THE mRNA EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN KELOID USING TOCOTRIENOL-RICH FRACTION IN PRIMARY HUMAN EPIDERMAL KERATINOCYTES AND PRIMARY HUMAN DERMAL FIBROBLAST CULTURES

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UNIVERSITI SAINS MALAYSIA
2012

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By

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Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

2012

ACKNOWLEDGEMENT

"In the name of Allah, Most Gracious, Most Merciful"

First and foremost it is a great pleasure for me put on records my sincerest gratitude and appreciation to my respectfull supervisor, Prof. Ahmad Sukari Halim. I also thanked my co-supervisor; Assc. Prof. Dr. Aida Hanum Ghulam Rasool from Department of Pharmacology, School of Medical Sciences and Assc. Prof. Dr. Shahrum Shamsuddin from School of Health Sciences, Universiti Sains Malaysia (USM). This thesis would not have been possible without their advice, supervision and support from the initial to the final level that enabled me to graduate. I would like to thank my Science officer, Mr Lau Hut Yee and Puan Cik Fareha Aleas for their support especially in purchasing and other financial affairs. I am grateful to School of Medical Sciences, Human Genome Centre, School of Health Sciences, National Tissue Bank and Tissue Engineering Laboratory (Unit Rawatan Kebakaran HUSM) for their supports in providing accommodation and various logistic assistance during this project was conducted. Due recognition is given to all the patients who willingly allowed and consented to the use of their skin biopsies for this research purposes. Special thanks to all medical officer and operation theater staffs of HUSM for their kind support in harvesting the skin biopsies during operation.

I am indebted to many of my colleagues who have supported me directly or indirectly. In my daily work I have been blessed with a friendly and helpful

colleagues especially Dr. Lim Chin Keong. I have a great guidance and assistance throughout this study and honestly, it was a magnificent experience for me to work together with him.

On a more personal note, I wish to express my deepest gratitude to my family especially my husband and my daughter for their love, understanding and moral support. Without their encouragement, I would not have finished this Master degree.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

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Appendix IX (E)

S. Mahirah Y, Lim CK, Nor Asiah MN, Mohamad Syaiful Bahri AR, AS Halim, S. Shaharum. (2008). The Expression of Cytokeratin 6, Involucrin, Heat Shock Protein 47 and Fibroblasts Surface Protein in Primary Culltured Human Skin In Vitro: A Model for Skin Imunocytochemistry. 13th National Conference on Medical Sciences, USM. Date: 22-23 rd May 2008

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S. Mahirah Y, A. S. Halim, S. Shaharum, A.H. Rasool. (2011). Cytotoxicity of Tocotrienol-rich Fraction (TRF) and the mRNA expression of inducible nitric oxide synthase (iNOS) in primary keloid cultures. 16th National Conference on Medical and Health Sciences, USM. Date:22-23 rd June 2011

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LIST OF ABBREVIATIONS

3T3	"3-day transfer, inoculum 3 x 10^5 cells" (cell line)
BSA-AGEs	Bovine serum albumin-advanced glycation end products
cDNA	Complimentary deoxyribonucleic acid
cHDF	Commercial Human Dermal Fibroblasts
cHEK	Commercial Human Epidermal Keratinocytes
CK6	Cytokeratin 6
cNOS	Constitutive Nitric Oxide Synthase
CO_2	Carbon Dioxide
DETA NONOate	Diethylenetriamine NONOate
DKSFM	Define Keratinocyte Serum Free Medium
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate.
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular Matric
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
FAM	6-carboxyfluorescein (fluorophores)
FBS	Fetal Bovine Serum
FRET	Förster or fluorescence resonance energy transfer
FSP	Fibroblasts Surface Protein

g	gravity
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HSc	Hypertrophic Scar
HScF	Hypertrophic Scar Fibroblasts
HSP 47	Human Surface Protein 47
iNOS	Inducible Nitric Oxide Synthase
IC_{50}	50% Inhibitory Concentration
LPS	Lipopolysaccharide
MEFs	Mouse Embryonic Fibroblasts
MGB	Minor Groove Binder
mRNA	Messenger Ribonucleic Acid
MTT	Tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-arboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NFQ	Non-fluorescent quencher
NO	Nitic Oxide
NOS	Nitric Oxide Synthase
NOS1	Neuronal Nitric Oxide Synthase
NOS3	Endothelial Nitric Oxide Synthase
PCR	Polymerase Chain Reaction
PMC	Pentamethyl-hydroxychromane
pHDF	Primary Human Dermal Fibroblasts
pHEK	Primary Human Epidermal Keratinocytes
pKHDF	Primary Keloid Human Dermal Fibrobalsts

pKHEK	Primary Keloid Human Epidermal Fibrobalsts
pNHDF	Primary Normal Human Dermal Fibrobalsts
pNHEK	Primary Normal Human Epidermal Fibrobalsts
PS	Penicillin Streptomycin
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
RT block	Reverse Transcriptase block
RT PCR	Reverse Transcriptase Polymerase Chain Reaction
SPSS	Statistical Package for the Social Sciences
T3s	Tocotrienols
TAMRA	Tetramethylrhodamine
TGF β	Transforming Growth Factor Beta
Tm	Annealing Temperature
TRF	Tocotrienol Rich Fraction
β-ME	Beta-Mercaptoethanol
ddH2O	Double distilled water
ft^2	Square feet
lb	Pound
kg	Kilogramme
%	Percentage
Mm	Milimeter
m^2	Square meters
in	Inches
Ca^{2+}	Calsium

~	Approximately
°C	Degree celcius
bp	Base pair
mM	Mili Molar
Mg^{2+}	Magnesium
h	Hour
µg	Microgram e
mL	Mililiter
µm	Micrometer
mg	Miligramme
nm	Nanometer
µM	Micromolar
Ct	Threshold Cycle Value
α	Alpha
β	Beta
γ	Gamma
δ	Delta

PENGEKSPRESAN mRNA NITRIK OKSID SINTASE TERARUH DALAM KELOID MENGGUNAKAN FRAKSI KAYA TOKOTRIENOL DALAM KULTUR PRIMER EPIDERMIS KERATINOSIT MANUSIA DAN KULTUR PRIMER DERMIS FIBROBLAS MANUSIA

ABSTRAK

Keloid dicirikan oleh pemendapan kolagen yang berlebihan yang boleh merosakkan tisu yang sihat. Penggunaan produk berasaskan Vitamin E- tokoferol secara meluas serta penggunaan tokotrienol (T3) berpotensi untuk merawat pelbagai penyakit kulit. Terdapat kemungkinan bahawa fraksi kaya tokotrienol (TRF) dapat mempengaruhi proses penyembuhan luka secara normal, pembentukan parut timbul dan keloid semasa proses keradangan. Walaubagaimanapun, terdapat kekurangan bukti saintifik yang mengesahkan keberkesanan terapi TRF dalam pencegahan parut. Oleh itu, kajian ini dijalankan untuk menilai kesan-kesan berfaedah TRF dalam penyembuhan luka dan rangsangan terhadap ekspresi mRNA nitrik oksid sintase teraruh (iNOS) dalam keratinosit kulit dan fibroblas keloid manusia. Kultur primer epidermis keratinosit manusia (pHEK) dan kultur primer dermis fibroblas manusia (pHDF) berjaya dihasilkan menggunakan kaedah penceraian sel. Pengesahan pHEK dan pHDF dijalankan dengan petanda Cytokeratin-6 (CK6), Involucrin, Heat Shock Protein-47 (HSP47) dan protein yang spesifik pada permukaan fibroblas (FSP) menggunakan analisis imunositokimia. Kesan TRF pada pHEK dan pHDF ditentukan menggunakan cerakin MTT. Pengekspresan mRNA iNOS di dalam hanya kultur primer normal dermis fibroblas manusia (pNHDF) dan kultur primer keloid dermis fibroblas manusia (pKHDF) yang dirawat dengan TRF di nilai menggunakan

PCR masa sebenar. Kultur primer normal epidermis keratinosit manusia (pNHEK) mencapai kadar pertumbuhan yang lebih tinggi berbanding kultur primer keloid epidermis keratinosit manusia (pKHEK). Sementara itu, pKHDF mempamerkan pertumbuhan linear dan mengekalkan pertumbuhan sel pada kadar yang lebih tinggi berbanding pNHDF. Kultur pHEK positif bagi kehadiran CK6 dan Involucrin manakala HSP47 dan FSP ditemui dalam kultur pHDF. TRF dari 2.85 μg /mL to180 μg /mL dan TRF (45 μg /mL 180 μg /mL) didapati menghalang pertumbuhan pHEK manakala bagi pHDF adalah pada 90 ug /mL pada 72 jam. Pada kepekatan yang lebih rendah (2.8-22.5 μg/mL), TRF meningkatkan pertumbuhan sel pHEK pada 24 dan 48 jam pengeraman tetapi tidak mempunyai kesan yang signifikan dalam jumlah sel hidup bagi pHDF pada semua selang masa. Kesan pengurangan dan perencatan pertumbuhan pada pHEK dan pHDF mungkin disebabkan oleh kesan perencatan proliferasi sel dan kesan antioksidan oleh TRF. Dalam kajian ini, TRF pada 2.8 μg/mL mengurangkan tahap mRNA iNOS pKHDF seperti dalam pNHDF pada 24 jam yang mungkin boleh merencatkan penghasilan nitrik oksid (NO) oleh pKHDF. Pengurangan tahap mRNA iNOS dalam pKHDF mungkin disebabkan TRF memiliki kesan anti oksidan dan anti-fibrogenik. Keputusan ini menunjukkan TRF mungkin memainkan peranan dalam intervensi keloid dengan menekan ekspresi mRNA iNOS.

THE mRNA EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN KELOID USING TOCOTRIENOL-RICH FRACTION IN PRIMARY HUMAN EPIDERMAL KERATINOCYTES AND PRIMARY HUMAN DERMAL FIBROBLAST CULTURES

ABSTRACT

Keloid is characterized by excess collagen deposition that can damage healthy tissue. With the widespread use of Vitamin E- tocopherols based product and the discovery of tocotrienol (T3) potential to treat various skin injuries; there are possibilities of tocotrienol-rich fraction (TRF) to intercede normal, hypertrophic, or keloid scarring during the inflammatory process. However, there is a lack of scientific evidence to validate the efficacy and the therapies of TRF in scar prevention. Thus, this study was carried out to evaluate the beneficial effects of TRF in wound healing and its possible stimulation towards mRNA expression of inducible nitric oxide synthase (iNOS) in keloid human skin keratinocytes and fibroblasts. Primary human epidermal keratinocytes (pHEK) and primary human dermal fibroblasts (pHDF) were successfully established using cell dissociation method. Verification of pHEK and pHDF was conducted using Cytokeratin-6 (CK6), Involucrin, Heat Shock Protein-47 (HSP47) and Fibroblast Surface Protein (FSP) markers using imunocytochemistry analysis. The effect of TRF on pHEK and pHDF were determined by using MTT assay. The mRNA expression of iNOS in primary normal human dermal fibroblasts (pNHDF) and primary keloid human dermal fibroblasts (pKHDF) treated with TRF was evaluated using real-time PCR. Primary normal human epidermal keratinocytes (pNHEK) achieved higher cellular growth

rate compared to primary keloid human epidermal keratinocytes (pKHEK). Whilst, pKHDF exhibited linear growth and sustained higher cellular growth rate compared to pNHDF. pHEK cultures were positive for the presence of CK6 and Involucrin whereas HSP47 and FSP were found in pHDF cultures. TRF ranged from 2.85 μg/ mL to180 μg/ mL. TRF (45 μg/ mL to 180 μg/ mL) was found to inhibit the growth of pHEK while for pHDF was at 90 ug/mL at 72 hours. At lower concentrations (2.8-22.5 μg/mL), TRF increased pHEK cell growth at 24 and 48 hours of incubation but have no significant effect in pHDF viability at all time intervals. TRF at 2.8 μg/mL has been found to reduce the mRNA expression of iNOS at 24 hours, which may in turn suppress the production of nitric oxide (NO) by pKHDF. The observed suppression and growth inhibition effect in pHEK and pHDF cultures may be caused by the antiproliferative and antioxidant activities of TRF. Whereas, the reduction of iNOS mRNA level in pKHDF may suggest that TRF possess antioxidant and antifibrogenic effect. This finding showed that TRF may play a role in keloid intervention by suppressing the mRNA expression of iNOS.

CHAPTER 1

INTRODUCTION

1.1 Research Background

The abnormal response to wound healing process may cause the over expression of fibroblasts and collagen synthesis. Overproduction of fibroblasts and collagen synthesis will rise above the surrounding skin to form a hypertrophic scar (HSc) or continue to extend beyond the site of injury to form a more serious scar which is known as keloid. Keloid and Hypertrophic scar (HSc) have been documented to happen in mankind since many years ago. With the development of modern technology and the increase in public awareness, efforts have been devoted to the better understanding and find effective treatment to control this problem globally.

Vitamin E has been known to be a major lipid soluble antioxidant in skin, and its use has been encouraged by some clinical practitioners for patients with various skin abrasions. Vitamin E is believed to have a great potential to accelerate wound healing process and improve the cosmetic appearance of scars (Chang *et al*., 2001). In contrast to tocopherols, another Vitamin E isomer (Tocotrienols (T3s)) are poorly studied. Only a few studies have shown that T3s exert more significant effect compared to tocopherols. Most of tocopherols originate from components of nuts and common vegetable oils, whereas T3s are found abundantly in palm oil, oat, barley, soybean and rice bran. Palm oil T3s is a complex natural phytonutrient that possess a higher antioxidant activity compared to tocopherols. Tocotrienol-Rich Fraction (TRF) derived from palm oil containing T3s fraction has been an economical and

efficient substitute for alpha tocopherol. TRF may lead to the induction of apoptosis in various types of cancer cells, such as breast, colon and hepatoma carcinoma cells. Currently, with the extensive used of Vitamin E- tocopherol based product and the discovery of potential use of T3s to treat variety skin modalities, TRF may play a role to intervene normal, hypertrophic, or keloid scarring by modulating the inflammatory response (Sen, 2009). However, there is lack of scientific evidence to validate the efficacy and the therapies of TRF in scar prevention.

The healing of skin requires enhanced and efficient production of healthy collagen for strength and to abbreviate the recovery time without scarring. However, the formation of collagen in injured crucial organs needs to be minimized to avoid fibrosis and successive loss of organ function. The reduction and the enhancement of this collagen formation are likely to have common regulatory mechanisms that remain to be explained. Collagen formation can be affected by multiple cellular and extracellular factors such as nitric oxide, growth factors and matrix metalloproteinases. Recently, studies showed that inducible nitric oxide synthase (iNOS) and nitric oxide (NO) play an important role in collagen formation during wound healing. Inhibition of NO synthase was found to decreased collagen synthesis in wound fibroblasts. Dermal fibroblasts from iNOS-knock out murine fibroblasts proliferated more slowly and synthesized less collagen (Schaeffer *et al.*, 1997).

Over the years, scar management has proven to be challenging and much more left to be learned. According to Sund (2000), about 100 million patients acquire scars every year in the developed world. These statistics resulted from 55 million elective surgical procedure and 25 million operations after trauma. Patient with significant abnormal scarring may face physical and cosmetic deformities, psychological stress and social consequences that might be related to substantial

emotional and financial costs. A lot of treatment modalities are carried out to prevent this abnormal wound healing but most of the methods are time consuming, expensive and sometime not effective. The expression, transcription, and function of the inducible isoform iNOS is induced by a variety of cytokines, growth factors and inflammatory stimuli on target cells which leads to high NO levels. Therefore, the regulation of iNOS occurs mainly at the gene level. Therefore, this study has explored the beneficial effects of TRF in wound healing and the possible stimulation towards mRNA expression of iNOS in normal and keloid human skin keratinocytes and fibroblasts. This fundamental understanding regarding scarring and the underlying mechanisms of the potential effect of TRF on wound management will have a vast implication on its potential pharmaceutical use.

1.1.1 Hypothesis

TRF is possibly used to inhibit the growth of cells and reduce the expression of iNOS mRNA expression in keratinocytes and fibroblasts cultures.

1.1.2 General objectives

The objective of this study is to assess the mRNA expression of iNOS in TRF treated primary epidermal human keratinocytes (pHEK) and primary dermal human fibroblast (pHDF) cultures from normal and keloid skin tissues.

1.1.3 Specific Objectives

1.1.3.1 To establish pHEK and pHDF culture system from normal skin and keloid scars.

1.1.3.2 To determine the effect of TRF in pHEK and pHDF of normal skin and keloid scar.

1.1.3.3 To verify the expression of iNOS in TRF treated pHDF of normal skin and keloid scar.

1.2 Literature review

1.2.1 Skin

The skin or cutaneous membrane covers the external surface of the body. It is the largest organ of the body in surface area and weight. The functions of skin include protection of the body against harmful environment and loss of plasma, prevention of entry by microorganism, regulation of body temperature and social interactive function (Wong and Cheng, 2009). Skin also plays a vital role as a barrier against environment and pathogens. Structurally, skin consists of two morphologically different layers that are derived from two different germ layers. The superficial layer is called epidermis and the deeper and thicker connective tissue layer is called dermis (Figure 1). In adults, the skin covers an area of about 2 m^2 (22 ft^2) and weighs 4.5- 5 kg (10-11 lb), about 16% of total body weight. It ranges in thickness from 0.5mm (0.02 in) on the eyelids to 4.0 mm (0.16 in) on the heels. However, over most of the body it is 1-2 mm (0.04 – 0.08 in) thick. The epidermis can vary in thickness depending on the body location, generally about 0.2 mm of thickness (Tortora and Grabowski 2003).

The epidermis is formed by stratified squamous epithelium and does not contain blood vessels. It consists of five layers, stratum corneum, lucidium, granulosum, spinosum and basale. The epidermis is a highly specialized organ made out of keratinocytes and residence melanocytes, Langerhans cells and Merkel cells. Dermis is situated between epidermis and subcutaneous fat; consist of network fibers of collagen, reticulin and elastin. Like other connective tissues, the dermal layer has three separate components, cells, fibers and amorphous ground substance. It supports the epidermis structurally and nutritionally (Hunter *et al*., 1995).

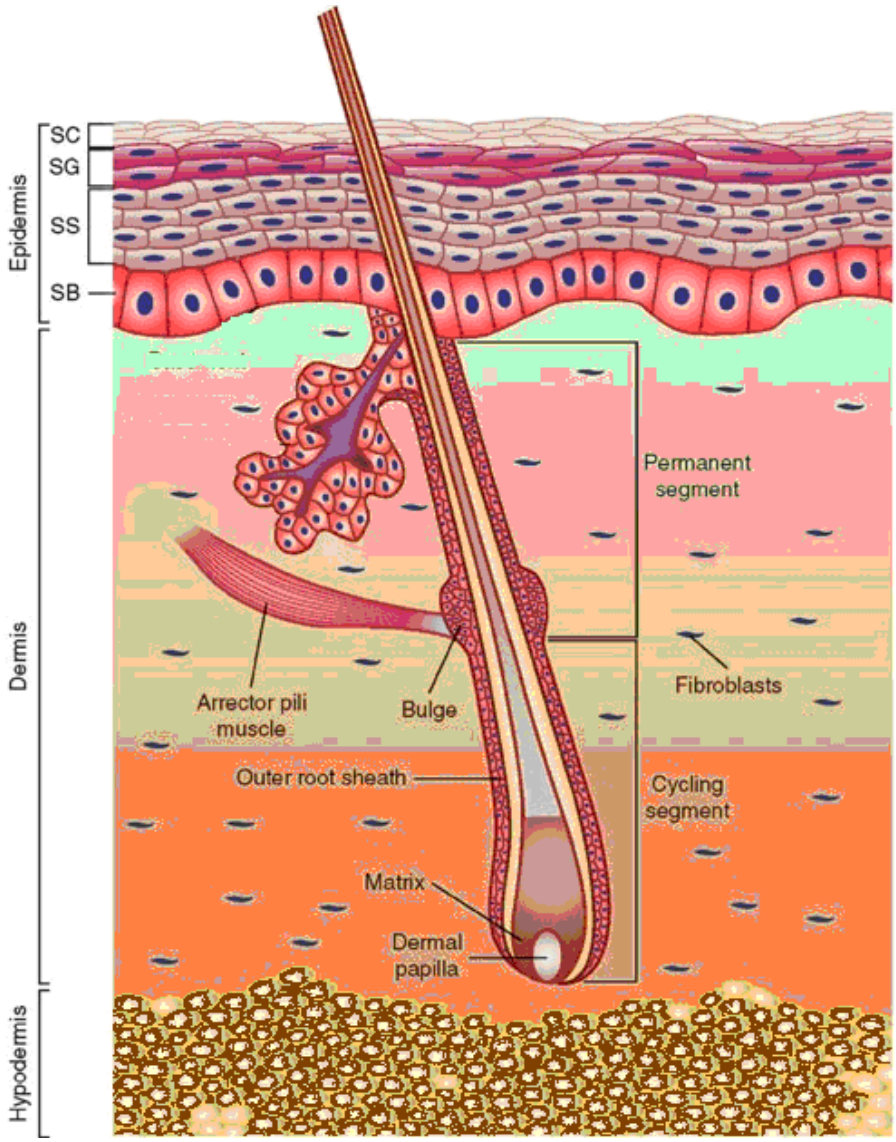

Figure 1: Anatomy of human skin. The structure of skin composed of three layers, starting with the epidermis, dermis, and hypodermis. The epidermis is primarily composed of stratified squamous epithelium of keratinocytes that is divided into four layers; stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB). Meanwhile, the dermis is primarily composed of fibroblasts from mesoderm origin. Adapted from Wong & Cheng: Skin Tissue Engineering. StemBook, ed. The Stem Cell Research Community, StemBook, doi/10.3824/stembook.1.44.1, http://www.stembook.org. March 31, 2009.

1.2.1.1 Keratinocytes

Keratinocytes are the largest population of epidermal cells in the skin (Figure 1). They attain their name through their function as warehouses for keratin, the protein that provides a physical barrier in the most superficial dead cells of the stratum corneum. Keratinocytes are formed through its stem cells located in the stratum basal and differentiates within the stratum spinosum (Jablonski, 2004). As more cells are produced in the stratum basal, cells above are forced upwards to the surface over a period of about 2 months. During transit, keratinocytes can lose some synthetic organelles (*e.g.* rough endoplasmic reticulum and Golgi apparatus). There is an elevated production of intracellular tonofilament, keratohyaline, cytoskeleton in connecting cytoplasm and desmosomes. Tonofilament and keratohyaline are able to combine in forming the matrix and keratin respectively at the level of the stratum corneum (Yamamoto and Lizuka, 1998).

Keratinocytes in stratum spinosum contain lamellar bodies which function to store complex carbohydrates, lipids and hydrolytic enzymes. The constituents are discharged into the intercellular space in the stratum corneum. They provide a complex glycoprotein adhesive between cells. Enzyme phosphatase acid shed in the stratum corneum will help to dissolve this attachment and permit shedding of the most superficial dead cells. Keratinocytes are also responsible in the production of lamellar granules, which perform as a water repellent sealant (Tortora and Grabowski, 2003). They are formed continuously in the basal layer and undergo a tightly regulated process of differentiation as they progress upwards and become part of the cornified epithelium. This maturation process consists essentially of

keratinization, but also involves a number of morphological and metabolic events as the cells differentiate and lose their nuclei (Cals-Grierson and Ormerod, 2004).

1.2.1.2 Fibroblasts

Fibroblast is the main cells in the dermal layer. Its extracellular matrix (ECM) secretions contribute to high tensile strength of the skin (Figure 1). It is involved in the formation of granulation tissue. Furthermore, it also play an important role in the production of collagen, elastin, fibronectin, sulfated and non-sulfated glycosaminoglycans and proteases in wound remodeling (James *at al*., 2006). Fibroblasts have a branched cytoplasm surrounding an elliptical, speckled nucleus having one or two nucleoli. Active fibroblasts can be recognized by their abundant rough endoplasmic reticulum. Inactive fibroblasts, which are called fibrocytes, are smaller and spindle shaped. They have a reduced number of rough endoplasmic reticulum.

Fibroblasts are a group or family of cells, which are present in virtually all tissues and arise from all three germ layers. Fibroblasts function in the deposition, maintenance, degradation and remodulation of the ECM. Fibroblasts have also generally been regarded as target cells of cytokines and growth factors. Hence, the use of any prospective biomaterial necessitates the need for knowledge of its interaction with fibroblasts (Radhika *et al*., 1999). Fibroblasts cultured in collagen gels have been reported to express a physiological behavior comparable to that exhibited *in vivo*. Fibroblasts become the predominant cells by the end of the first week of healing. They originate from nearby connective tissue cells. The stimulus for subsequent fibroblasts proliferation and collagen synthesis are induced by growth

factors. The fibroblasts migrate into the wound forming adhesive contact with the fibrin strands from the initial wound clot and with collagen fibers and fibronectin. The fibroblasts activity depends on the adequacy of the local oxygen supply. The fibroblasts proliferate and migrate along the fibronectin, crossing the wound and establishing a lattice for collagen synthesis (Porras, Reyes and Mustoe, 1994).

1.2.2 Correlation between Keratinocytes and Fibroblasts

The biological mechanisms for the normal healing process toward an excessive reparative response are largely unidentified. Obviously, the most visible feature of the hypertrophic scar (HSc) is the excessive deposition of collagen, suggesting that the equilibrium of collagen synthesis and degradation is out of control. The cell predominantly present in HSc is the fibroblasts, the cell that is playing an important role in collagen synthesis (Gu *et al*., 2005 and Nedelec *et al*., 2000).

Excessive biosynthesis of extracellular matrix proteins (ECM) by fibroblasts has been suggested as one of the potential contributing factors leading to the accumulation of excessive matrix. Comparatively, hypertrophic scar fibroblasts showed a higher collagen type-I and type-III mRNA expression and produced additional collagen than normal skin fibroblasts (Zhang *et al*., 1995). Excessive matrix accumulation may occur in the case of increased synthesis of extracellular matrix proteins or declined in the matrix degradation. Reduced collagenase activity of HSc fibroblasts may be responsible for excessive accumulation of collagen, possibly due to activity of collagenase inhibitors such as α2-macroglobulin which was found in the extracellular matrix of HSc (Ghahary *et al*., 1996). According to

Sawicki *et al*., 2005, epidermal growth factors may play a vital role in the process of hypertrophic scarring under specific conditions as occurring during delayed wound healing. This is supported by observation that able to control proliferation of fibroblasts to synthesize growth factors, which in turn will stimulate keratinocyte proliferation (Werner *et al*., 2007), fibroblasts collagen synthesis and collagenase activity (Garner, 1998).

1.2.3 Wound Healing

Wound healing is a well-ordered and highly coordinated process involving inflammation, cell proliferation, matrix deposition, and tissue remodeling (Figure 2). After injury, the formation of new tissues begins with re-epithelialization and followed by the granulation of tissue formation. The latter process encompasses the accumulation of macrophage, in growth of fibroblast, formation of matrix and also angiogenesis (Clark, 1996).

Throughout the inflammatory phase, blood clots form in wound and loosely unite the wound boundaries. This deep wound healing phase involved the inflammation; a vascular and cellular reaction that facilitate to eliminate microbes, foreign materials and dying tissues in preparation for repair process. The vasoconstriction followed by active vasodilatation increased permeability of blood vessels enhanced the deliverance of helpful cells (Broughton and Rohrich, 2005). These include the phagocytic white blood cells called neutrophils, monocytes (which develop into macrophages that phagocytize microbes) and mesenchymal cells (which develop into fibroblasts). In the migratory phase, the clots become scab, and epithelial cells migrate beneath the scab to bridge the wound.

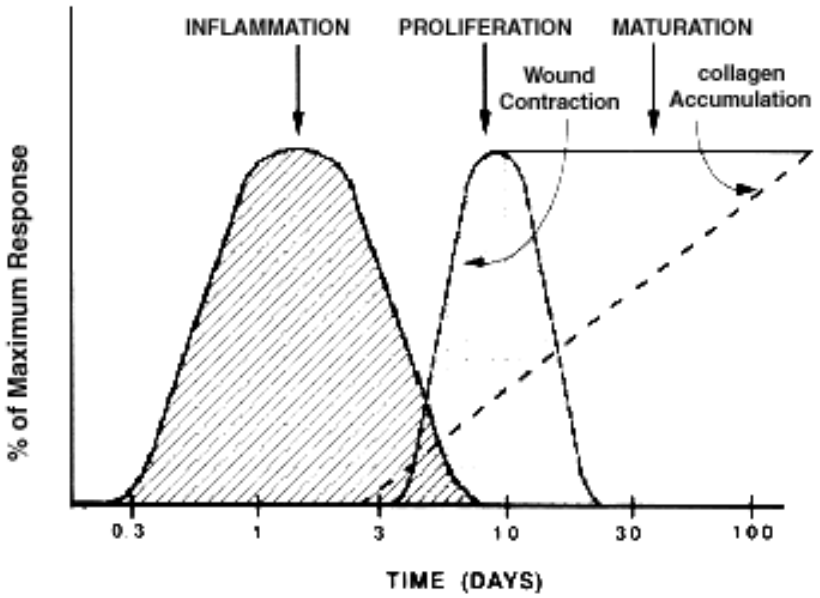

Figure 2: The phase of cutaneous wound repair. Adapted from Clark, (1996). The molecular and cellular biology of wound repair.

In the granulation tissue phase, fibroblasts migrate along fibrin threads and start to synthesize scar tissue and damaged blood vessels begin to regrow. This is followed by the proliferative phase which is recognized by the extensive growth of epithelial cells beneath the scab, deposition of collagen fibers in random pattern by fibroblasts, and continued growth of blood vessels. Finally, in the maturation phase in which the scab sloughs off once the epidermis has been reinstate to normal thickeness. Collagen fibers will be more organized, fibroblasts decreased in number and blood vessel are restored to normal (Tortora and Grabowski, 2004).

Successful wound healing is the result of a complex interaction between stimulatory and regulatory cytokine, growth factors, the fibroblast, epithelial cells and endothelial cells that populate the wound. The purpose of wound healing is to restore the functions of skin, such as protection of the body against harmful environmental entities, prevention of entry of microorganisms and loss of plasma, the regulation of body temperature, the processing and interpretation of environmental information through the neurosensory system and social-interactive function (William and Phillips, 1996).

1.2.4 Hypertrophic scar and Keloid

The ability to heal wounds by scar tissue formation is essential for the survival of human. A preferred scar is one that matures rapidly without contracture or increase in width and without forming more collagen than necessary for its strength. However, in most instances particularly following burn injury, scarring becomes a real concern and a clinical challenge in the treatment of patients. The scars are itchy, painful, and unsightly and interfere with function and daily activities. These are the most common reasons for patients requesting plastic and reconstructive surgery. The effective treatment and prevention remain elusive.

Both HSc and keloids resulted from an exaggerated and perpetuated fibrotic phase of the wound healing response (Uitto, 1991). HSc and keloids are the type of scarring which can be characterized by the excessive collagen synthesis and deposition of fibroblasts produced during the wound healing process, particularly after severe thermal injury (Castagnoli *et al*., 1997). However, the exact mechanism involved in the scarring remains unclear. HSc is common; they are wide, often red, itchy and even painful. HSc is a tumor-like lesion which is elevated above the level of surrounding skin. The edges are usually prominent and end abruptly. They are typically raised scars that remain within the boundaries of the original wound and frequently raised spontaneously after the initial injury (Bayat *et al*., 2003) (Figure 3 a).

Ultrastructurally keloids are more disorganized than HSc. In contrast to HSc, keloids extend beyond the original wound margin and invade the surrounding normal skin in a way that is site specific (Mafong and Ashinoff, 2000). A keloid continues to grow over time, does not regress spontaneously, and almost invariably recurs after

(a)

(b)

Figure 3: The appearance of HSc and keloid from patients of this study before surgery. (a) HSc (b) keloid. Both scars resulted from delayed wound healing. Keloid has a more prominent features compared to HSc.

simple excision. Keloids are characterized as collagenous, cutaneous lesions and do not regress spontaneously. Keloids are wide, raised and deep scars with excessive fibrous tissue that resulted from skin injury (Figure3b). Keloid formation is characterized by an extended period of fibroblasts proliferation and elevated rate of collagen synthesis with a decreased rate of collagen degradation (Orimolade *et al*., 2011).

The increased metabolic activity of HSc and keloids is reflected in the elevated glycolytic enzyme activity, the fibronectin deposition and the collagen mRNA expression. Fibroplasia in these abnormal scars continues beyond the third week of post injury. The scars remain immature, with an abnormally high content of type-III collagen and a disorganized pattern of collagen deposition.

An array of treatments modalities has been introduced to manage HSc and keloid (Su *et al*., 1998 ; Mustoe *et al*., 2002). Alster *et al*. (1997) and Niessen *et al*. (1999) presented comprehensive reviews on the nature and treatment modalities of hypertrophic scar and keloids. An international panel of clinicians recently reviewed the available literature for an evidence based analysis of the treatment for cutaneous scarring and recommended useful approaches for the clinicians where evidence was lacking (Mustoe *et al*., 2002). At present, accepted modalities for the prevention of hypertrophic scarring include silicone occlusive therapy (Cruz-Korchin, 1996; Ahn *et al.*, 1991; Lyle *et al*., 2001), steroid injections (Tang, 1992) and pressure garments (Linares *et al*., 1993). Pressure garment is a widely used therapy where the patients need to continuously wear the elastic compression of garments for months. Under pressure, collagen fibers in the dermis will modify the disorganized orientation and will adopt a more parallel arrangement which is the characteristic of normotrophic

healing. The scar becomes less vascular, expresses less glycosamnioglycans and has less collagen deposition (Akkermans, 1999). Therefore in the treatment with pressure garments, it is believed that pressure controls collagen synthesis to the levels as in the normal scar tissue.

1.2.5 Primary cell culture

Tissue culture was first devised at the beginning of this century as a useful method for studying the behavior of animal cells in free systemic variation that might arise in the animal during normal homeostasis and under the stress of an experiment (Cruz *et al*., 2009). As the name implies, the technique is elaborated first with undisaggregated fragments of tissue and growth was restricted to the migration of cells from tissue fragments, with occasional mitoses in the outgrowth. Since culturing cells from primary tissues conquered the field for more than 50 years, it is not surprising that the name 'tissue culture' has remained in spite of the fact that the most explosive expansion in this area in the second half of the 20^{th} century utilized cell culture (Freshney, 2010).

Cell culture refers to a culture derived from dispersed cells taken from original tissue of a primary culture or from an established cell line or cell strain by enzymatic, mechanical or chemical desegregation. According to Sultan and Haagsman (2001), uniqueness of primary cell cultures is the possibility to amend the metabolic and regulatory pathways of cells of interest and to delineate the pysiological effects of various compounds and drugs in a controlled way. The refinement of this experimental tool can be expected to accelerate species-specific research. Cells isolation of several cell types from the same tissue biopsies would

constitute a significant advantage in the identification of the cells that responsible for dysfunction in skin diseases (Abdel Naseer *et al*., 2005).

1.2.5.1 Keratinocytes cultures

Before 1974, there had been many attempts to cultivate human epidermal cells in culture. The growth obtained was very limited and inadequate to permit satisfactory subcultivation (Green, 2004). Essentially no basic or applied work could be done on co-culture of human keratinocytes with other variety of cells. Rheinwald (1974) had started his culture work on mouse teratoma, a germ line tumor growing as a transplanted tumor to differentiate into a number of somatic tissues. When he put cells disaggregated from a tumor into a culture, colonies of different appearance arose including an unusual-looking epithelial cell type together with a background of teratomal fibroblasts. Once lethally irradiated 3T3 cells had been introduced, the epithelial cells grew rapidly in culture system. The 3T3 cells thus had been widely applied in supporting the growth of the epithelial cells in a culture. This made it possible to isolate clones of the epithelial cells and study their behavior. It became obvious that some of these clones were keratinocytes, the principal cell type of all stratified squamous in epidermis (Rheinwald and Green, 1975). With the subsequent improvements in cultivation, keratinocytes became the most cultivable of human diploid cell types (Barrandon and Green, 1987). The ability to culture human keratinocytes is a great importance to the clinician and the research workers.

According to some studies, human keratinocytes cultures become important studies of keratins and their numerous disease-producing mutations (Cooper *et al*., 1985; Moll *et al*., 1982; Wu *et al*., 1982). Furthermore, the cross-linked envelopes of

terminal differentiation can also be studied. Recent improvements and simplification of the method of preparing the cultures for grafting are likely to expand the use of cultured autologous keratinocytes in the treatment of wound (Pellegrini, 1999 and Ronfard *et al*., 2000).

1.2.5.2 Fibroblasts cultures

The nutritional constitution of the medium, the presence of inducer or repressor substances and the interaction of cells with the substract control the expression of specialized functions in a culture. Most of the cell lines grow well at pH 7.4. Although the optimum pH for cell growth varies relatively among different cells strain, some normal fibroblast cell lines performed best at pH 7.4 to 7.7, and transformed cells may do better at pH 7.0 to 7.4 (Freshney, 2000). Fibroblasts can give rise to other cells, such as bone cells, fat cell, and smooth muscle cells. All of these cells are of mesodermal origin. The fact that fibroblasts can easily proliferate makes them a popular cell type for cell cultures in biological research. Notably, mouse embryonic fibroblasts (MEFs) are often used as "feeder cells" in human embryonic stem cell research (Reubinoff *et al*., 2000; Ludwig *et al*., 2006). However, many researchers are gradually phasing out MEF's in favor of culture media with precise defined ingredients of exclusive human derivation.

Skin cultures of fibroblasts and keratinocytes are also available for cytotoxicity and inflammation research such as Episkin™ (Saduc) and Epiderm™ (Mat Tek) and from Advanced Tissue Sciences. These products are prepared by combining keratinocytes with dermal fibroblasts and supported with a nylon collagen net called “skin equivalent” (Freshney, 2000).

The ability of fibroblasts in the granulation tissue to differentiate into myofibroblasts is not only seen as the start of wound contraction but is also the cause of scar formation, contractures and hypertrophy. In culture, myofibroblasts are characterized by slower growth rates, presence of stress fibers and larger stellate cell morphology. In comparison, fibroblasts have higher growth rate and spindle shape morphology with predominant fillamentous actin (Lamme, 1999).

1.2.5.3 Scar keratinocytes and fibroblasts culture

Dermal fibroblasts play a major role in scar formation and have been used *in vitro* for a variety of wound repair studies to uncover the mechanism of fibrosis. Often, *in vitro* fibroblasts exhibit characteristic typical of their *in vivo* phenotypes. For instance keloid fibroblasts continue to produce high levels of collagen, fibronectin, elastin, and proteoglycan *in vitro* and show aberrant responses, compared with normal fibroblasts, to metabolic modulators such as glucocorticoids, hydrocortisone, growth factors and phorbol esters (Tuan *et al*., 1991). The altered response of keloid fibroblasts to these metabolic modulators are thought to attribute to the pathogenesis of keloid formation. Fibroblasts from hypertrophic scars also display a moderate elevation in collagen production *in vitro*. However, their responses to the metabolic modulators are similar to normal fibroblasts (Russell *et al*., 1995).

1.2.6 Immunocharacterization of keratinocytes and fibroblasts

Detection of antigens in cultured cells is generally referred to as immunocytochemistry. Immunocytochemistry is the demonstration of a cellular constituent *in situ* by detecting specific antibody-antigen interactions where the antibody has been tagged with a visible label. The visual marker may be a fluorescent dye, colloidal metal, hapten, radioactive marker or an enzyme. Considerable attention has been focused on cell markers development for cells detection especially cell specialized from stem cells. Despite the diversity of techniques now available to the researcher, ideally, maximal signal strength along with minimal background or non-specific staining are required to give optimal antigen demonstration. Central to these techniques is the use of an antibody to link a cellular antigen specifically to a stain that can be readily visualized with a microscope. A commonly used technique is immunocytochemical analysis of a cell with their specific markers or antigen. The keratinocytes are the major cell found in skin epidermal while fibroblasts in dermal. Cytokeratin 6 (CK6), involucrin, heat shock protein 47 (HSP 47) and fibroblasts surface protein (FSP) are among the markers to be used to identify cells from human skin epidermal and dermal (Strutz *et al*., 1995)

1.2.7 Nitric Oxide (NO)

Nitric Oxide (NO, formula N=O) is a simple, inorganic gaseous free radical whose predominant functions are that of a messenger and effectors molecule. In mammals, NO is synthesized by a family of enzymes referred to as the nitric oxide synthase (NOS). NO originally identified as endothelium-derived relaxing factor, cell types such as macrophages, endothelial cells, neurons, hepatocytes, fibroblasts, epithelial cells, keratinocytes, smooth muscle cells, cardiac myocytes and many more have been shown to produce NO (Geller and Billiar, 1998).

Nitric Oxide also has been revealed to possess several physiologic and pathophysiologic activities. These include smooth muscle relaxation or vasolidation, anti-microbial activities, immune regulation, anti-proliferation and neurotransmission. The biosynthesis of NO is mediated by NO synthases (NOS), beta-nicotinamide adenine dinucleotide phosphate (reduced form)-dependent enzymes that catalyze the oxidation of one of the guanidine nitrogen atoms of L-arginine with molecular oxygen to form NO and L- citrulline (Geller and Billiar, 1998). NO synthesis is catalyzed by one of three isoforms of NOS: NOS I (neuronal or nNOS), NOS III (endothelial eNOS) and NOS II (inducible or iNOS). Expression of both the constitutive as well as the inducible isoform has been shown in several cells resident, including keratinocytes and fibroblasts. NO also enhanced the production of collagen type 1 by normal fibroblast at the post-translational level *in vitro* study (Witte *et al*., 2000). The inhibition of NOS by competitive inhibitors has been shown to reduce collagen synthesis (Schaffer *et al*., 1997).

1.2.8 Inducible nitric oxide (iNOS)

iNOS is one of three key enzymes generating NO from the amino acid L-arginine. iNOS-derived NO plays an important role in numerous physiological and pathophysiological conditions for example to mediate vasolidation, angiogenesis, inflammation re-epithelization, collagen deposition and various immune responses. Three isoforms of NOS have been distinguished by cloning their genomic and complementary DNA in several mammalian species including human. Activities of two of these isoforms are dependent on elevated intracellular Ca^{2+} and exogenous calmodulin and are constitutively expressed (cNOS). cNOS appears to be restricted to endothelial cells, while another is expressed in neurons (cNOS) of the central and peripheral nervous system and in skeletal muscles. The third isoforms, iNOS is independent of elevated intracellular Ca^{2+} and possess its own calmodulin as a tightly bound subunit. This isoform is not expressed under normal conditions but can be induced in many cell types by inflammatory cytokines and bacterial lipopolysaccharide (LPS) (Wang *et al*., 1996).

Expression of the inducible isoform has been shown in several cell residents in the skin, including keratinocytes and fibroblasts. In humans, as in other mammals, many types of cells have been shown to synthesize NO and express NOS. Under normal conditions, fibroblasts are responsible for tissue integrity and extracellular matrix metabolism by producing structural (*e.g.,* collagens) and functional (*e.g.,* collagenase) proteins. In damaged tissues, activated fibroblasts migrate towards the wound site, proliferate and produce more extracellular matrix proteins, express NOS and synthesize NO, suggesting that they may participate in regulating the inflammatory, healing process of wound and scar tissue remodeling (Wang *et al.*,

1996). Jorens *et al.*, 1992 also reveals that iNOS plays important role in wound healing through its vasodilatation and anti-proliferative effect**.**

1.2.9 Vitamin E

Vitamin E was discovered in 1922 in green leafy vegetables by University of California researchers, Herbert Evans and Katherine Bishop. In 1936, it was discovered that vitamin E was abundant in wheat germ oil. Two years later, it was chemically synthesized for the first time. Vitamin E then emerged as an essential, fat soluble nutrient that functions as an antioxidant in the human body. Vitamin E is an essential component of the human diet and is synthesized exclusively by photosynthetic organisms. It is essential because human body cannot produce its own Vitamin E. Vitamin E represents a generic term of two groups of chemically related, lipid-soluble molecules that collectively known as tocochromanols; the tocopherols and tocotrienols (Falk and Munne Bosch, 2010). It consists of eight naturally occurring isomers, a family of four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ homologues (Packer *et al*. 2001).

A double blind and randomized trial assessing the influence of topical Vitamin E or steroid on scar formation in burn patient reported by Jenkins *et al*., 1986, did not demonstrate any beneficial effect of either on scar thickness, range of motion or ultimate cosmetic appearance. Therefore, the benefits of vitamin E on surgical wound healing specifically scar formation have not been reliably demonstrated. However, a study by Gray (2003) discovered that Vitamin E may help to minimize the damage and potential healing of wounds from source of radiation. According to Mckay and Miller (2003), vitamin E may have alternative effects on

different types of wounds with the presence of other nutrients. the combined action Limited scope of the study regarding the effect of Vitamin E on the prevention and treatment of scars leads to a lot of unanswered questions.

1.2.10 Tocotrienols (T3s) and Palm oil

T3s is one of the two sub groups of molecules belonging to Vitamin E family derived from locally produced palm oil. T3s are found in high concentrations (800-1500ppm) in crude palm oil extracted from the fruit of *Elaeis guineensis.* T3s are the primary form of Vitamin E in the seed endosperm of most monocotyledone including a few important cereal grains such as wheat, rice and barley (Sen *et al.,* 2005). T3s differ structurally from tocopherols by the presence of the unsaturated and isoprenoid chain possessing three double bonds (Figure 4). Interestingly, besides having strong antioxidant activity, it also has other characteristics such as a hypocholesterolemic, anti-atherogenic, antithrombic, anticarcinogenic and immunomodulatory actions (Qureshi *et al*., 2002; Khanna *et al*., 2005b; Unchern *et al*., 2003). T3s has been found to have a better antioxidant properties compared to α-tocopherol (Osakada *et al*., 2004; Mazlan *et al*., 2006). According to Saito *et al*., 2004, T3s posses a higher antioxidant activity compared to α-tocopherol due to its uniform distribution in the membrane lipid bilayer. The unsaturated side chain of T3s allows for more efficient penetration into tissue that have saturated fatty layers such as brain and liver (Suzuki *et al*., 1993). T3s showed improved absorption ability through the skin and incorporate into cell membrane for the prevention of oxidative damaga when applied topically (Yoshida *et al*., 2003).