic chronic diabetic foot ulcers: a prospective randomized study. Diabetes Care *26*, 2378–2382.

Khanna, S., and Wallace, W. (2002). Would Healing: oxygen and emerging therapeutics. Columbus, Ohio, September 12-15, 2002. Antioxid. Redox Signal. *4*, 961–963.

Kim, H.S., Kim, N.H., Kim, J., and Cha, I.H. (2013). Inducing re-epithelialization in skin wound through cultured oral mucosal keratinocytes. J. Korean Assoc. Oral Maxillofac. Surg. *39*, 63–70.

Kindwall, E.P., Kindwall, E.P., and Whelan, H.T. (2008). Hyperbaric medicine practice (Flagstaff, AZ: Best Pub. Co).

Kingsley, A. (2001). A proactive approach to wound infection. Nurs. Stand. R. Coll. Nurs. G. B. 1987 *15*, 50–54, 56, 58.

Kirsner, R.S., and Eaglstein, W.H. (1993). The wound healing process. Dermatol. Clin. *11*, 629–640.

Knighton, D.R., Silver, I.A., and Hunt, T.K. (1981). Regulation of wound-healing angiogenesis-effect of oxygen gradients and inspired oxygen concentration. Surgery *90*, 262–270.

Knighton, D.R., Hunt, T.K., Scheuenstuhl, H., Halliday, B.J., Werb, Z., and Banda, M.J. (1983). Oxygen tension regulates the expression of angiogenesis factor by macrophages. Science *221*, 1283–1285.

Knighton, D.R., Halliday, B., and Hunt, T.K. (1984). Oxygen as an antibiotic. The effect of inspired oxygen on infection. Arch. Surg. Chic. Ill 1960 *119*, 199–204.

Kondo, S., Kono, T., Sauder, D.N., and McKenzie, R.C. (1993). IL-8 gene expression and production in human keratinocytes and their modulation by UVB. J. Invest. Dermatol. *101*, 690–694.

Korn, H.N., Wheeler, E.S., and Miller, T.A. (1977). Effect of hyperbaric oxygen on second-degree burn wound healing. Arch. Surg. Chic. Ill 1960 *112*, 732–737.

Kostarnoy, A.V., Gancheva, P.G., Logunov, D.Y., Verkhovskaya, L.V., Bobrov, M.A., Scheblyakov, D.V., Tukhvatulin, A.I., Filippova, N.E., Naroditsky, B.S., and Gintsburg, A.L. (2013). Topical bacterial lipopolysaccharide application affects inflammatory response and promotes wound healing. J. Interferon Cytokine Res. Off. J. Int. Soc. Interferon Cytokine Res. *33*, 514–522.

Krasner, D.D., Rodeheaver, G., Woo, K., and Sibbald, G. (2012). Chronic Wound Care 5 (Cork: BookBaby).

Kuffler, D.P. (2011). The role of hyperbaric oxygen therapy in enhancing the rate of wound healing with a focus on axon regeneration. P. R. Health Sci. J. *30*, 35–42.

Kupcsik, L. (2011). Estimation of cell number based on metabolic activity: the MTT reduction assay. Methods Mol. Biol. Clifton NJ *740*, 13–19.

Kupetsky, E.A., Mathers, A.R., and Ferris, L.K. (2013). Anti-cytokine therapy in the treatment of psoriasis. Cytokine *61*, 704–712.

Ladizinsky, D., and Roe, D. (2010). REVIEW New Insights Into Oxygen Therapy for Wound Healing. Wounds *22*, 294.

Lahat, N., Bitterman, H., Yaniv, N., Kinarty, A., and Bitterman, N. (1995). Exposure to hyperbaric oxygen induces tumour necrosis factor-alpha (TNF-alpha) secretion from rat macrophages. Clin. Exp. Immunol. *102*, 655–659.

Laplante, A.F., Germain, L., Auger, F.A., and Moulin, V. (2001). Mechanisms of wound reepithelialization: hints from a tissue-engineered reconstructed skin to long-standing questions. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *15*, 2377–2389.

LaVan, F.B., and Hunt, T.K. (1990). Oxygen and wound healing. Clin. Plast. Surg. *17*, 463–472.

Lee, C.-C., Chen, S.-C., Tsai, S.-C., Wang, B.-W., Liu, Y.-C., Lee, H.-M., and Shyu, K.-G. (2006). Hyperbaric oxygen induces VEGF expression through ERK, JNK and c-Jun/AP-1 activation in human umbilical vein endothelial cells. J. Biomed. Sci. *13*, 143–156.

Lee, C.-H., Lee, L., Yang, K.-J., and Lin, T.-F. (2012). Top-cited articles on hyperbaric oxygen therapy published from 2000 to 2010. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *39*, 1089–1098.

Li, J., Chen, J., and Kirsner, R. (2007). Pathophysiology of acute wound healing. Clin. Dermatol. *25*, 9–18.

Lin, Z.-Q. (2003). Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. J. Leukoc. Biol. *73*, 713–721.

Lin, H.-I., Chu, S.-J., Perng, W.-C., Wu, C.-P., Lin, Z.-Y., and Huang, K.-L. (2008). Hyperbaric oxygen attenuates cell growth in skin fibroblasts cultured in a high-glucose medium. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *16*, 513–519.

Lipsky, B.A., and Berendt, A.R. (2010). Hyperbaric Oxygen Therapy for Diabetic Foot Wounds: Has hope hurdled hype? Diabetes Care *33*, 1143–1145.

Löndahl, M., Katzman, P., Nilsson, A., and Hammarlund, C. (2010). Hyperbaric oxygen therapy facilitates healing of chronic foot ulcers in patients with diabetes. Diabetes Care *33*, 998–1003.

Loryman, C., and Mansbridge, J. (2008). Inhibition of keratinocyte migration by lipopolysaccharide. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *16*, 45–51.

Luo, B.-H., Carman, C.V., and Springer, T.A. (2007). Structural basis of integrin regulation and signaling. Annu. Rev. Immunol. *25*, 619–647.

Marieb, E.N. (2000). Human anatomy and physiology (San Francisco; London: Longman).

Martin, P. (1997). Wound healing--aiming for perfect skin regeneration. Science *276*, 75–81.

Martini, F., Nath, J.L., and Bartholomew, E.F. (2012). Fundamentals of anatomy & physiology (San Francisco: Benjamin Cummings).

Martins-Green, M., Petreaca, M., and Wang, L. (2013). Chemokines and Their Receptors Are Key Players in the Orchestra That Regulates Wound Healing. Adv. Wound Care *2*, 327–347.

Mast, B.A., and Schultz, G.S. (1996). Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *4*, 411–420.

Mehm, W., Pimsler, M., Becker, R., and Lissner, C. (1988). Effect of Oxygen on in vitro Fibroblast Cell Proliferation and Collagen Biosynthesis.

Micallef, L., Belaubre, F., Pinon, A., Jayat-Vignoles, C., Delage, C., Charveron, M., and Simon, A. (2009). Effects of extracellular calcium on the growth-differentiation switch in immortalized keratinocyte HaCaT cells compared with normal human keratinocytes. Exp. Dermatol. *18*, 143–151.

Moen, I., Øyan, A.M., Kalland, K.-H., Tronstad, K.J., Akslen, L.A., Chekenya, M., Sakariassen, P.Ø., Reed, R.K., and Stuhr, L.E.B. (2009). Hyperoxic Treatment Induces Mesenchymal-to-Epithelial Transition in a Rat Adenocarcinoma Model. PLoS ONE *4*, e6381.

Mortensen, C. (2008). Hyperbaric oxygen therapy. Curr. Anaesth. Crit. Care *19*, 333–337.

Mouren, S., Souktani, R., Beaussier, M., Abdenour, L., Arthaud, M., Duvelleroy, M., and Vicaut, E. (1997). Mechanisms of coronary vasoconstriction induced by high arterial oxygen tension. Am. J. Physiol. *272*, H67–H75.

Mustoe, T. (2004). Understanding chronic wounds: a unifying hypothesis on their pathogenesis and implications for therapy. Am. J. Surg. *187*, 65S – 70S.

Mustoe, T.A., Pierce, G.F., Thomason, A., Gramates, P., Sporn, M.B., and Deuel, T.F. (1987). Accelerated healing of incisional wounds in rats induced by transforming growth factor-beta. Science *237*, 1333–1336.

Mustoe, T.A., O'Shaughnessy, K., and Kloeters, O. (2006). Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis. Plast. Reconstr. Surg. *117*, 35S – 41S.

Nakamura, Y., Kano, R., Hasegawa, A., and Watanabe, S. (2002). Interleukin-8 and Tumor Necrosis Factor Alpha Production in Human Epidermal Keratinocytes Induced by Trichophyton mentagrophytes. Clin. Vaccine Immunol. *9*, 935–937.

Nauta, T.D., van Hinsbergh, V.W.M., and Koolwijk, P. (2014). Hypoxic signaling during tissue repair and regenerative medicine. Int. J. Mol. Sci. *15*, 19791–19815.

Nazario, J., and Kuffler, D.P. (2011). Hyperbaric oxygen therapy and promoting neurological recovery following nerve trauma. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *38*, 345–366.

Nestle, F.O., Kaplan, D.H., and Barker, J. (2009). Psoriasis. N. Engl. J. Med. *361*, 496–509.

Ngo, M.A., Sinitsyna, N.N., Qin, Q., and Rice, R.H. (2007). Oxygen-Dependent Differentiation of Human Keratinocytes. J. Invest. Dermatol. *127*, 354–361.

Niezgoda, J.A., Cianci, P., Folden, B.W., Ortega, R.L., Slade, J.B., and Storrow, A.B. (1997). The effect of hyperbaric oxygen therapy on a burn wound model in human volunteers. Plast. Reconstr. Surg. *99*, 1620–1625.

Niinikoski, J. (1969). Effect of oxygen supply on wound healing and formation of experimental granulation tissue. Acta Physiol. Scand. Suppl. *334*, 1–72.

Niinikoski, J., Hunt, T.K., and Dunphy, J.E. (1972a). Oxygen supply in healing tissue. Am. J. Surg. *123*, 247–252.

Niinikoski, J., Grislis, G., and Hunt, T.K. (1972b). Respiratory gas tensions and collagen in infected wounds. Ann. Surg. *175*, 588–593.

Ninikoski, J., Heughan, C., and Hunt, T.K. (1972). Oxygen tensions in human wounds. J. Surg. Res. *12*, 77–82.

Nissen, N.N., Polverini, P.J., Koch, A.E., Volin, M.V., Gamelli, R.L., and DiPietro, L.A. (1998). Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. Am. J. Pathol. *152*, 1445–1452.

Oh, S., Lee, E., Lee, J., Lim, Y., Kim, J., and Woo, S. (2008). Comparison of the effects of 40% oxygen and two atmospheric absolute air pressure conditions on stress-induced premature senescence of normal human diploid fibroblasts. Cell Stress Chaperones *13*, 447–458.

Oztas, E., Kiliç, A., Ozyurt, M., Korkmaz, A., and Başustaoğlu, A. (2001). Effect of hyperbaric oxygen and penicillin in a murine model of streptococcal myositis. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *28*, 181–186.

Pack, P.E. (2001). Anatomy and physiology (New York: Wiley).

Padgaonkar, V., Giblin, F.J., Reddan, J.R., and Dziedzic, D.C. (1993). Hyperbaric Oxygen Inhibits the Growth of Cultured Rabbit Lens Epithelial Cells Without Affecting Glutathione Level. Exp. Eye Res. *56*, 443–452.

Pakyari, M., Farrokhi, A., Maharlooei, M.K., and Ghahary, A. (2013). Critical Role of Transforming Growth Factor Beta in Different Phases of Wound Healing. Adv. Wound Care *2*, 215–224.

Park, J., Jung, J., Kim, H., Bae, I.-H., Kim, D.-Y., Lee, T.R., and Shin, D.W. (2016). Hypoxia leads to abnormal epidermal differentiation via HIF-independent pathways. Biochem. Biophys. Res. Commun. *469*, 251–256.

Pastar, I., Stojadinovic, O., Yin, N.C., Ramirez, H., Nusbaum, A.G., Sawaya, A., Patel, S.B., Khalid, L., Isseroff, R.R., and Tomic-Canic, M. (2014). Epithelialization in Wound Healing: A Comprehensive Review. Adv. Wound Care *3*, 445–464.

Patel, V., Chivukula, I.V., Roy, S., Khanna, S., He, G., Ojha, N., Mehrotra, A., Dias, L.M., Hunt, T.K., and Sen, C.K. (2005). Oxygen: from the benefits of inducing VEGF expression to managing the risk of hyperbaric stress. Antioxid. Redox Signal. *7*, 1377–1387.

Paulo, M.S.L., Santos, F.T.B. dos, Rocha, P.G., Silva, M. de B., Cintra, L.C., Motta, L.L. da, Errera, F.I.V., Paulo, D.N.S., and Nunes, T.A. (2013). Immunoexpression of proliferating cell nuclear antigen (PCNA) in spleen of splenectomized rats with preservation of inferior pole, submitted to hyperbaric oxygenation. Acta Cirúrgica Bras. Soc. Bras. Para Desenvolv. Pesqui. Em Cir. *28*, 691–695.

Plafki, C., Peters, P., Almeling, M., Welslau, W., and Busch, R. (2000). Complications and side effects of hyperbaric oxygen therapy. Aviat. Space Environ. Med. *71*, 119–124.

Pollock, J.S., Webb, W., Callaway, D., Sathyanarayana, O'Brien, W., and Howdieshell, T.R. (2001). Nitric oxide synthase isoform expression in a porcine model of granulation tissue formation. Surgery *129*, 341–350.

Priestley, J. (1775). An Account of Further Discoveries in Air. By the Rev. Joseph Priestley, LL.D. F. R. S. in Letters to Sir John Pringle, Bart. P. R. S. and the Rev. Dr. Price, F. R. S. Philos. Trans. R. Soc. Lond. *65*, 384–394.

Rabeony, H., Petit-Paris, I., Garnier, J., Barrault, C., Pedretti, N., Guilloteau, K., Jegou, J.-F., Guillet, G., Huguier, V., Lecron, J.-C., et al. (2014). Inhibition of Keratinocyte Differentiation by the Synergistic Effect of IL-17A, IL-22, IL-1α, TNFα and Oncostatin M. PLoS ONE *9*, e101937.

Raja, null, Sivamani, K., Garcia, M.S., and Isseroff, R.R. (2007). Wound re-epithelialization: modulating keratinocyte migration in wound healing. Front. Biosci. J. Virtual Libr. *12*, 2849–2868.

Rappolee, D.A., Mark, D., Banda, M.J., and Werb, Z. (1988). Wound macrophages express TGF-alpha and other growth factors in vivo: analysis by mRNA phenotyping. Science *241*, 708–712.

Reaven, E.P., and Cox, A.J. (1968). Behavior of adult human skin in organ culture. II. Effects of cellophane tape stripping, temperature, oxygen tension, pH and serum. J. Invest. Dermatol. *50*, 118–128.

Rennekampff, H.O., Hansbrough, J.F., Kiessig, V., Doré, C., Sticherling, M., and Schröder, J.M. (2000). Bioactive interleukin-8 is expressed in wounds and enhances wound healing. J. Surg. Res. *93*, 41–54.

Rezvani, H.R., Ali, N., Serrano-Sanchez, M., Dubus, P., Varon, C., Ged, C., Pain, C., Cario-André, M., Seneschal, J., Taïeb, A., et al. (2011). Loss of epidermal hypoxia-inducible factor-1α accelerates epidermal aging and affects re-epithelialization in human and mouse. J. Cell Sci. *124*, 4172–4183.

Rocco, M., Antonelli, M., Letizia, V., Alampi, D., Spadetta, G., Passariello, M., Conti, G., Serio, P., and Gasparetto, A. (2001). Lipid peroxidation, circulating cytokine and endothelin 1 levels in healthy volunteers undergoing hyperbaric oxygenation. Minerva Anestesiol. *67*, 393–400.

Rodriguez, P.G., Felix, F.N., Woodley, D.T., and Shim, E.K. (2008). The role of oxygen in wound healing: a review of the literature. Dermatol. Surg. Off. Publ. Am. Soc. Dermatol. Surg. Al *34*, 1159–1169.

Rollins, M.D., Gibson, J.J., Hunt, T.K., and Hopf, H.W. (2006). Wound oxygen levels during hyperbaric oxygen treatment in healing wounds. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *33*, 17–25.

Rosenberger, C., Solovan, C., Rosenberger, A.D., Jinping, L., Treudler, R., Frei, U., Eckardt, K.-U., and Brown, L.F. (2007). Upregulation of hypoxia-inducible factors in normal and psoriatic skin. J. Invest. Dermatol. *127*, 2445–2452.

Ross, C., Alston, M., Bickenbach, J.R., and Aykin-Burns, N. (2011). Oxygen tension changes the rate of migration of human skin keratinocytes in an age-related manner. Exp. Dermatol. *20*, 58–63.

Rossol, M., Heine, H., Meusch, U., Quandt, D., Klein, C., Sweet, M.J., and Hauschildt, S. (2011). LPS-induced cytokine production in human monocytes and macrophages. Crit. Rev. Immunol. *31*, 379–446.

Rovee, D.T., and Maibach, H.I. (2004). The epidermis in wound healing (Boca Raton: CRC Press).

Saba, A.A., Kaidi, A.A., Godziachvili, V., Dombi, G.W., Dawe, E.J., Libcke, J.H., and Silva, Y.J. (1996). Effects of interleukin-6 and its neutralizing antibodies on peritoneal adhesion formation and wound healing. Am. Surg. *62*, 569–572.

Said, H.K., Hijjawi, J., Roy, N., Mogford, J., and Mustoe, T. (2005). Transdermal sustained-delivery oxygen improves epithelial healing in a rabbit ear wound model. Arch. Surg. Chic. Ill 1960 *140*, 998–1004.

Sander, A.L., Henrich, D., Muth, C.M., Marzi, I., Barker, J.H., and Frank, J.M. (2009). In vivo effect of hyperbaric oxygen on wound angiogenesis and epithelialization. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *17*, 179–184.

Sapirstein, A., and Bonventre, J.V. (2000). Specific physiological roles of cytosolic phospholipase A(2) as defined by gene knockouts. Biochim. Biophys. Acta *1488*, 139–148.

Sawamura, D., Meng, X., Ina, S., Sato, M., Tamai, K., Hanada, K., and Hashimoto, I. (1998). Induction of keratinocyte proliferation and lymphocytic infiltration by in vivo introduction of the IL-6 gene into keratinocytes and possibility of keratinocyte gene

therapy for inflammatory skin diseases using IL-6 mutant genes. J. Immunol. Baltim. Md 1950 *161*, 5633–5639.

Scheitza, S., Bonifas, J., and Blömeke, B. (2012). Variable NAT1 enzyme activity in long-term cultured human HaCaT keratinocytes. J. Toxicol. Environ. Health A *75*, 471–477.

Schreiner, M., Peiser, M., Briechle, D., Stahlmann, R., Zuberbier, T., and Wanner, R. (2007). A loose-fit coculture of activated keratinocytes and dendritic cell-related cells for prediction of sensitizing potential. Allergy *62*, 1419–1428.

Schreml, S., Szeimies, R.M., Prantl, L., Karrer, S., Landthaler, M., and Babilas, P. (2010). Oxygen in acute and chronic wound healing. Br. J. Dermatol. *163*, 257–268.

Schultz, G.S., Sibbald, R.G., Falanga, V., Ayello, E.A., Dowsett, C., Harding, K., Romanelli, M., Stacey, M.C., Teot, L., and Vanscheidt, W. (2003). Wound bed preparation: a systematic approach to wound management. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *11 Suppl 1*, S1–S28.

Semenza, G.L. (2001). Regulation of hypoxia-induced angiogenesis: a chaperone escorts VEGF to the dance. J. Clin. Invest. *108*, 39–40.

Sen, C.K. (2003). The general case for redox control of wound repair. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *11*, 431–438.

Sen, C.K., Khanna, S., Babior, B.M., Hunt, T.K., Ellison, E.C., and Roy, S. (2002). Oxidant-induced vascular endothelial growth factor expression in human keratinocytes and cutaneous wound healing. J. Biol. Chem. *277*, 33284–33290.

Sharifipour, F., Baradaran-Rafii, A., Idani, E., Zamani, M., and Jabbarpoor Bonyadi, M.H. (2011). Oxygen therapy for acute ocular chemical or thermal burns: a pilot study. Am. J. Ophthalmol. *151*, 823–828.

Sharifipour, F., Panahi-Bazaz, M., Idani, E., Malekahmadi, M., and Feizi, S. (2012). Normobaric oxygen therapy for scleral ischemia or melt. J. Ophthalmic Vis. Res. *7*, 275–280.

Sheffield, P.J. (1998). Measuring tissue oxygen tension: a review. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *25*, 179–188.

Sheikh, A.Y., Gibson, J.J., Rollins, M.D., Hopf, H.W., Hussain, Z., and Hunt, T.K. (2000). Effect of hyperoxia on vascular endothelial growth factor levels in a wound model. Arch. Surg. Chic. Ill 1960 *135*, 1293–1297.

Siddiqui, A., Galiano, R.D., Connors, D., Gruskin, E., Wu, L., and Mustoe, T.A. (1996). Differential effects of oxygen on human dermal fibroblasts: acute versus chronic hypoxia. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *4*, 211–218.

Silver, I.A. (1969). The Measurement of Oxygen Tension in Healing Tissue. In Progress in Respiratory Research, F. Kreuzer, ed. (S. Karger AG), pp. 124–135.

Singer, A.J., and Clark, R.A. (1999). Cutaneous wound healing. N. Engl. J. Med. *341*, 738–746.

Smith, B.M., Desvigne, L.D., Slade, J.B., Dooley, J.W., and Warren, D.C. (1996). Transcutaneous oxygen measurements predict healing of leg wounds with hyperbaric therapy. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *4*, 224–229.

So, T., Ito, A., Sato, T., Mori, Y., and Hirakawa, S. (1992). Tumor necrosis factor-alpha stimulates the biosynthesis of matrix metalloproteinases and plasminogen activator in cultured human chorionic cells. Biol. Reprod. *46*, 772–778.

Sogabe, Y., Abe, M., Yokoyama, Y., and Ishikawa, O. (2006). Basic fibroblast growth factor stimulates human keratinocyte motility by Rac activation. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *14*, 457–462.

Song, P.I., Park, Y.-M., Abraham, T., Harten, B., Zivony, A., Neparidze, N., Armstrong, C.A., and Ansel, J.C. (2002). Human keratinocytes express functional CD14 and toll-like receptor 4. J. Invest. Dermatol. *119*, 424–432.

Staiano-Coico, L., Higgins, P.J., Schwartz, S.B., Zimm, A.J., and Goncalves, J. (2000). Wound fluids: a reflection of the state of healing. Ostomy. Wound Manage. *46*, 85S – 93S; quiz 94S – 95S.

Stücker, M., Struk, A., Altmeyer, P., Herde, M., Baumgärtl, H., and Lübbers, D.W. (2002). The cutaneous uptake of atmospheric oxygen contributes significantly to the oxygen supply of human dermis and epidermis. J. Physiol. *538*, 985–994.

Suh, D.Y., and Hunt, T.K. (1998). Time line of wound healing. Clin. Podiatr. Med. Surg. *15*, 1–9.

Sula, B., Ekinci, C., Uçak, H., Uçmak, D., Akkurt, Z.M., Aktaş, A., Arica, M., and Deveci, E. (2016). Effects of hyperbaric oxygen therapy on rat facial skin. Hum. Exp. Toxicol. *35*, 35–40.

Tarnuzzer, R.W., and Schultz, G.S. (1996). Biochemical analysis of acute and chronic wound environments. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *4*, 321–325.

Terraneo, L., Virgili, E., Caretti, A., Bianciardi, P., and Samaja, M. (2014). In vivo hyperoxia induces hypoxia-inducible factor-1α overexpression in LNCaP tumors without affecting the tumor growth rate. Int. J. Biochem. Cell Biol. *51*, 65–74.

Teshigawara, K., Hosaka, T., Yamaguchi, M., Terada, E., Kisyuku, Y., Fukunaga, K., Hirata, Y., Jambaldorj, B., Harada, N., Sakai, T., et al. (2010). Long-term treatment

with hyperbaric air improves hyperlipidemia of db/db mice. J. Med. Investig. JMI *57*, 224–231.

Thackham, J.A., McElwain, D.L.S., and Long, R.J. (2008). The use of hyperbaric oxygen therapy to treat chronic wounds: A review. Wound Repair Regen. Off. Publ. Wound Heal. Soc. Eur. Tissue Repair Soc. *16*, 321–330.

Thom, S.R. (1993). Functional inhibition of leukocyte B2 integrins by hyperbaric oxygen in carbon monoxide-mediated brain injury in rats. Toxicol. Appl. Pharmacol. *123*, 248–256.

Thom, S.R. (2009). Oxidative stress is fundamental to hyperbaric oxygen therapy. J. Appl. Physiol. Bethesda Md 1985 *106*, 988–995.

Tibbles, P.M., and Edelsberg, J.S. (1996). Hyperbaric-oxygen therapy. N. Engl. J. Med. *334*, 1642–1648.

Tompach, P.C., Lew, D., and Stoll, J.L. (1997). Cell response to hyperbaric oxygen treatment. Int. J. Oral Maxillofac. Surg. *26*, 82–86.

Tortora, G.J., and Grabowski, S.R. (2003). Principles of anatomy and physiology (New York: Wiley).

Uhl, E., Sirsjö, A., Haapaniemi, T., Nilsson, G., and Nylander, G. (1994). Hyperbaric oxygen improves wound healing in normal and ischemic skin tissue. Plast. Reconstr. Surg. *93*, 835–841.

Vann, R.D., Butler, F.K., Mitchell, S.J., and Moon, R.E. (2011). Decompression illness. The Lancet *377*, 153–164.

Varghese, M.C., Balin, A.K., Carter, D.M., and Caldwell, D. (1986). Local environment of chronic wounds under synthetic dressings. Arch. Dermatol. *122*, 52–57.

Velnar, T., Bailey, T., and Smrkolj, V. (2009). The wound healing process: an overview of the cellular and molecular mechanisms. J. Int. Med. Res. *37*, 1528–1542.

Al-Waili, N.S., and Butler, G.J. (2006). Effects of hyperbaric oxygen on inflammatory response to wound and trauma: possible mechanism of action. ScientificWorldJournal *6*, 425–441.

Wallace, H.J., and Stacey, M.C. (1998). Levels of tumor necrosis factor-alpha (TNF-alpha) and soluble TNF receptors in chronic venous leg ulcers--correlations to healing status. J. Invest. Dermatol. *110*, 292–296.

Warriner, R.A., and Hopf, H.W. (2012). The effect of hyperbaric oxygen in the enhancement of healing in selected problem wounds. Undersea Hyperb. Med. J. Undersea Hyperb. Med. Soc. Inc *39*, 923–935.

Weir, L., Robertson, D., Leigh, I.M., Vass, J.K., and Panteleyev, A.A. (2011). Hypoxia-mediated control of HIF/ARNT machinery in epidermal keratinocytes. Biochim. Biophys. Acta *1813*, 60–72.

Weisz, G., Lavy, A., Adir, Y., Melamed, Y., Rubin, D., Eidelman, S., and Pollack, S. (1997). Modification of in vivo and in vitro TNF-alpha, IL-1, and IL-6 secretion by circulating monocytes during hyperbaric oxygen treatment in patients with perianal Crohn's disease. J. Clin. Immunol. *17*, 154–159.

Werdin, F., Tennenhaus, M., Schaller, H.-E., and Rennekampff, H.-O. (2009). Evidence-based management strategies for treatment of chronic wounds. Eplasty *9*, e19.

Werner, S., and Grose, R. (2003). Regulation of wound healing by growth factors and cytokines. Physiol. Rev. *83*, 835–870.

Wilson, A.P.R. (2004). Surgical wound infection as a performance indicator: agreement of common definitions of wound infection in 4773 patients. BMJ *329*, 720–0.

Wolk, K., Witte, E., Wallace, E., Döcke, W.-D., Kunz, S., Asadullah, K., Volk, H.-D., Sterry, W., and Sabat, R. (2006). IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. Eur. J. Immunol. *36*, 1309–1323.

Wu, L., Brucker, M., Gruskin, E., Roth, S.I., and Mustoe, T.A. (1997). Differential effects of platelet-derived growth factor BB in accelerating wound healing in aged versus young animals: the impact of tissue hypoxia. Plast. Reconstr. Surg. *99*, 815–822; discussion 823–824.

Wu, L., Xia, Y.P., Roth, S.I., Gruskin, E., and Mustoe, T.A. (1999). Transforming growth factor-beta1 fails to stimulate wound healing and impairs its signal transduction in an aged ischemic ulcer model: importance of oxygen and age. Am. J. Pathol. *154*, 301–309.

Xia, Y.P., Zhao, Y., Tyrone, J.W., Chen, A., and Mustoe, T.A. (2001). Differential activation of migration by hypoxia in keratinocytes isolated from donors of increasing age: implication for chronic wounds in the elderly. J. Invest. Dermatol. *116*, 50–56.

Xu, J., and Clark, R.A. (1996). Extracellular matrix alters PDGF regulation of fibroblast integrins. J. Cell Biol. *132*, 239–249.

Zamboni, W.A., Browder, L.K., and Martinez, J. (2003). Hyperbaric oxygen and wound healing. Clin. Plast. Surg. *30*, 67–75.

Zhang, Z., McGoron, A.J., Crumpler, E.T., and Li, C.-Z. (2011). Co-culture based blood-brain barrier in vitro model, a tissue engineering approach using immortalized cell lines for drug transport study. Appl. Biochem. Biotechnol. *163*, 278–295.

Zhao, L.L., Davidson, J.D., Wee, S.C., Roth, S.I., and Mustoe, T.A. (1994). Effect of hyperbaric oxygen and growth factors on rabbit ear ischemic ulcers. Arch. Surg. Chic. Ill 1960 *129*, 1043–1049.

(1996). The molecular and cellular biology of wound repair (New York: Plenum Press).

(2001). The biology of the skin (New York: Parthenon Pub. Group).

(2007). Wound care: a collaborative practice manual for health professionals (Philadelphia, PA: Lippincott Williams & Wilkins).

(2008). Molecular biology of the cell (New York: Garland Science).

Appendix

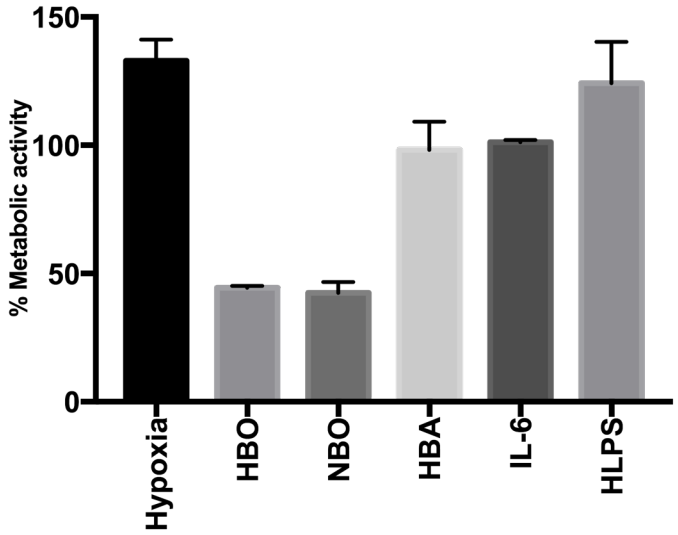

Figure 8-1 The metablic activity of HaCaT cells under chronic hypoxia, HBO, HBA, IL-6 and HLPS conditions.

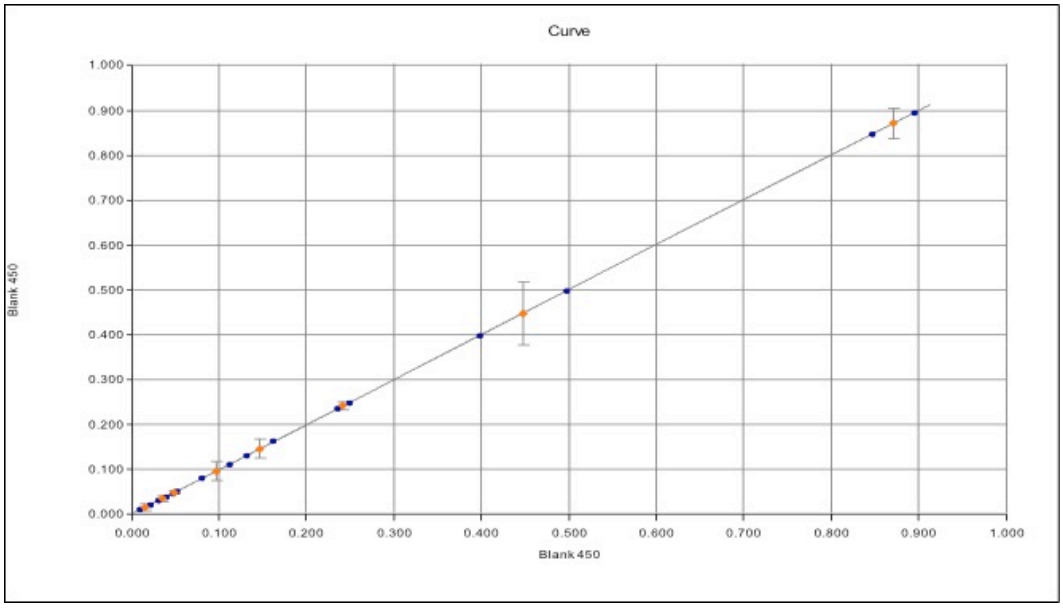

Figure 8-2 the standard curve of TNF-α

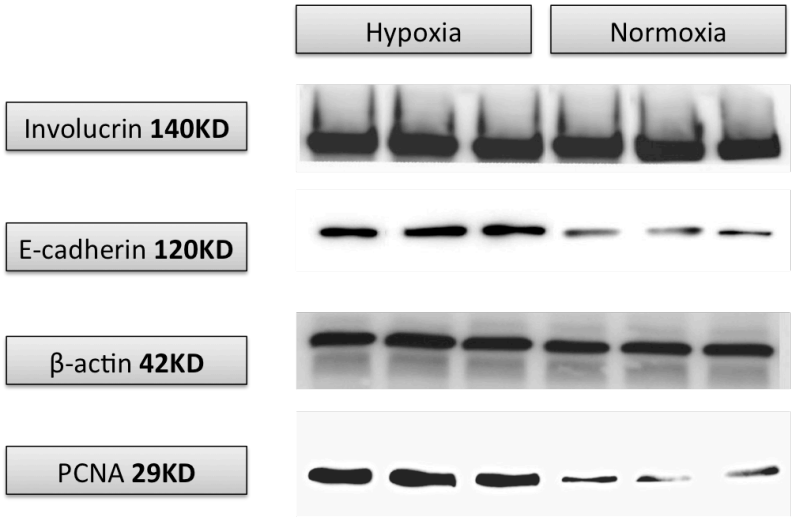

Figure 8-3 the expressions of involucrin, E-cadherin and PCNA in HaCaT cells analysed by Western blot. HaCaT cells were exposed to hypoxic and normoxic conditions at 1 ATA for 72 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control.

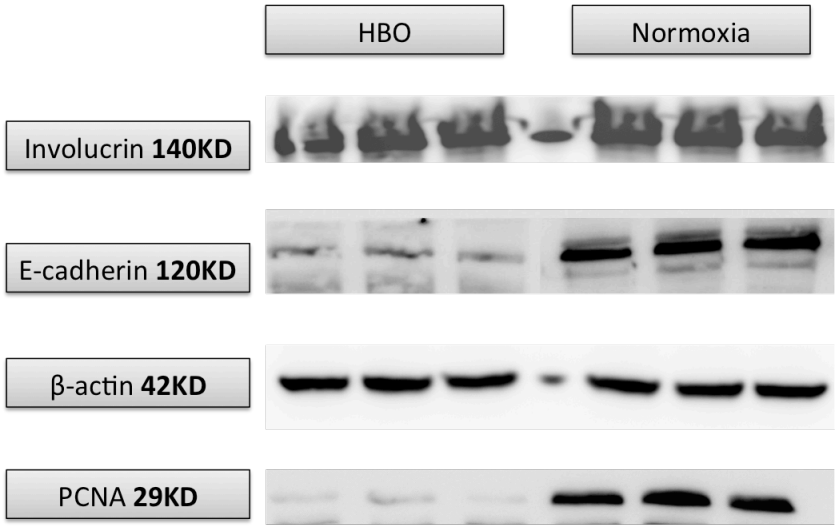

Figure 8-4 the expressions of involucrin, E-cadherin and PCNA in HaCaT cells analysed by Western blot. HaCaT cells were exposed to HBO at 3 ATA and normoxic conditions at 1 ATA for 48 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control

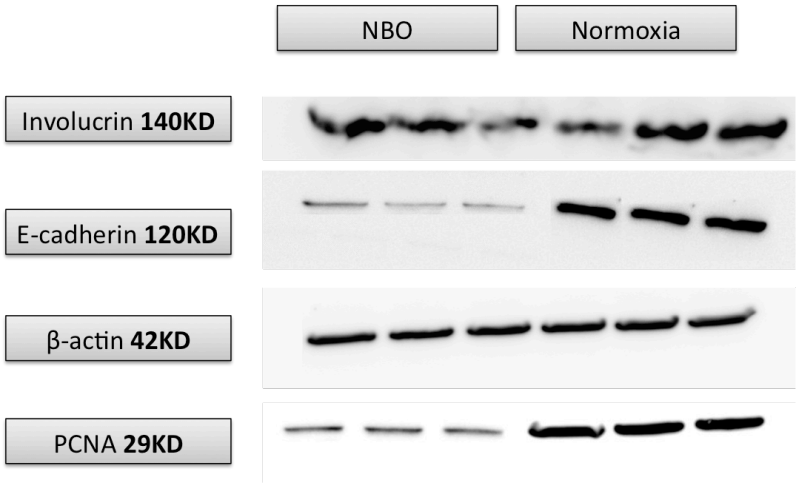

Figure 8-5 the expressions of involucrin, E-cadherin and PCNA in HaCaT cells analysed by Western blot. HaCaT cells exposed NBO and normoxic conditions for 48 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control.

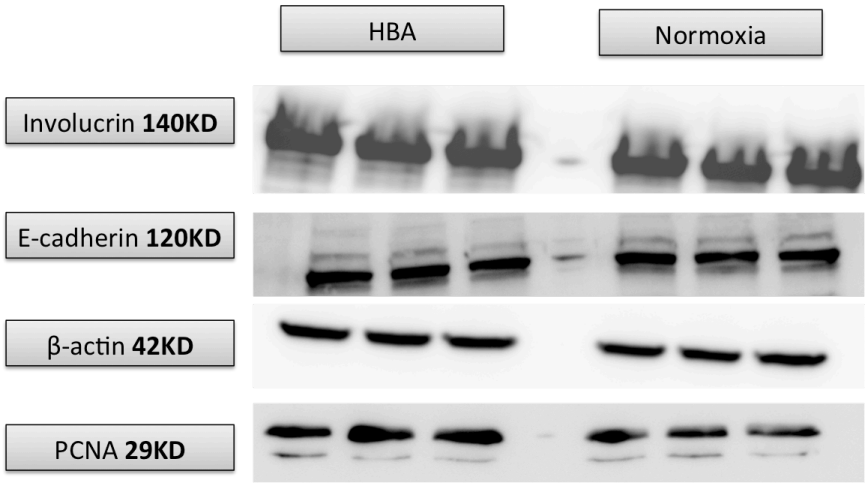

Figure 8-6 the expressions of involucrin, E-cadherin and PCNA in HaCaT cells analysed by Western blot. HaCaT cells exposed to HBA and normoxic conditions for 48 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control.

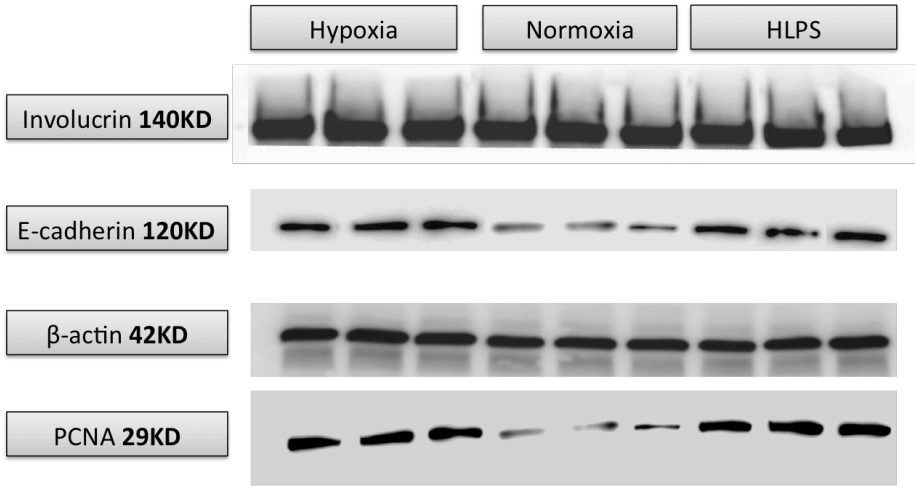

Figure 8-7 the expressions of involucrin, E-cadherin and PCNA in HaCaT cells analysed by Western blot. HaCaT cells treated with LPS 100μg/ml and exposed to hypoxic condition at a modular chamber for 72 hours and then, Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control.

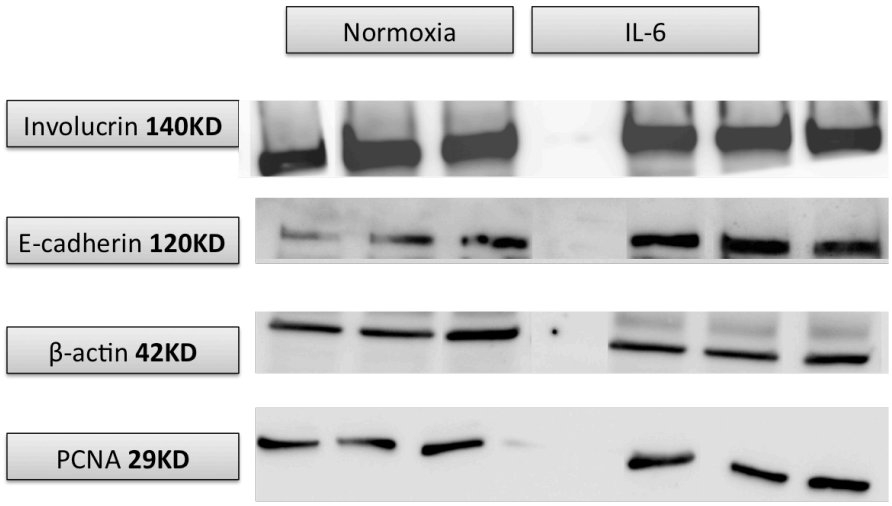

Figure 8- the expressions of involucrin, E-cadherin and PCNA in HaCaT cells analysed by Western blot. HaCaT cells treated with IL-6 (50pg/ml) under normoxic conditions at 1 ATA for 60 hours. Proteins were extracted from the cells and separated through SDS-PAGE. β-actin expression was used as a loading control.