A Comparative In-vitro Study of the Viability of Cultured Primary Normal Human Epidermal Keratinocytes on Cellular and Acellular Human Amniotic Membrane

By

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CERTIFICATE

This is to certify that the dissertation entitled 'A COMPARATIVE IN-VITRO STUDY OF THE VIABILITY OF CULTURED PRIMARY NORMAL HUMAN EPIDERMAL KERATINOCYTES ON CELLULAR AND ACELLULAR HUMAN AMNIOTIC MEMBRANE' is the bonafide record of research work done by MOHD YUSRI BIN AHMAD FOZI during the period from July 2009 to October under my supervision.

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ABSTRACT

Skin is the largest organ in human body. It plays important role to regulated and maintain human body homeostasis. Based on this condition, burned skin needs rehabilitation in order to reduce and avoid disturbances of body homeostasis. This study aimed to assess the ability of Human Amniotic Membrane (HAM) as scaffold for the growth of keratinocytes in-vitro. The HAM's epithelium had been removed using enzymatic technique namely dispase and trypsin-EDTA. De-epithialized HAM (also known as an acellular of HAM) was used as the main scaffold for the cultured primary normal human epidermal keratinocytes (pNHEK). Cell viability was compared between de-epithialized HAM and HAM without de-epithialization (also known as cellular HAM) for the three consequence days. Epithelium of HAM was successfully removed via dispase and trypsin-EDTA, as confirmed by histochemical and cytochemical analysis. De-epitheliazed HAM sustained better cell viability only at the first 24 hours compared with the HAM without de-epithialization. However, viable cells were deserved only at 48 hours post-seeding of pNHEK on the HAM without de-epithialization. In conclusion, HAM can be de-epithialized using enzymatic technique. De-epithialized HAM and HAM without de-epithialization can sustain the growth of pNHEK. Result of this study will use to produces specific kind of HAM for healing of skin burn.

ABSTRAK

Kulit adalah organ terbesar pada tubuh badan manusia. Ia memainkan peranan yang penting dalam mengawal atur dan mengekalkan homeostasis badan manusia. Kepentingan struktur ini menjadikan kulit yang telah mengalami kelecuran mestilah segera dipulihkan agar tidak mengganggu dan merencatkan proses normal di dalam badan. Berdasarkan kepentingan inilah kajian ini dijalankan untuk menilai keupayaan pertumbuhan dan pembiakan sel keratin di atas membran amnion manusia (HAM). Normal HAM diselaputi oleh satu lapisan epithelium. Oleh itu, lapisan epithelium ini perlu disingkirkan dahulu dengan menggunakan teknik tindakan enzim iaitu kombinasi tindakan enzim dispase dan trypsin-EDTA. HAM yang telah dibuang epithelium ini akan digunakan sebagai tapak pembiakan dan pertumbuhan sel keratin. Sel-sel keratin diambil dan diproses dari epidermal normal keratin manusia (pNHEK). Perbandingan akan dibuat untuk menilai keupayaan dan kebolehan sel keratin untuk hidup di antara HAM yang berepithelium dan HAM yang telah dibuang epithelium dalam hari berturutan. Daripada teknik yang telah dioptimakan, epithelium telah berjaya disingkirkan daripada HAM dengan menggunakan kombinasi tindakan enzim dispase dan trypsin-EDTA. Perbandingan yang telah dibuat bagi menilai keupayaan pNHEK untuk membiak dan tumbuh di atas HAM menunjukkan pNHEK berupaya hidup hanya untuk 24 jam yang pertama selepas dikultur bagi HAM yang telah dibuang epithelium. Bagi HAM yang berepithelium, pNHEK berupaya untuk hidup dan tumbuh selepas 24 jam dikultur tetapi hanya bertahan selama 24 jam sahaja. Sebagai kesimpulan, epithelium telah berjaya dibuang dari HAM dengan menggunakan teknik enzimatik. Sel pNHEK berupaya untuk membiak dan tumbuh di atas kedua-dua

permukaan HAM. Hasil kajian ini membolehkan produk amnion yang lebih khusus dapat dihasilkan untuk mempercepatkan pemulihan kulit yang mengalami kelecuran.

CONTENT

LIST OF ABBREAVIATION

pNHEK	Primary normal human epidermal keratinocytes
%	Percent
H&E	Hematoxycillin and eosin
CK	Cytokeratine staining
HAM	Human amniotic membrane
cm^2	Centimeter square
mL	illiliter
mm	millimeter
µL	microliter
µm	micrometer
UV	Ultraviolet
rpm	Round per minute
^oC	Degree Celsius
CO_2	Carbon dioxides
PGE	Prostaglandin E
NaOH	Sodium hypochlorite
ddH_2O	sterile distilled water
RT	Room temperature
TNF	Tumour necrosis factors
IL	Iinterleukin
Ach	Acetylcholine

AMT	Amniotic membrane transplantation
HAEC	Human amniotic epithelial cells
EGF	Epidermal growth factor
KGF	Keratinocytes growth factors
HUSM	Hospital Universiti Sains Malaysia
PCT	Progenitor cell target
PBS	Phosphate-buffered saline
DMEM	Dulbecco's Modified Eagle's medium
FBS	Fetal bovine serum
KGM	Keratinocytes growth media
EDTA	Ethylenediaminetetraacetic acid

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CHAPTER 1

INTRODUCTION

1.1 Introduction of skin

Skin is the largest organ of the integumentary system. There are covered external human body surface which has a surface area of between 1.5-2.0 meters square, mostly between 1-2 mm thick (Flynn and Woodhouse, 2009). It comprised about one sixth of total body weight. The average square inch (6.5 cm^2) of skin holds 650 sweat glands, 20 blood vessels, 60,000 melanocytes, and more than a thousand nerve endings. The skin made up of multiple layers of epithelial tissues, and guards various body organs (Sembulingam and Sembulingam, 2005). Its plays an important role in body homeostasis likes water and temperature regulation, protecting against pathogens, sensation, and vitamin D synthesis. Skin has pigmentation, or melanin, provided by melanocytes, which absorb some of the potentially dangerous ultraviolet radiation (UV) in sunlight (Balasubramani *et al.*, 2001).

The skin is composed of three primary layers:

1.1.1 Human skin epidermis

The epidermis is the outer layer of the skin which provides waterproofing and serves as a barrier to infection. It has no blood vessels, and cells in the deepest layers are nourished by diffusion from blood capillaries extending to the upper layers of the dermis. The total thickness of the epidermis is usually about 0.1 – 0.5 mm (MacNeil, 2008). The

epidermis consists of three types of cells; keratinocytes, melanocytes and langerhans cells.

1.1.1.1 Keratinocytes

Keratinocytes are the predominant type of cells (90%) in the epidermis. These cells are arranged in stratified layers which able to produce the protein keratins, which provide some of the rigidity of the outer layers of the skin (Flynn and Woodhouse, 2009). At the dermal-epidermal junction, there is a single layer of keratinocytes with a small number of interspersed melanocytes (approximately 1/30) which called the stratum basale. This basal layer of keratinocytes is also called the stratum germinativum, because there is new keratinocytes are generated by cell proliferation. Three types of keratinocytes in the stratum basale have been defined by kinetic analysis such are stem cells, transient-amplifying cells and committed cells. Stem cells, which represent about 10% of the basal cell population may able to generate daughter cells from mitosis, either from stem cells themselves or transient-amplifying cells, whereas the transient-amplifying cells which represent about 40% of the basal cell population, replicate with much higher frequency than stem cells, but are capable of only a few population doublings. Transient-amplifying cells will produce daughter cells that are committed to terminally differentiate. These committed cells detach from the basement membrane, differentiate, and ultimately cease to proliferate as they migrate toward the skin surface, where they are sloughed off as dead, cornfield cells called squamus (Stratum corneum). This process of a living cell's evolution, called keratinization, takes about 4 weeks (MacNeil, 2008).

Keratinocytes was isolated from the skin and then in vitro cultured, which was further developed into small sheets of cells which called cultured epithelial autografts (CEA's) (Rheinwald and Green, 1977). At the early 1980s, small sheets of keratinocytes were being used to treat patients with extensive burns injuries (MacNeil, 2008). The keratinocyte cell technology used to create CEA's has recently been taken further by forming cell suspensions that can be sprayed onto wound sites with or without fibrin, e.g. CellSprays (Navarro *et al.*, 2000).

1.1.1.2 Melanocytes

Melanocytes are cells in low abundance in the epidermis that produce the pigment melanin. The pigment made in melanocytes is transferred to the cells of the hair or epidermis. The melanin granules are injected into (or ingested by) the keratinocyte cells. There, the melanin granules accumulate around the nucleus of each keratinocyte. Melanin absorbs harmful ultraviolet (UV) light before the UV radiation can reach the nucleus. Melanin protects the DNA in the nucleus from UV radiation damage. When melanin is produced and distributed properly in the skin, dividing cells are protected from mutations that might otherwise be caused by harmful UV light. Differences in skin color are due mostly to differences in the types and amount of pigment in our keratinocytes. Skin darkening (tanning) from sun exposure is caused by the movement of existing melanin into keratinocytes, and by increased production of melanin by the melanocyte (Flynn and Woodhouse, 2009). During embryonic development these cells migrate from the neural crest into the skin.

1.1.1.3 Langerhan cells

Langerhans cells is a star shaped resident immune cells, which also known as macrophages. A macrophage is a cell that protects our body from infection. Macrophages will break up or destroy (phagocytes) the invading organisms (Sauder and Pastore, 1993). These macrophages process the invading organisms and present antigens to the T-lymphocytes. The T-lymphocytes are immune-system cells which ultimately identify a substance as foreign or dangerous to the body.

1.1.2 Human skin dermis

The dermis (or corium) is typically 3–5 mm thick and is the major component of human skin, which serves as a location for the appendages of skin. It is composed of a network of connective tissue, predominantly collagen fibrils providing support and elastic tissue providing flexibility, embedded in a mucopolysaccharide gel (Balasubramani *et al.*, 2001). The dermis consists largely of fibroblast in which is part of the mesenchyme. The fibroblasts produce the collagens and elastins that make skin very durable (Metcalfe and Ferguson, 2007).

1.1.3 **Hypodermis** (subcutaneous adipose layer)

Subcutaneous tissue is the innermost layer of the skin located under the dermis and consisting mainly of fat. Subcutaneous fat acts as a shock absorber and heat insulator, protecting underlying tissues from cold and mechanical trauma (Metcalfe and Ferguson, 2007). Sweat glands and minute muscles attached to hair follicles originate in subcutaneous tissue.

1.2 Skin burns

Burns are a very common injury to the skin. It may be due to heat, light, radiation, friction, chemical or electrical causes. In burn injuries, the trauma and subsequent repair relies greatly on the extension and the depth of the wound (Balasubramani *et al.*, 2001). Minor burns will heal without much medical attention, but severe burns require special care to prevent infection and reduce the severity of scarring. Burn are classify to first-, second-, third- or fourth-degree burn. However for surgical intervention, the burns are described on their depth of injuries as superficial, superficial partial-thickness, deep partial-thickness, or full-thickness. There are relates to the epidermis, dermis and subcutaneous layers of skin and is used to guide treatment and predict outcome.

1.2.1 Superficial thickness burns

There are known as first-degree burn which is usually limited to redness (erythema), a white plaque and minor pain at the site of injury. These burns involve only the epidermis. Most sunburn can be included as first-degree burns. The skin may be very tender to touch.

1.2.2 Partial thickness – superficial burns

Before this, there are known as second-degree burn which manifest as erythema with superficial blistering of the skin, and can involve more or less pain depending on the level of nerve involvement. Second-degree burns involve the superficial (papillary) dermis and may also involve the deep (reticular) dermis layer.

1.2.3 Partial thickness – deep burns

Old term known as third-degree burn which occur when the epidermis is lost with damage to the subcutaneous tissue. Burn victims will exhibit charring and extreme damage of the epidermis, and sometimes hard eschar will be present. Third-degree burns result in scarring and victims will also exhibit the loss of hair shafts and keratin. These burns may require grafting.

1.2.4 Full thickness burns

The most severe is the fourth-degree burn which involves all three layers of skin (epidermis, dermis, and fat layer). It damaged muscle, tendon, and ligament tissue, thus result in charring and catastrophic damage of the hypodermis. It will appear white and leathery and tend to be relatively painless. In some instances the hypodermis tissue may be partially or completely burned away as well as this may result in a condition called compartment syndrome, which threatens both the life and the limb of the patient. Grafting is required if the burn does not prove to be fatal.

1.3 Skin tissue engineering

The development of tissue engineering and tissue banking contributed more benefit to the process of wound healing especially for treating burn (MacNeil, 2007). There are providing more material and techniques to enhance healing process especially for surgical applications. In theory, tissue-engineered skin could function as a skin replacement, as a matrix material into which the host (keratinocytes) might grow, or in other hand may act as a source of chemical stimulants to healing (pharmacologic agent)

(Eaglstein and Falanga, 1998). The current treatment modalities involve multiple stages like excision of injured tissue, subsequent to stabilizing the patient. Then, it was followed by grafting which involves application of skin substitutes which are either split thickness autografts, allografts (homografts), xenografts (heterografts) or their refined versions (Balasubramani *et al.*, 2001).

Now, there are various product of tissue-engineered on market. It is like Apligraf AlloDerm, Biobrane, Dermagraft, Epicel as well as Integra (Pham *et al.*, 2007). However, the long-term fate of these tissue-engineered skins still unknown, but the original investigational protocols allowed investigators to treat the skin ulcers with multiple and sequential applications of these agents during the initial weeks of therapy, suggesting that they might be working as pharmacologic agents rather than as tissue replacements. Although the human cells and animal matrix materials used in these product, there are various test needed in order to avoid transmission of viruses such as hepatitis and HIV, the possibility of long term toxicities cannot be totally excluded (Eaglstein and Falanga, 1998).

Understanding how to manipulate the relevant skin cells (or progenitor cells) using lessons learned from foetal and adult wound repair as well as mechanisms of mammalian regeneration will be integral in developing the next generation of tissue-engineered skin substitutes (Metcalfe and Ferguson, 2007).

1.4 Human amniotic membrane (HAM)

For skin substitute, there are the need for biological scaffold. Previously, surgeons used human amniotic membrane (HAM) and fibrin glue to provide an ideal platform for growth and proliferation of viable cells. It is a stable basement membrane and having varies cytokine and others signaling molecules which may enhance the growth of various cell lines (Maral *et al.*, 1999). It is like prostaglandin E (PGE), transforming growth factors-α, tumour necrosis factors (TNF), interferon, interleukin (IL)-4, IL-8, acetylcholine (ACh), catecholamine and neurotrophic factors.

HAM is able to reduce occurrence of exudates, adhesions, drying and scaring in defect surface. With angiogenic activity, there were improve the tissue vascularity. It is accelerated by various growth factors. HAM is easy biodegradable.

HAM is derived from the fetal membranes sac. It is constituted of the inner amniotic membrane made of single layer of amnion cells fixed to the collagen-rich mesenchyme 6 to 8 cells thick loosely attached to chorion. It is composed of 3 layers: a single epithelial layer, thick basement membrane, and avascular stroma. Besides, it contains of fibroblast and other growth factors collagen types III and V. Collagen types IV and VII are similar to corneal epithelial basement membrane as well as fibronectin and laminin.

HAM is believed to be nonimmunogenic. Antibodies or cell-mediated immune response to amniotic membrane have not been demonstrated, suggesting low antigenicity. Therefore, the use of systemic immunosuppressives in amniotic membrane transplantation (AMT) is not required. In contrast, chorion provokes neovascularization and typical rejection phenomenon. The use of amnion components in tissue repair