IN VITRO AND *IN VIVO* STUDY OF PRIMARY EPIDERMAL ALLOGRAFTS VERSUS XENOGRAFTS TRANSPLANTED ON FULL THICKNESS WOUND IN RABBITS

NORLAILY BINTI HANIFI

UNIVERSITI SAINS MALAYSIA

2021

IN VITRO AND *IN VIVO* STUDY OF PRIMARY EPIDERMAL ALLOGRAFTS VERSUS XENOGRAFTS TRANSPLANTED ON FULL THICKNESS WOUND IN RABBITS

by

NORLAILY BINTI HANIFI

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

January 2021

ACKNOWLEDGEMENTS

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

Firstly, I would like to express my utmost gratitude to my supervisor, Professor Dato' Dr. Ahmad Sukari Bin Abdul Halim, for his guidance and countless support for me throughout years of my studies. Apart from that, he has been the important person who encouraged me to finish up my study despite my financial problem in settling the study fees. I also like to thank my co-supervisor; Associate Professor Dr. Thirumulu Ponnuraj Kannan, Associate Professor Dr. Rapeah Suppian and Dr. Thin Thin Win @ Safiya Yunus for giving me the supportive ideas and comments in different parts of the study according to their expertise. I also would like to acknowledge my former supervisorfor the first yearof my studies, Dr Aravazhi Ananda Dorai who had moved to a private hospital, for his ideas and guidance in starting up my research. I am most indebted to the staff members of the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia, especially Mr. Md Faizul Ismail Che Adam, Mr. Nur Muhammad and Dr. Noziah for their help rendered during conduction of animal experiments. To all my collegues and staff at Reconstructives Science Unit, especially Cik Fareha Aleas, thank you so much and may Allah pay your kindness in helping me throughout my study. To my family, my husband Mohd Amir Husni and my three wonderful daughters Kakak, Adik and Baby for giving me the strength to pursue my will and vision for the better future of our family. I love you all very much, you are my golden virtues. Lastly, I wouldlike to acknowledge the Universiti Sains Malaysia Research University Grant (No.1001/PPSP/813042) for funding this research study and My Brain15, Postgraduate Scholarship Programme from MOHE for awarding the scholarship.

TABLE OF CONTENTS

Chapter 3: Materials and Methods

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

CEA	Cultured epidermal autograft
CEAllo	Cultured epidermal allograft
CEXeno	Cultured epidermal xenograft
MHC	Major histocompatibility complex
BPE	Bovine pituitary extract
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate Buffered Saline
mg	Milligram
mL	Milliliter
µg	Microliter
EDTA	Ethylenediaminetetraacetic acid
DMSO	Dimethyl sulphoxide
PCR	Polymerase chain reactions
BSA	Bovine serum albumin
FBS	Fetal bovine serum
FCS	Fetal Calf Serum
TBS	Tris-buffered saline
NaCl	Sodium chloride
KCl	Potassium chloride
IHC	Immunohistochemistry
HRP	Horseradish peroxidase

DAB	Diaminobenzidine
LSAB	Labeled streptavidin biotin
PCK-26	Pan-Cytokeratin 26
ARASC	*Animal Research And Service Centre*
kg	Kilogram
cm	Centimetre
ICC	Immunocytochemical
H&E	Hematoxylin and Eosin
DPX	Distyrene, plasticizer and xylene
PBMC	Peripheral blood mononuclear cells
MAb	Monoclonal antibody
FACS	Fluorescence-activated cell sorting
SD	Standard deviation
APCs	Antigen presenting cells

LIST OF APPENDICES

KAJIAN SECARA *IN VITRO* DAN *IN VIVO* TERHADAP EPIDERMIS PRIMER ALLOGRAF MELAWAN XENOGRAF YANG DI TAMPAL KE ATAS LUKA BERKETEBALAN PENUH PADA ARNAB

ABSTRAK

Penampalan kulit telah berkembang sebagai satuaplikasi yang penting dalam pembedahan rekonstruktif. Penampalan kulit mempercepatkan penyembuhanluka dan juga dapat mengurangkan kehilangan cecair tanpa sedar yang disebabkan oleh luka terbakar dan juga luka yang lain. Ia juga dapat mengurangkan penghasilan parut dan mempercepatkan kosmesis. Pelbagai laporan yang berbeza mengenai tahap ketahanan tisu keratinosit dari allogenik dan xenogenik menyebabkan kajian mendalam perlu dilakukan untuk mengkaji peranannya dalam penyembuhan luka. Tujuan kajian ini adalah untuk mengkaji potensi penyembuhan luka berketebalan penuh menggunakan epidermis primer dari allograf dan xenograf pada arnab. Kulit dari arnab dan tikus di semai dan dikulturkan secara *in vitro* dengan supplemen pertumbuhan yang spesifik. Dua luka berketebalan penuh (2cm x 2cm) dihasilkan pada bahagian belakang setiap arnab. Kultur epithelium allograf dari arnab dan xenograft dari tikus di semburkan terus ke tempat luka yang baru dihasilkan itu bersama fibrin dengan menggunakan alat Tissomat (Baxter, Austria). Selepas empat minggu, arnab-arnab itu dikorbankan dan tisu biopsy diambil dari setiap luka yang telah sembuh itu untuk kajian histologikal yang seterusnya menggunakan pewarnaan Hematoxilin dan Eosin, pewarnaan imunohistokimia dan pewarnaan histokimia. Keputusan dari kajian ini mendapati kedua-dua graf menunjukkan penyembuhan yang baik dan tiada berlakunya penolakan pada

graf-graf tersebut. Parut yang terhasil selepas luka itu sembuh juga agak memuaskan dan sembuh dengan formasi parut yang sedikit. Penilaian immunologikal melalui analisis mengalir sitometri telah menyokong jangkaan akhir dari penyelidikan ini dengan menunjukkan bahawa tiada berlakunya penolakan immunologikal. Kesimpulannya, epidermis primer allograf dan xenograf berpotensi untuk di jadikan sebagai kulit gentian untuk menggantikan kulit rosak. Ini dapat mengatasi kekurangan kawasan kulit derma pada pesakit sendiri terutamanya bagi kes-kes luka kebakaran.

IN VITRO AND *IN VIVO* STUDY OF PRIMARY EPIDERMAL ALLOGRAFTS VERSUS XENOGRAFTS TRANSPLANTED ON FULL THICKNESS WOUND IN RABBITS

ABSTRACT

Skin grafting has been evolving as an important application in reconstructive surgery. Skin grafting accelerates healing of wounds as well as reduces insensible fluid loss from burns and other wounds. It also reduces the formation of scars and accelerates cosmesis. Mixed reports on the survival of allogenic and xenogenic keratinocytes require further substantiation to determine their role in wound healing. The purpose of this study was to investigate the full thickness wound healing potential using primary epidermal allografts versus xenografts in rabbits. The rabbit and rat skins were harvested and cultured *in vitro* using specific growth media. Two full thickness wounds (2cm x 2cm) were created on the dorsum of the rabbits. Cultured epithelial allograft from the rabbits and xenograft from the rat were sprayed onto the freshly created wounds along with fibrin using the Tissomat device (Baxter, Austria). After four weeks, the rabbits were euthanized and skin biopsies were taken from each healed wound and subjected to histological evaluation using Haematoxylin and Eosin staining, immunohistochemical staining and also histochemical staining. The result from this study revealed that both graft showed favorable healing outcomes and healed without graft rejection. The scars that formed after the wound healed also desirable and healed with less scar formation. The immunological evaluation by flow cytometry analysis support the expected outcome of the study by showing there was no immunological rejection occurred. In

conclusion, the primary epidermal allograft and xenograft have the potential to be used as askin substitute to replace skin defects. This can overcome the limitation of autologous skin donor site especially in burn cases.

CHAPTER 1

INTRODUCTION

1.1 Rationale of the study

Epidermis has very important functions such as protecting the organism against environment hazards e.g., microbes and stress, and keeping fluids inside the body (Jensen *et al*., 2003). Although skin grafting has evolved into an essential component of reconstructive surgery, when larger parts of the body are lost, skin replacement remains a challenging task (Adams & Ramsey., 2005).The limited availability of autologous normal skin donor site for grafting has therefore been in the focus of research. Hence, temporary and permanent skin substitutes have been developed to avoid pain and potential complications of harvesting. Hence, this animal study was conducted to investigate the full thickness wound healing potential using primary epidermal allograft and xenograft to overcome this problem. The purpose of this study was to compare the different grafts as to which is better to be used as skin substitute based on histological and immunological characteristics after the wound healed following transplantation onto the wound. This animal study is an important tool as a stepping stone for clinical studies on humans as the results of animal study can be extrapolated before being tested on humans. According to a study by Eaglestein *et al.*, (1997), skin substitutes from allogeneic origin was not rejected, because the Langerhans cells, which play a major immunologic role, are lost during serial culture of keratinocytes (Eaglestein *et al.*, 1997). The sustainability of an off-shelf skin substitute could be tested by looking at the features of the scars after the wound healed. Studies have shown that the resultant scar

after transplantation with cultures of keratinocytes was favourable in comparison to those wound not treated by cultured cells (Atiyeh & Costagliola, 2007). It is expected that both grafts allograft and xenograft, have the potential to re-epithelialise. However, allograft is more suitable to be used as skin substitute. Meanwhile, the preparation of xenografts is more cost effective and donors are more readily available. This gives it an added advantage when compared to allografts for use in human patients. A skin substitute could be filed for the off the shelf skin replacement which would benefit numerous patients as it can cut down the waiting time for wound healing.

1.2 General objective

To investigate the full thickness wound healing potential using primary epidermal allografts versus xenografts in rabbits (*Oryctolagus cuniculus*).

1.3 Specific objectives

1.3.1 To isolate and characterize primary epidermal keratinocytes from rabbits (*Oryctolagus cuniculus*) and rats (*Rattus norvegicus*).

1.3.2 To evaluate histologically the full thickness wound healing potential of transplanted epidermal allograft and xenografts using keratinocyte specific markers.

1.3.3 To study the scarring formation by measuring the collagen accumulation of the transplanted grafts by using special stain (Masson-Trichrome).

1.3.4 To evaluate the immunoreactivity of the transplanted grafts using flow cytometry.

1.4 Hypothesis

Primary epidermal allograft from rabbits have better full thickness wound healing potential than primary epidermal xenograft and there will be no rejection in allograft and xenograft that is transplanted.

CHAPTER 2

LITERATURE REVIEW

2.1 Structure and function of the Skin

The skin covers the external surface of the human body. It is the largest organ of the body in surface area and weight. In adult, the skin occupies an area of 1.5 to 2 m^2 and total weight of skin can reach 20 kg, about 16% of total body weight. It varies in thickness from 0.3 to several cm in thickness. The thinnest sites are the eyelids (a few cells thick) and scrotum. The thickest are the soles and palms because they contain stratum lucidum the extra layer of the epidermis (Yousef *et al*., 2017)). Human skin layers consist of two main parts; epidermis and dermis (Figure 2.1). The outer superficial, thinner portion is the epidermis, which is composed of epithelial tissue that contains dead cells that are continually shed off. The deeper, thicker underlying part consist of connective tissue is the dermis. The basement membrane zone is at the junction of the dermis and epidermis. Beneath the skin is a variable amount of subcutaneous fatty tissue called hypodermis (Tortora & Grabowski, 2003). The skin is the first protective part of the body that help avoid damage to the body from outside influences. The skin protects the body from water loss and from injury due to bumps, chemicals, sunlight or microorganisms (germs). It helps to control body temperature and become a sensor to inform the brain of changes in the immediate environment. It also synthesizes vitamin D from the sunlight.

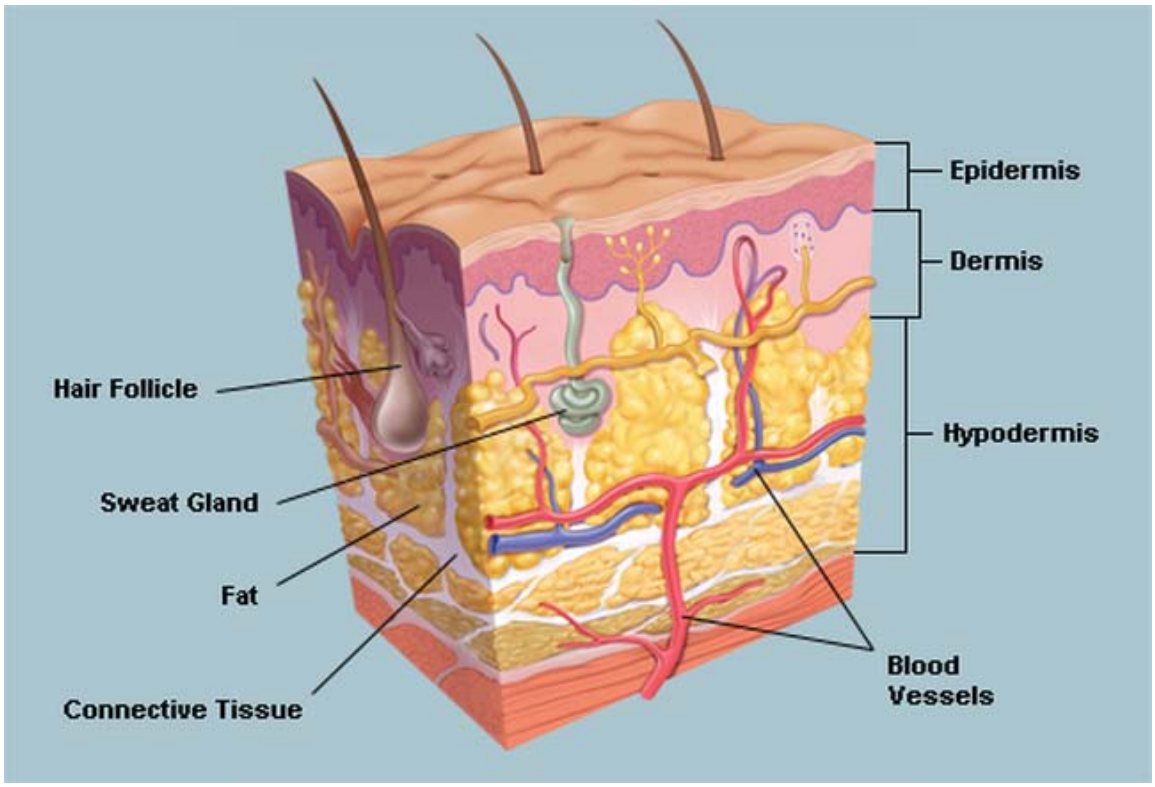

Figure 2.1 Structure of human skin

Skin structure showing the layers of epidermis, dermis and hypodermis. Epidermis is the outermost layer that provides waterproof barrier and give our skin tone. The second layer, dermis consists of tough connective tissues, hair follicle and also sweat glands. The deeper layer is the hypodermis (subcutaneous tissue) where it contains fat and connective tissues. Adapted from Tortora & Grabowski, (2003).

2.1.1 Epidermis

The epidermis plays an impressive role in protecting the organism against surroundings threat, e.g., microbes and stress and retain the fluids inside the body (Jensen *et al.*, 2003). It has an undulating surface with cross-crossing ridges and valleys, with invaginations due to follicles and sweat duct ostia. The epidermis contains four principal types of cells; keratinocytes, melanocytes, Langerhans cells and Merkel cells.

The keratinocytes make up about 90% of the epidermal cells. Meanwhile, the melanocytes constitute about 8% of the epidermal cells, which produce the brown-black pigment melanin that contribute to skin colour. Melanin is important in protecting the skin damaged from ultraviolet (UV) light. The Langerhans cells constitute a small fraction of the epidermal cells. It plays an important role in triggering immune responses against microbes that invades the skin. The Merkel cells are outnumbered from the others, located in the deepest layer of the epidermis. It connects with the tactile disc to detect variable touch sensations (Tortora & Grabowski, 2003).

The epidermis is composed of four layers in most regions of the body (Figure 2.2), while five layers in the regions where it is exposed to rigid fractions such as fingertips, palms and soles. The outermost layer is the stratum corneum, which consists of layers of flattened dead keratinocytes. The second layer stratum lucidum is found only in the thick skin. The middle is stratum granulosum, where a protein called keratohyalin is found. It is composed of three to five layers of flattened keratinocytes that undergo apoptosis. The stratum spinosum consists of 8 to 10 layers of keratinocytes packed closely together. In this layer, the Langerhans cells and the melanocytes are found. The deepest layer is the stratum basale. It is arranged in a single row of cuboidal or columnar keratinocytes, composed mainly of proliferating and non-proliferating keratinocytes. Some cells are the stem cells that play important roles in producing new keratinocytes through cell division.

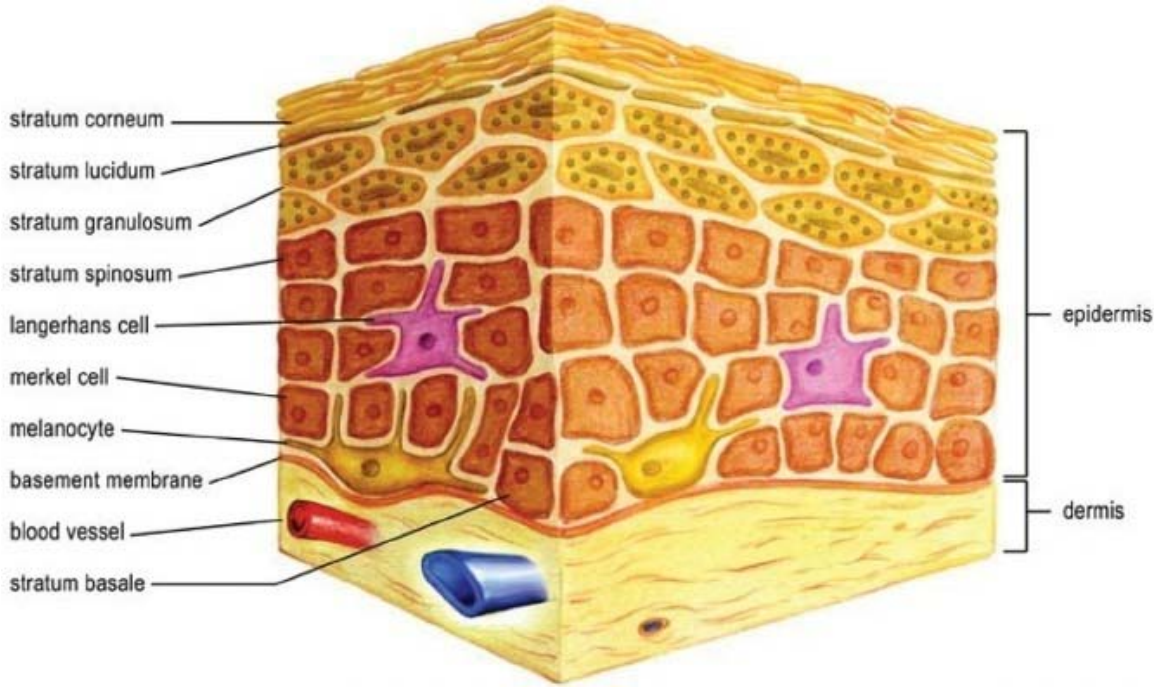

Figure 2.2 The epidermal layer

The epidermis consists of five layers that are stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale. This figure was adapted from Farage *et al.*, (2007).

2.1.2 Keratinocytes

About 95% of epidermal cells are the keratinocytes that construct the skin surface and apparently regenerate in a month. The pace of the skin regeneration will be higher if the epidermis is wounded or being invaded by certain skin infection. Keratinocytes can be isolated from skin biopsies and are able to undergo expansion *in vitro* which can be used in patients with deep dermal burns as cultured epidermal autograft (CEA) (Dedovic *et al*., 1998).

In wound healing condition, the keratinocytes are triggered and turn into hyper proliferative cells which secrete extracellular matrix components and signalling polypeptides (Freedberg *et al*., 2001). Keratinocytes form in the deep basal layer of the skin and progressively move upwards. First, it forms squamous cells, then flattens out and differentiates towards the anuclear horny cell of the stratum corneum, the top layer of the epidermis. The cells are flat and scale-like shape (squamous) and contain keratin. The keratins are arranged in overlapping layers so that it is thick and tough and acts as waterproof for the skin surface (Rheinwald & Green, 1975). An early observation in the field of skin biology was that the epidermal keratinocytes could be grown in culture. As opposed to many other cell types that require transformation to be cultured effectively, epithelial cells taken directly from the skin can be passaged for many generations when cultured in the presence of a fibroblast feeder layer (Islam & Zhou, 2007).

2.2 Overview of wound healing

Wound healing is a complex and dynamic process that can last for days and even months (Sagliyan *et al*., 2012). It is a complicated process formed by migration and proliferation of cells such as fibroblasts, endothelium and epithelial cells as well as migration of cells originating from bloods such as thrombocytes, macrophages and neutrophils (Germain *et al*., 1994; Hunt *et al*., 2000, Mohammad *et al*., 2008 Mutsaers *et al*., 1997). Analysing the events of normal wound healing is a necessary prerequisite for understanding the pathological processes. Normal wound healing occurs in three phases (Figure 3). First phase is where haemostasis and inflammation occurs, followed by

fibroplasia and proliferation or cellular phase and lastly the maturation and remodelling phase (Abu Al-Basal, 2010; Emami *et al*., 2006; Gunay *et al*., 2005).

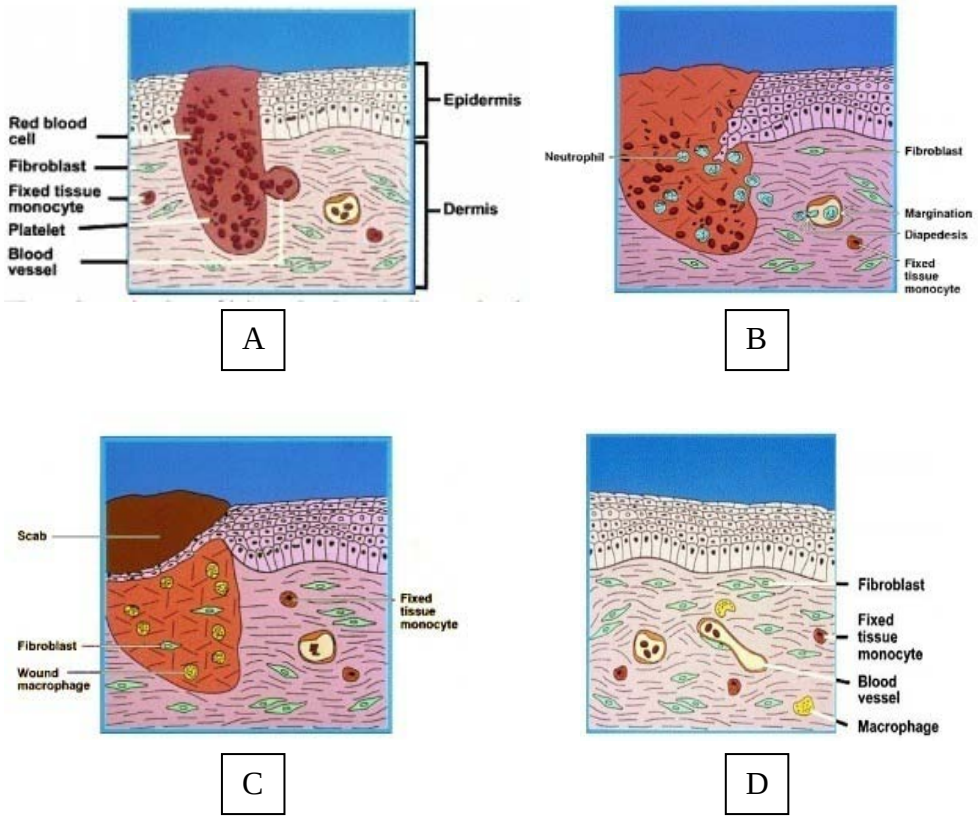

Figure 2.3 Wound healing process

(A) Process when the injury occurs, the tissue disrupted and the blood clot formed at the wound site. (B) First day following the injury, where the inflammation phase started. The migration of the neutrophil to the wound site attached with the endothelial cells and changed shape to move through the cell junction. (C) Activated macrophages will move to the wound site along with the fibroblast thus beginning the proliferative phase. (D) Remodelling phase where the extracellular matrix components were continued to be synthesised and degraded until new equilibrium is initiated. Adapted from Diegelmann & Evans, (2004).

2.2.1 Inflammation phase

The inflammation phase is the first stage in wound healing. It is the body's natural response to trauma. After the injury occurred, a blood clot is formed at the wound site and loosely merges the wound edges. During this phase, vascular and cellular responses will be triggered that involves in eliminating the bacteria, microbes, foreign material and also dying tissue in preparation of skin repair. This will lead in rising of exudate levels and appearance of maceration surrounding the wound area. At this stage, the characteristics sign of inflammation can be seen, such as erythema, heat and redness, oedema, pain and functional disturbance (Koh & DiPietro, 2011).

2.2.2 Proliferation phase

The second wound healing stage, proliferation, is where the wound starts to be rebuilt with new, healthy granulation tissue. Epithelialisation, fibroplasia, and angiogenesis occur during the proliferative phase. The mixture of extracellular matrix components with fibroblasts and also collagen helps to strengthen the wound site. This process happened after 3, 4 days of the injury. The wound continues to grow stronger as the fibroblasts repeatedly being organized to aid in the development of the new tissue and accelerate the healing process. The colour of the granulation tissue is an indicator of the health of the wound whether it is healthy (reddish or pinkish colour) or had been contaminated or inadequate blood delivery to the wound bed (dark brown colour). The blood vessels must receive a sufficient supply of nutrients and oxygen to aid in the wound healing process called the angiogenesis process (Hunt *et al*., 2000).

2.2.3 Remodelling phase

This process is the longest process in wound healing where it lasts from 21 days to even a year or longer. At this stage, the fibroblast will transform to myofibroblast and bind to the extracellular matrix components to form linkages. The wound begins to contract and collagen level start to be produced. Meanwhile, the extracellular matrix components continue to be synthesised and degrade until the equilibrium of the wound is reached. The epidermal cells will grow over the connective tissue to close the wound (Young & McNaught, 2011).

2.2.4 Scar formation

The repair of wounds in adult skin is comprised of an intricate series of overlapping phases, including inflammatory, proliferative, and scar production/remodelling phases (Singer & Clark, 1999). The ultimate result of the repair process in adults is a scar, an overproduction of disorganized collagen, which acts to patch the break in the tissue (Ehrlich, 2016). Although the intention of scar tissue production during the wound healing process is to increase the tensile strength of the skin in the wound area, the strength of scar tissue never surpasses 70% the strength of the original tissue (Levenson *et al*., 1965). There are two types of scars that form after the wound healed, that is the hypertrophic scar, which remain under boundaries of original wound and the keloid scar, which extends beyond boundaries of the original wound. In addition to being weaker than normal skin, excessive scar tissue can lead to a number of serious problems by impairing skin function in the area where it is present. Added to the psychosocial consequences of disfigurement that arises in patients with

large scars on exposed areas, severe scarring as a result of burns, for instance, can lead to restricted joint movement, while scarring following surgeries to repair cleft lips and palates in children can lead to impaired midfacial growth (Ross, 1987; Tredget *et al*., 1997). Abnormal scar formation is also the source of fibrotic diseases such as keloids and hypertrophic scars that occur in the skin (English & Shenefelt, 1999; Brissett & Sherris, 2001).

2.3 Skin grafting

Skin graft is a piece of living skin that is fixed or patched onto damaged part of a body in a surgery (Oxford, 2012), while skin grafting is the surgical procedures that involves removing, transferring and transplanting the skin to other sites of the body. Healthy skin is taken from any area of the body, especially buttock or inner thigh where it is hidden by clothes. This surgery is done due to the excessive loss of skin due to trauma, skin infections, burns, bed sores or ulcers on the skin that would not heal well for prolonged time. There are two types of skin grafting that are, split-level thickness graft and full-thickness skin graft. Split-level thickness graft is where it involves removal of two top layers of the skin that is epidermis and dermis, while, full-thickness skin graft is the addition of removal of muscle and blood vessels along with the epidermis and dermis. There are 3 types of grafts that are autograft, allograft and xenograft. Autograft is the tissue or organ that being taken from the same donor, but in different part of the body. Allograft is the tissue or organ from a donor to another recipient of the same species with a different genotype. Meanwhile, xenograft is the tissue or organ that is taken from donor from different species than the recipient, for

example the skin sample taken from monkey to transplant on human. Previous study by Cheng *et al*., (2017) has stated that murine full thickness skin transplantation is a well-established *in vivo* model to study alloimmune response and graft rejection. The wound healing study also had been done using different types of animals such as athymic mice and rats, hamster, guinea pigs, rabbits and also porcine model according to the scientific artifices (Ramos *et al*., 2008). Over the years, skin grafting had been the focus in handling wide variety of skin problems, but problem occur in handling extensive burns where there is lack availability of donor sites from which autografts skin was taken (Hefton *et al*., 1983). In order to overcome this problem, researchers have tried to use autologous cultured epidermal cells. The result showed that the grafted cultured epithelial subsist *in vivo* and retrieve an epidermal structure same as healed autografts (O'Connor *et al*., 1981).

2.4 Graft rejection

Graft or transplant rejection is a process that occurred when the recipient's immune systems strike the transplanted tissue or organ (Pradue & Corosella, 2006).It is known as the immunological destruction of transplanted organs or tissues. The rejection might happen in two set process. Firstly, it occurs within 10 days after transplant and secondly, within one week after the second graft with same antigenicity as the first is being transplanted (Kanitakis *et al*., 2003). The immune system is the best defensive mechanism that involves in combating the foreign agents. Graft rejection may be based on cell-mediated and antibody- mediated immunity intervene between cells of the graft by histoincompatible recipient. Skin graft rejections might occur associated with an

impressive inflammatory immune response leading to the abolishing of the donor cells (Benichou *et al*., 2011). The studies of immunological mechanisms that involve in allogeneic graft rejection were developed from the research of skin transplantation. The fundamental studies of skin grafting were performed during wartime, first in humans (Billingham & Medawar, 1951)and then in animals (Billingham *et al*., 1953). The main problem in skin grafting was the graft rejection. This problem had become the stepping stone in cell culture studies to overcome the rejection that occurred after the graft transplant.

2.5 Culture of skin epithelial cells

Cell culture is the technique where the researcher removes, grows and maintains cells under controlled condition in favourable artificial environment outside of a living animal or so called *in vitro* (Carter & Shieh, 2015). Before the cell is cultivated, it might be removed from the tissue directly and maintained by enzymatic or mechanical environment, or it might be derived from cell line that has already been established. However, cell culture studies are only possible because of the research by Harisson in 1907. He developed an *in vitro* method to restrain live cells in laboratory (Breslin & O'Driscoll, 2013). The cells segregated from donors, animals or plants are known as the primary cells. Primary cell culture specifies the stage after the cells are isolated from the tissue and proliferated under appropriate conditions until it reach confluence. The cells are sub cultured until it is ready to be used for next process by changing the vessel with fresh growth medium to afford more room for the cells to grow. In the past three decades, the skin epithelial cells had been successfully cultured. The cells cultured retain

the normal skin epithelial cell characteristics and properties *in vivo* (Karasek, 1965). Autologous cultured epidermal cells have been used in studies to increase the availability of material for grafting (O'Connor *et al*., 1981). From the studies, they found that the epidermal structure of the grafted cultured epithelia survived *in vivo* and similar to that seen with healed autografts.

2.6 Animal model for full thickness wound healing

Wound healing is complicated processes that occurs as a chain of events. Therefore, the use of animal and cellular models is very important to analyse this situation and it has even been reported that the skin wound healing between experimental animals and humans had some significant difference (Graham, 2004). Researchers have used animal model to study the new method, protocol and progress of diseases. The effectiveness of new developed drug or treatment are also being tested on animal model to know how safe it is before being used or tested in humans. There are a few guidelines for use of animal in scientific research. In our institution, Universiti Sains Malaysia (USM) also has these guidelines when handling animal studies. All experiments should incorporate the 3Rs: replacement, reduction and refinement (Russell *et al*., 1959) when conducting the procedures and protocol. This is done to ensure that the researchers follow the good scientific and ethical practices for acceptance of the positive result from the experimental studies (Smith *et al.*, 2018).

Animal model is needed to test the scientific hypotheses by comparing what is expected to occur with what actually occurs (Shanks *et al*., 2009). In this regard, animal and cellular models are very useful although they have significant differences in skin

wound healing compared to humans (Graham, 2004). In this study, rabbit model was used. Rabbits are significantly larger than the commonly used laboratory animals such as, mice and rats, but remain easy to handle and inexpensive (Ramos *et al*., 2008). The rabbit skin is formed in two parts that are epidermis and dermis. The epidermis is very thin and formed in three layers consisting of stratum basale, stratum spinosum and stratum corneum (Yagci *et al*., 2006). The rabbit represents an excellent animal model for the study of rejection responses to cultured keratinocyte grafts transplant for three reasons. First, the small size enables large numbers of subjects to be practically and inexpensively housed for prolonged periods. Second, a high fecundity and rapid sexual maturation allows for the development of inbred strains. Third, the rabbit's major histocompatibility complex (MHC) displays a higher degree of homology with human MHC than the murine or canine models (Breidahl *et al*., 1990).

With regards to human clinical studies, animal model gave added advantages by allowing the study of post- graft in small intervals without limitations of the quantity of the biopsy. Many involved aspects of the wound healing process in skin regeneration of cultured keratinocytes graft also could be controlled and monitored by using the animal models (Gragnani *et al*., 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and reagents

3.1.1 Skin sample

Skin samples from 1 adult male New Zealand White rabbit and 1 adult male Sprague Dawley rat were excised from the dorsum site of the animals under sterile conditions.

3.1.2 Chemical and reagents

1. Pre-anaesthetic medication; atropine (1mg/kg i/m) xylazine (5mg/kg i/m) and ketamine (35mg/kg i/m)

   It is the drug that is usually administered prior to induction of anaesthesia for sedation if the animal is to be operated under the effect of local or regional analgesia, or for smooth induction and smooth recovery from general anaesthesia, and reduction of the anaesthetic dose.

2. Povidone iodine

   Povidone-iodine is a broad-spectrum antiseptic for topical application in the treatment and prevention of infection in wounds.

3. CnT-57 (CELLnTEC, Switzerland)

   Formulated for isolation and proliferation of mouse and human keratinocytes. CnT-57 is a BPE-containing keratinocyte medium. CnT-57 is a liquid culture medium kit including both basal medium and supplements. It does not contain

antibiotics/antimycotics. The basal medium contains amino acids, minerals, vitamins and organic compounds, but is protein free.

4. CnT-03 (CELLnTEC, Switzerland)

   CnT- 03 is formulated for rat epidermal keratinocytes and vaginal epithelium cells. CnT- 03 is a progenitor cell targeted liquid culture medium kit including both basal medium and supplements. CnT-03 is a fully defined formulation. It does not contain antibiotics-antimycotics. The basal medium contains amino acids, minerals, vitamins and organic compounds, but is protein free.

5. Dulbecco's Modified Eagle Medium (DMEM) (Gibco®Life Technologies, USA)

   This basal medium contains 4500mg/mL glucose, L-glutamine and pyridoxine hydrochloride for supporting the growth of various mammalian cells.

6. 1 x Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco®Life Technologies, USA)

   DPBS is used to provide a buffer system to maintain cell culture media in the physiological pH range, or to irrigate, transport, or dilute fluid while maintaining cell tonicity and viability.

7. Antibiotic-antimycotic (Gibco®Life Technologies, USA)

   This solution contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin and 25 µg/mL of Fungizone®. The antibiotics penicillin and streptomycin prevent bacterial contamination of cell cultures due to their effective combined action against gram-positive and gram-negative bacteria. Fungizone® (amphotericin B) prevents fungal contamination of cell cultures due to its inhibition of multi-cellular fungus and yeast.

8. Dispase (Gibco® Life Technologies, USA).

Dispase, or neutral protease, is a metalloenzyme produced by Bacillus Polymyxa which has been classified as an amino-endopeptidase. This enzyme has proven to be a rapid and gentle agent for the harvest and transfer of normal diploid cells and cell lines. In addition, dispase has been shown to effectively separate cell clumps, as well as cells from intact tissue without significantly affecting cell membrane integrity or viability.

9. Trypsin, Trypsin EDTA (Gibco® Life Technologies, USA)

Trypsin solutions are used widely for dissociation of tissues and cell monolayers. The biochemical assays performed on trypsin 1:250 determine both trypsin-specific activity at the level of certain co-purified enzymes that influence cell removal and viability. All trypsin solutions are prepared from porcine parvovirus-tested and mycoplasma-tested materials.

10. TrypLE™ Express (Invitrogen™, USA)

TrypLE™ reagents are highly purified, recombinant cell-dissociation enzymes that replace porcine trypsin. These reagents are ideal for dissociating attachment-dependent cell lines in both serum-containing and serum-free conditions, and can be directly substituted for trypsin without protocol changes.

11. Dimethyl sulphoxide (DMSO) (Sigma-Aldrich®, USA)

DMSO is a polar aprotic solvent used in chemical reactions, in polymerase chain reactions (PCR) and as a cryoprotectant vitrification agent for the preservation of cells, tissues and organs. DMSO is used in cell freezing media to protect cells from ice crystal induced mechanical injury. It is used for frozen storage of

primary, sub-cultured, and recombinant heteroploid and hybridoma cell lines; embryonic stem cells (ESCs), and hematopoietic stem cells. DMSO is frequently used in the combinations with BSA or foetal bovine serum (FBS).

12. Foetal bovine serum (FBS) (Gibco® Life Technologies, USA)

    Foetal Bovine Serum (FBS) is the most widely used growth supplement for cell culture media due to its high content of embryonic growth promoting factors.

13. Tris-buffered saline (1X TBS) (Thermo-Scientific™, USA)

    TBS is supplied as a lyophilized powder, and when dissolved in one litre distilled or deionised water, it becomes 0.05M TBS, pH 8.0 (0.238M NaCl; 0.0027M KCl) with 0.05% Tween 20.

14. Cold methanol (Sigma-Aldrich®, USA)

    Cold methanol issued for fixation and permeabilization of cells in order to ensure free access of the antibody to its antigen.

15. IHC Select® HRP/DAB Kit (Chemicon® Merck, USA)

    This kit contains 1X washing solution, blocking reagent, Streptavidin-HRP Conjugate, Chromogen, Counterstain reagents.

16. Absolute alcohol

    Absolute alcohol is a common name for the chemical compound ethanol. Ethanol is a colourless liquid with molecular formula C_2H_5OH.

17. Hydrogen Peroxide (Sigma-Aldrich®, USA)

    Hydrogen peroxide (H_2O_2) is used as a bleach, disinfectant, to make propellants, and as an oxidizer.

18. DakoCytomation LSAB+ System-HRP Kit (Dako, USA)

These kits are for use with both rabbit and mouse primary antibodies. The biotinylated link antibody in the kits is produced in goat. No blocking step for reducing background staining caused by protein-protein interaction is required, the enzyme-conjugated streptavidin is provided in prediluted form.

3.1.3 Antibodies

1. Pan-Cytokeratin, PCK-26 (Abcam, Germany)
2. Biotinylated Goat anti-Mouse (Abcam, Germany)
3. Anti-pan Cytokeratin antibody [C-11] (Abcam, Germany)

3.1.4 Wound dressing

1. Mepilex® (Molnlycke, Sweden)

   Mepilex is a soft and conformable foam dressing for a wide range of acute and chronic wounds. Mepilex minimizes pain and wound or skin damage at dressing change. Mepilex can be cut to fit making it ideal for difficult to dress areas.

2. 3M Coban Self-Adherent Bandage (3M, United States)

   A self-adherent elastic wrap that functions like a tape, but sticks only to itself. Available in sterile and nonsterile styles, and in a variety of widths and colours to meet application needs.

3.2 Methodology

3.2.1 Experimental animals

Nine adult male New Zealand White rabbits were used in this study with one used as the subjects for harvesting skin sample for allograft. Another eight were divided into two groups, four for allografts and another four for xenografts. The rabbits were aged between 6months to 1 year and weighed between 2-3 kg. Meanwhile, one Sprague Dawley rat aged 3 months with weight of about 250gram was used as subjects for harvesting xenograft skin sample.

3.2.2 Ethical approval

This study was approved by the Animal Ethics Committee, Universiti Sains Malaysia (USM/ Animal Ethics Approval/ 2010/ (54) (192) (Appendix A).

3.2.3 Harvesting of skin sample

The experiment was conducted in the Animal Research and Service Centre (ARASC) USM, Health Campus. Skin samples from one adult male New Zealand White rabbit and one adult male Sprague Dawley rat were taken under sterile conditions. The animals were given a pre-anaesthetic dose of atropine (1mg/kg i/m) and xylazine (5mg/kg i/m) and later anaesthetized using ketamine (35mg/kg i/m). The hairs on the dorsum of the animals were clipped using an electrical clipper and coated with 10% povidone iodine as an antiseptic. Full thickness skin biopsies in the size range of 2cm x 2cm were harvested under sterile techniques on the dorsum of the animals. The skin sample harvested from the rabbits and rats were placed in the transport medium

comprising of CnT Basal Medium and 1% antibiotic-antimycotic solution. This has been shown in Figure 4. The skin biopsies were taken to the laboratory for keratinocyte cell culture (which will act as allografts for the rabbits and xenograft for the rats). The animals were then euthanized according to the animal ethics standard protocol.

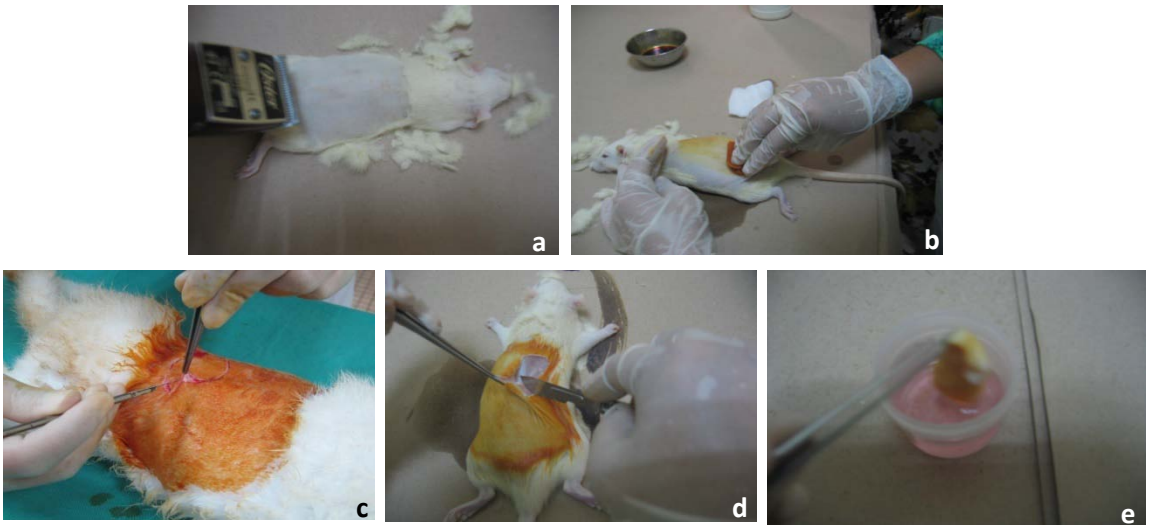

Figure 3.1 Harvesting skin sample
(a) Shaving the dorsum of rabbit and rat (b) Cleaning the wound site on the dorsum of rabbit and rat. (c) Incision of the skin. (d) Harvesting the skin sample. (e) Transporting the skin sample in transport media before processing.

3.2.4 Isolation and characterisation of primary epidermal keratinocytes

3.2.4(a) Isolation of rabbit and rat primary epidermal keratinocytes

Optimization of the isolation of rabbit and rat primary epidermal keratinocytes was done until the desirable result was established. The rabbit and rat skin biopsy specimens were transferred into a petri dish containing 70% ethanol and washed for 3 min (washing too long in 70% ethanol causes cell death) to wash away the blood

followed by a wash in DPBS into 15ml centrifuge tube for 5 min. Then, the skin were transferred into clean petri dish containing CnT Basal Medium and minced into small pieces using scalpel and blades. The minced pieces were placed in a centrifuge tube containing 0.6% dispase and 1% antibiotic-antimycotic solution overnight at 4°C. After overnight in dispase, the specimen was transferred to a new petri dish for separating the epidermis layer and dermis layer. Then, the epidermis was transferred into 50ml centrifuge tube. 10ml of pre-warmed Trypsin solution was added and the specimens were incubated at 37°C for 10-15 min. Later, 10ml solution of DMEM with 10% FBS was added to deactivate the enzyme trypsin activities. The specimens were then carefully pipetted and filtered using 70 µm nylon cell strainer (BD Biosciences, USA) to separate the keratinocytes and the epidermis layer. The suspension of keratinocytes was then centrifuged at 1500 rpm for 5 min. The supernatant was aspirated and fresh keratinocyte medium, CnT-57for rabbit keratinocytes and CnT-03 for rat keratinocytes were added to the cell pellet and gently re-suspended. The cells were counted using haemocytometer and the cell viability was estimated using 1% trypan blue. The viable cells were cultured at 4×10^4 cells/cm^2 in T-25 culture flasks (Nunc, Australia). The cells were incubated at 37°C and 5% CO_2 prior to use or cryopreserved in liquid nitrogen for longer time storage. The cell morphology of the keratinocytes was analysed using inverted microscope (Leica Microsystem, Germany).