STUDY ON THE EFFECTS OF

NATURAL CREAMS INFUSED WITH SEA CUCUMBER IN THE ABRASION WOUND HEALING OF SPRAGUE DAWLEY RATS

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UNIVERSITI SAINS MALAYSIA

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by

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for the degree of
Master of Science

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DECLARATION

I hereby declare that I am the sole author of this thesis in title "Study on the Effects of Natural Creams Infused With Sea Cucumber in the Abrasion Wound Healing of *Sprague Dawley Rats*". I declare that the thesis is being submitted to Universiti Sains Malaysia (USM) for the purpose of the award of Master of Science in Medical Research. This thesis is the result of my own research under supervision of Dr. Jahangir bin Kamaldin except as cited in the references. The thesis has being accepted for the respective study and is not concurrently submitted in candidature of any other degree.

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	Analysis of variance
DEET	N , N-dietyl-m-toluamide
EPA	Eicosapentaenoic acid
NPCB	National Pharmaceutical Control Bereau
OECD	Economic Cooperation and Development
PMN	Polymorphonuclear neutrophil
PDGF	Platelet Derived Growth Factor
TGF-β	Transforming Growth Factor
NPCB	National Pharmaceutical Control Bereau
ECM	Epithelial Cell Migration
cm	Centimeter
BC	Base cream formulation containing excipient only
BCG	Base cream infused with 1% w/w sea cucumber powder
BCGD	Base cream infused with 1% w/w sea cucumber powder and 12% w/w DEET

ABSTRACT

The study aims to evaluate the effects of the healing process on the abrasion wound by base natural cream formulation (BC), sea cucumber infusion in BC named as BCG formulation and presence of DEET in the BCGD formulation in comparison to normal saline which is a standard antiseptic treatment solution for abrasion wound. There is much lacking of data on efficacy of products that based on infusion of dried tissue of sea cucumber. For the topical treatment on skin, the study employed method described in the OECD Test Guideline 402, Acute Dermal Toxicity Test. Whereas, for the abrasion wounding and dosing, it was based on published method by Bhagavathula *et al.*, (2009) and Lateef *et al*., (2005). The 10 days of post treatment observation found that any of the three cream formulation namely as BC, BCG and BCGD or the standard normal saline treatment resulted complete wound healing process without any inflammation or infection within the 10 days of post treatment observation. The complete abrasion wound healing process using the Sprague Dawley Rat model can be visually can be segregated into five phases which are reddish-phase, opaque-phase, sticky-phase, dry-phase and hairy-phase. Overall, at the end of tenth day, all the four treatments namely standard normal saline solution, creams of BC, BCG and BCGD showed no significant difference in inducing the complete skin epithelization of the abraded wound ($P \geq 0.05$). Nevertheless, comparing the three formulations, the wound treated with BCG cream led to significantly ($P<0.05$) faster complete epithelization by day-4 post treatment. Whereas, in term of hair budding, among the three cream formulations, only BCG cream showed adequate hair budding by day 6 on par with the standard normal saline without significant difference (P

≥ 0.05). The presence of DEET in the BCGD cream significantly ($P < 0.05$) delayed the hair budding process but eventually resolved by day-8. Overall, the study suggest that the infusion of 1% w/w sea cucumber powder alone in the cream formulation has improved the abraded wound healing process by speeding the skin epithelization and encouraging the hair budding processes within the 10 days of post treatment.

ABSTRAK

Kajian ini bertujuan untuk mengkaji kesan proses penyembuhan luka lelasan menggunakan rawatan krim asas, krim asas yang mengandungi gamat dan krim asas yang mengandungi gamat beserta DEET berbanding rawatan mengguna cecair salina yang merupakan rawatan antiseptik standard dalam penyembuhan luka lelasan. Data mengenai keberkesanan produk gamat yang berasaskan gamat kering adalah sangat terhad. Untuk proses menyapu krim pada kulit tikus kaedah kajian yang digunakan mengikut Garis Panduan Ujian OECD 402, Akut Dermal Ketoksikan Ujian. Manakala proses luka lelasan dan dos berdasarkan kaedah yang diterbitkan oleh Bhagavathula *et al.*, (2009) and Lateef *et al*., (2005). Selepas pemerhatian selama 10 hari selepas rawatan, kajian mendapati bahawa semua krim yang terpilih yang dinamakan sebagai BC, BCG dan BCGD atau rawatan menggunakan cecair salina mampu menyembuhkan luka sepenuhnya tanpa menyebabkan sebarang keradangan atau jangkitan dalam tempoh 10 hari pemerhatian selepas rawatan. Proses penyembuhan luka lelasan ke atas tikus Sprague Dawley boleh dibahagikan kepada lima fasa iaitu fasa-merah, fasa-legap, fasa-melekit, fasa-kering dan berbulu. Secara keseluruhan, pada akhir tempoh pemerhatian, kesemua empat rawatan iaitu cecair salina, krim BC, BCG dan BCGD menunjukkan tiada perbezaan yang signifikan dalam proses peghasilan kulit ($P \geq 0.05$). Walau bagaimanapun, membandingkan tiga rumusan krim tersebut, luka yang dirawat dengan krim BCG menunjukkan penghasilan kulit yang ketara ($P < 0.05$) iaitu pada hari yang keempat selepas rawatan. Manakala, untuk proses penunasan rambut, hanya BCG krim menunjukkan penunasan rambut yang mencukupi iaitu pada hari yang keenam selepas rawatan setanding dengan rawatan menggunakan cecair salina tanpa perbezaan yang ketara ($P \geq 0.05$). Kehadiran DEET dalam krim BCGD menunjukkan penangguhan penunasan rambut yang ketara ($P < 0.05$)

namun mampu menunjukkan penunasan rambut pada hari yang ke 8 selepas rawatan. Secara keseluruhan, kajian menunjukkan bahawa penyerapan 1% serbuk gamat di dalam formulasi krim mampu mempercepatkan proses penyembuhan luka lelasan dengan mempercepatkan penghasilan kulit dan menggalakkan proses penunasan rambut dalam tempoh 10 hari selepas rawatan.

CHAPTER 1

INTRODUCTION

Skin is the biggest organ in the body which consist of dermis and epidermis layer. The dermis comprise of four layers which are stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Breitkreutz *et al.*, 2013; Mikesh *et al.*, 2013; Venus *et al.*, 2011). Skin is a physical barrier that provide a protection from environment by forming an efficient invasion barrier for exogenous molecules. It also performs several vital functions which include temperature regulation, protection of underlying structures, avoiding dehydration or water resistance, vitamin D production, sensation, immune function, barrier against pathogens, mating, attraction and appearance. Epidermis is the component parts that provides defence from the surroundings. The dermis is very permeable once the epidermis is absence (Venus *et al.*, 2011).

A wound is demarcated as damage to the normal anatomical structure and function. It range from a simple disruption in the epithelial integrity of the skin to a deeper extending into subcutaneous tissue (Dewangan *et al.*, 2012; Meenakshi *et al.*, 2006; Velnar *et al.*, 2009; Young & McNaught, 2011). Wound can be classified into acute and chronic wound according to the time taken for the healing process to complete (Majumdar, 2005; Velnar *et al.*, 2009).

Wound healing is a progression of restoration of skin and other soft tissues (Malviya & Jain, 2009; Nayak & Pereira, 2006). It is essential to have a correct and efficient wound healing management. Many efforts have been done with an emphasis

on new therapeutic approaches and the advance of technologies for acute and chronic wound management (Velnar *et al.*, 2009).

It is more difficult to manage chronic wound which when the healing process does not progress in a timely and orderly manner (Velnar *et al.*, 2009). After injury, an inflammatory response take place and the cells below the dermis activate collagen production in the connective tissue. Later, the epithelial tissue is regenerated. There are stages to the process of wound healing which are coagulation, inflammation, proliferation and remodelling (Guo & DiPietro, 2010; Nayak & Pereira, 2006; Rosana *et al.*, 2014; Velnar *et al.*, 2009).

Sea cucumber have been reported to have branched chain fatty acids in high levels believed to be responsible for the potential role of sea cucumber in wound healing effects (Zhong *et al.*, 2007). Besides that, sea cucumber have antimicrobial (Omran & Allam, 2013) . It also contain some active antioxidant substances such as phenolic compounds which is supposed to play a vital part in wound healing progression (Zhong *et al.*, 2007). Thus sea cucumber is effective in accelerating healing process as antioxidants promote healing and the prevention of microbial contamination of wounds facilitates repair. In Malaysia, sea cucumber or "gamat" is a traditional medicine and has been practiced to treat various conditions for 300 years (Barathi *et al.*, 2013) as well as for healing various internal and external wounds (Fredalina *et al.*, 1999). Ridzwan *et al.* (2003) also reported evidence indicating the sea cucumber capability accelerates wound healing.

Sea cucumbers are cylinder-shaped invertebrates that survive in a range of sea floor habitats from warm tropical waters to cold deep sea trenches (Zhong *et al.*, 2007). It was believed to be an essential food in the Indo-Pacific region including The Philippines, Malaysia, Japan, Korea, and China (Zhong *et al.*, 2007). China is the

leading sea cucumber producer worldwide with sea cucumber farming and ranching being a main part of its aquaculture industry (Zhong *et al.*, 2007). Especially the Chinese communities, Malaysians consider sea cucumber as a sea food delicacies. It is one of the essential sea products and sell in high prices. It is also used in traditional medicinal ointment manufactured goods named locally as 'Minyak Gamat' (Woo *et al.*, 2013).

Current preclinical efficacy data on wound healing is based on extracts of sea cucumber wet tissue (Barathi et al, 2013; Fredalina et al,1999). While, some of the wound healing product in the market is based on infusion of dried tissue of sea cucumber. However, there is much lacking of data on efficacy of such products. Besides that, here is an idea to produce a hybrid cream product that combine the benefit of skin healing using sea cucumber and the mosquito repellent based on DEET. Thus there is a need to determine its effect on wound healing. Moreover, the minimum standard practice of treating abrasion wound is by applying normal saline. Therefore a cream product infused with sea cucumber should at least performed on par with normal saline treatment.

The objectives of the study are:

a) To illustrate the healing activity of the abraded rat's skin after standard treatment with normal saline and selected cream formulation.

b) To determine the effect of excipient in the base natural cream formulation on healing process of abraded wound.

c) To determine the effect of sea cucumber infused in the base natural cream formulation on wound healing process of abraded wound.

d) To determine the effect of sea cucumber infused natural cream formulation containing DEET on healing process of abraded wound.

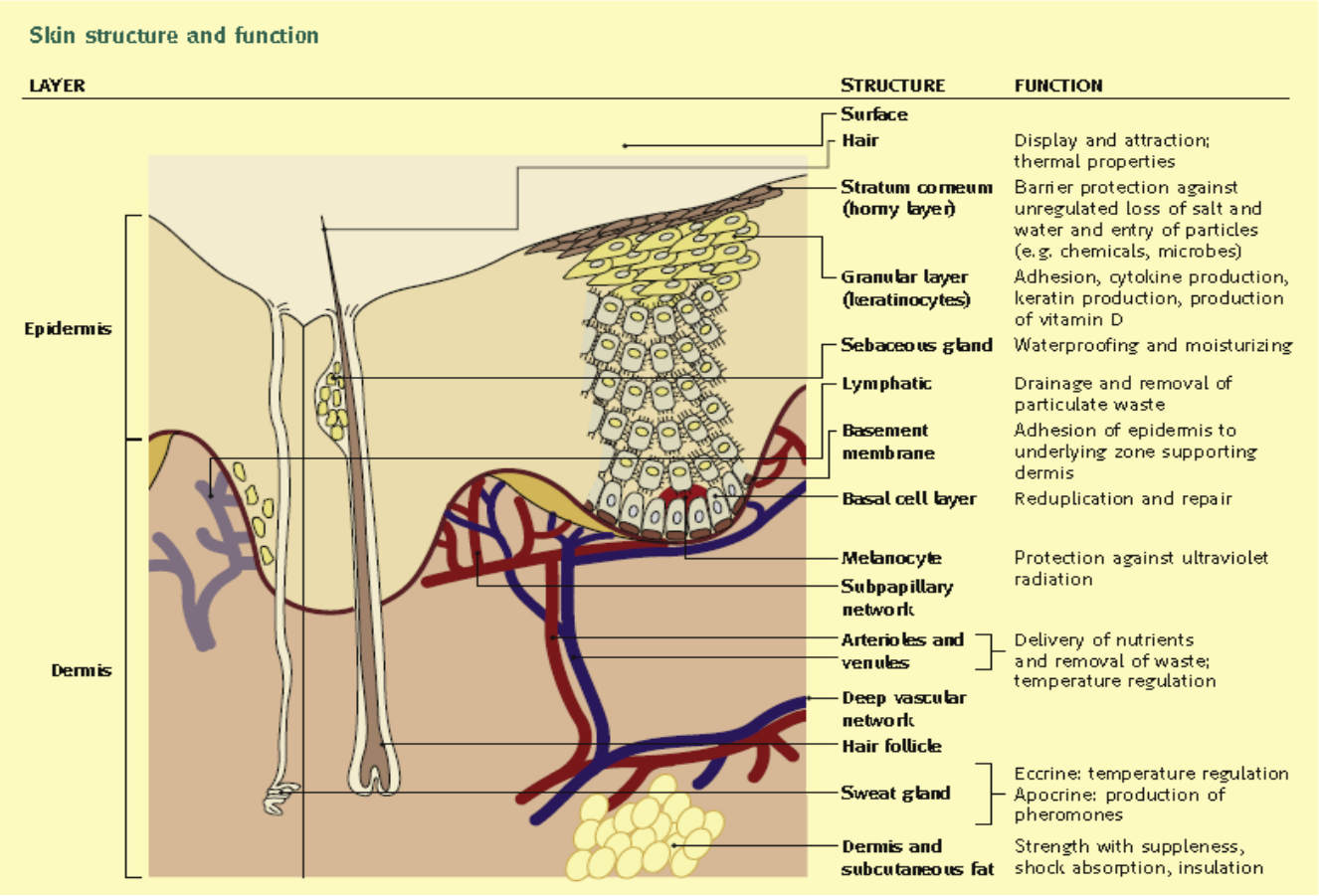
CHAPTER 2

LITERATURE REVIEW

2.1. Skin anatomy and physiology

The integument is said to be the biggest organ in the body covering 16% of total body weight considering about 5 kg with a surface area of 2 m^2 (Godin & Toutin, 2007; Venus *et al.*, 2011). It consists of two main layers which are epidermis and dermis (Breitkreutz *et al.*, 2013; Mikesh *et al.*, 2013; Venus *et al.*, 2011). In between the epidermis and dermal layer there is a basement membrane comprises of a thin layer of extracellular matrix. The dermal layer consists of two regions which are papillary dermis and reticular dermis. The papillary region is composed of thin collagen fibers and loose areola tissue whereas the reticular dermis is consist of thicker, denser collagen fibers, receptor, sweat glands, sebaceous glands and blood vessel (Mikesh *et al.*, 2013).

Epidermis represents the outer layer of an organism. It act as a protection barrier to penetration of foreign substance to the underlying vascular dermis (Venus *et al.*, 2011). The epidermis can be separated into four compartments namely stratum basale, stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Breitkreutz *et al.*, 2013; Venus *et al.*, 2011). Below are the description of each stratum as illustrated in Figure 2.1:



Source: Venus *et al.* (2011)

Figure 2.1: Skin structure and its function

- **Stratum basale (basal cell layer)**

In this layer, the melanocyte is present and comprises of 5-10% of the cell population. It is one cell thick.

- **Stratum spinosum**

Langerhans cells are present in this layer. As the basal cells move towards the surface it form a layer of polyhedral cells connected by desmosomes and seen as 'prickles' under the microscope.

- **Stratum granulosum**

In this layer, the lipid contents is discharge from to cells into the intercellular space plays a vital role for the barrier function and intercellular cohesion within the stratum corneum. Keratinocytes are found in the granular layer which contains intracellular granules of keratohyalin.

- **Stratum corneum**

The outermost layer of the epidermis is stratum corneum in which the cells travelled from stratum granulosum. The cells in this layer are called corneocytes. The nuclei and cytoplasmic organelles have been lost (Venus *et al.*, 2011)

2.2. Skin damage

A wound is defined as damage or disruption to the normal anatomical structure and function of skin (Velnar *et al.*, 2009). Another definition by Meenakshi *et al.* (2006) which state that wound can be physical, chemical or thermal injuries that cause an opening or breaking in the integrity of the skin. In other words, wound is a break in the epithelial integrity of the skin and may be accompanied by disruption of the structure and function of underlying normal tissue and may also result from a contusion, haematoma, laceration or an abrasion (Dewangan *et al.*, 2012; Young & McNaught, 2011). It range from a simple break in the epithelial integrity of the skin or deeper extending into subcutaneous tissue. It might include destruction to other structures such as tendons, muscles, vessels, nerves, parenchymal organs and bone (Velnar *et al.*, 2009).

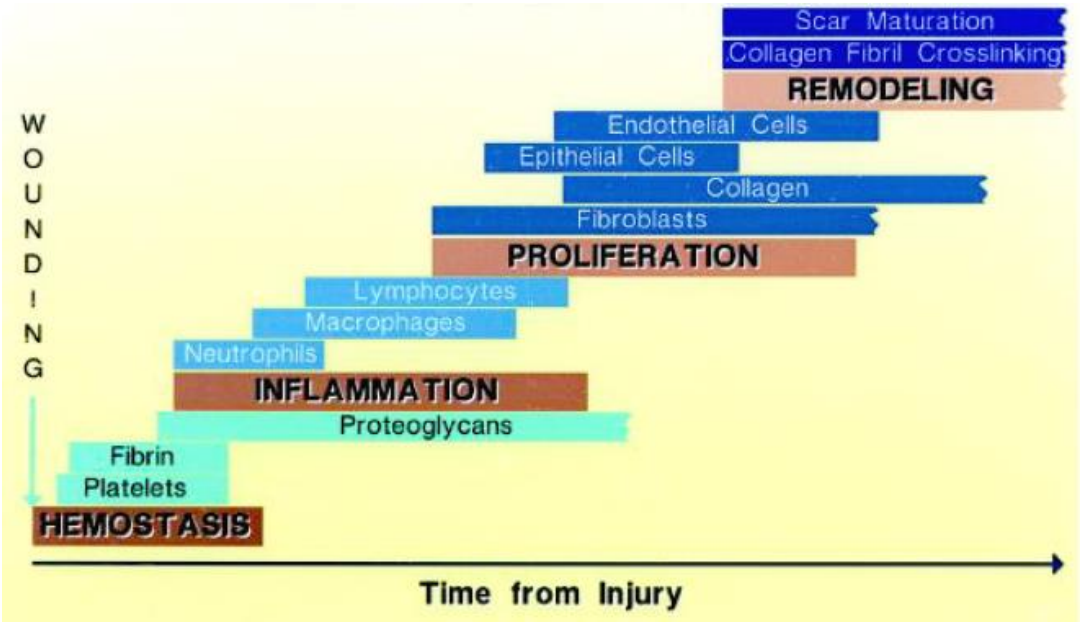
Wounds can be categorized as acute and chronic according to their healing time frame (Majumdar, 2005; Velnar *et al.*, 2009). Wounds that repair themselves ordinarily by following a timely and orderly pathway with the end result of both functional and anatomical restoration are classified as acute wounds. The time sequence of healing commonly ranges from 5 to 10 days or within 30 days. Acute wounds can be result of traumatic loss of tissue or from a surgical procedure. Chronic wound is the skin impairment that unsuccessful to heal through the normal stages of healing and cannot be repair in an orderly and timely manner. The healing process of chronic wound is incomplete and disturbed by various factors that prolong one or more stages of healing. The factors include infection, necrosis, tissue hypoxia, exudate and superfluous levels of inflammatory cytokines (Velnar *et al.*, 2009).

Wounds can happen as part of a pathological process, accidental or intentional etiology (Young & McNaught, 2011) that begin externally or internally within the complicated organ (Velnar *et al.*, 2009).Wound can be categorized into three namely open wound, closed wound and miscallenous wound. Open wound comprises of incisional, laceration, abrasion, amputation, puncture, penetration and gunshot. Types of closed wound are bruised, hematoma and crush injury. Miscallenous wound includes burns, chemical wounds, bite and stings, electrical wounds, radiation dermatitis or necrosis, cancer wounds, infective, chancre and pressure ulcers (Velnar *et al.*, 2009).

2.3 Wound healing

Wound healing is the process of reconstituting the integrity of the skin (Manuskiatti & Maibach, 1996) through connective tissue matrix synthesis (Pradhan *et al.*, 2009). Following damage, multiple cellular and extracellular pathways are activated in a regulated manner with the target of restoring tissue integrity (Young & McNaught, 2011). An appropriate methods for wound healing are vital for the rebuilding of disrupted anatomical continuity and disturbed function of the skin (Meenakshi *et al.*, 2006).

The process of normal wound healing as illustrated by Diegelmann & Evans, (2004) as shown in Figure 2.2 can be categorized into four stages which consist of coagulation and haemostasis, inflammatory, proliferation, and remodeling phase characterized by scar tissue formation which eventually determines the strength and appearance of the tissue (Guo & DiPietro, 2010; Rosana *et al.*, 2014; Velnar *et al.*, 2009). In normal healing process, all four phases must occur in timely and orderly



Source: Diegelmann and Evans (2004).

Figure 2.2: The sequence of events during normal wound healing

manner. Many factors can hinder with one or more phases of this process which causing impaired wound healing (Guo & DiPietro, 2010).

Wound healing begins with the inflammation stage that involves two processes. Regeneration of injured tissue by parenchymal cells which leave no residual trace of the previous injury and the second is replacement by connective tissue. Fibroplasia usually in its permanent state constitutes a scar. Both regeneration and fibroplasia are determined by similar mechanisms encompassing cell differentiation and cell-matrix interactions. Orderly regeneration of the epithelial tissue of skin and viscera requires the continuous presence of basement membrane (BM). The epithelized extracellular matrix functions as an extra-cellular scaffold for accurate regeneration of pre-existing structure and for recruitment of cells require for fibroplasia (Marcandetti & Cohen, 2002).

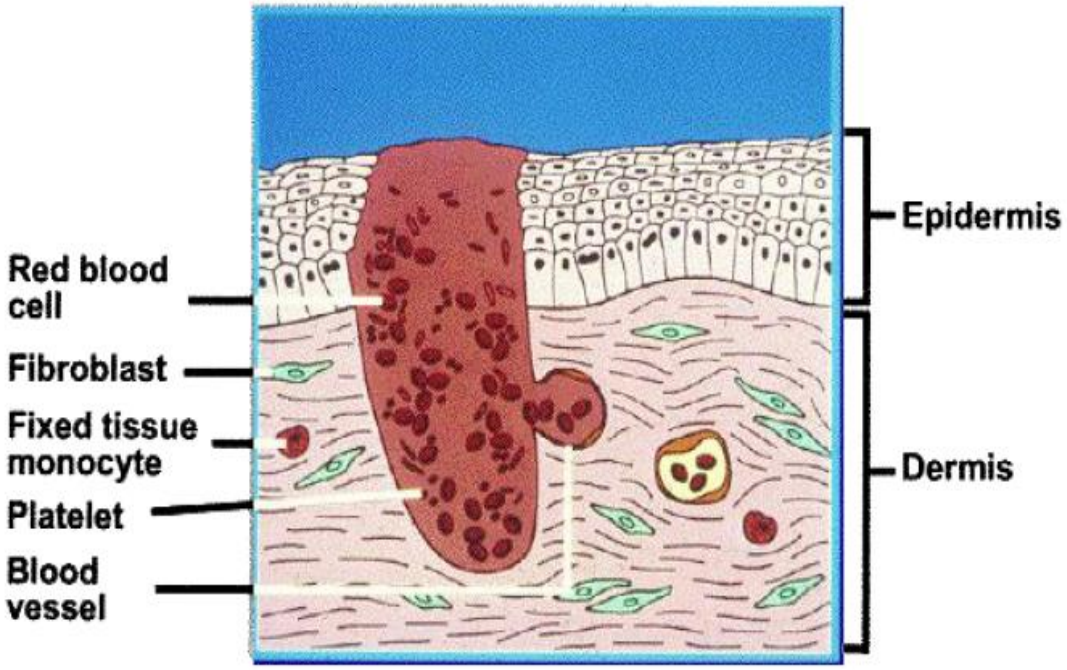
2.3.1 Coagulation and haemostasis phase

Following injury, coagulation and haemostasis as illustrated in Figure 2.3 takes place in the wound (Broughton *et al.*, 2006; Majumdar, 2005; Velnar *et al.*, 2009). Exposure of blood to the wound collagen after injury causes deregulation of platelet and activation of Hageman factor. This process activates kinin, clotting cascades and plasmin generation. These serve to strengthen the original injury signal and lead to clot formation which unites the wound edges. It also lead to high number of mitogens and chemoattractants at the site of wound (Majumdar, 2005).

The principal target of this particular mechanisms is to avoid exsanguinations. It is a process that is important to protect the vascular system by ensuring that it stay intact so that the function of the vital organs remains unharmed. Secondly, as a long term effect which is to deliver a matrix for invading cells that are needed in the later phases of healing. A dynamic balance between endothelial cells, thrombocytes, coagulation,

and fibrinolysis regulates haemostasis and determines the amount of fibrin deposited at the wound site influencing the progress of the healing processes (Velnar *et al.*, 2009).

Together with haemostatic events, the coagulation cascade is activated through extrinsic and intrinsic pathways. It leads to platelet accumulation and clot formation to prevent high level of blood loss. The cytoplasm of platelets contains α-granules filled with growth factors and cytokines such as platelet derived growth factor (PDGF). It transform growth factor-β (TGF-β), epidermal growth factor and insulin-like growth factors (Velnar *et al.*, 2009). These molecules act as promoters in the wound healing cascade by stimulating and attracting neutrophils, macrophages, endothelial cells and fibroblasts (Broughton *et al.*, 2006; Velnar *et al.*, 2009).



Note: At the time of injury, the tissue is disrupted and the platelets adhere to the exposed collagen and to each other. The platelets release clotting factors, PDGF and TGF-ß to initiate the repair process.

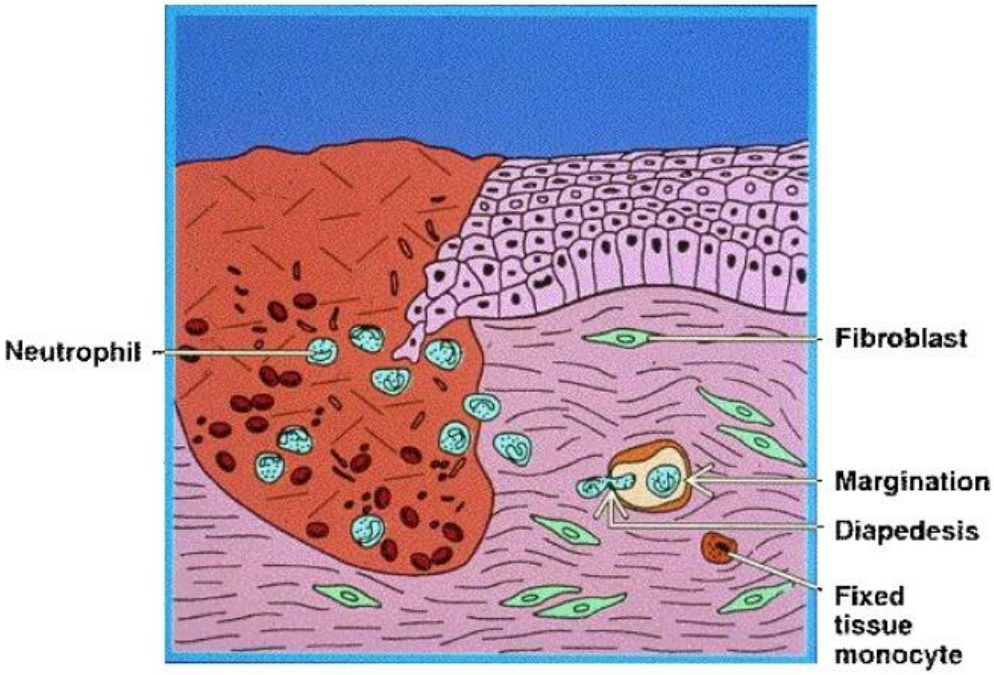
Source: Diegelmann and Evans (2004)

Figure 2.3: Coagulation and Haemostasis Phase.

2.3.2 Inflammatory phase

Shown in Figure 2.4, the production of kinins and prostaglandins leads to vasodilatation and increased small vessel permeability in the wound. This results in oedema cause pain and swelling which occurs early after injury. Within 6 hours, circulating immune cells start to accumulate in the wound (Majumdar, 2005; Velnar *et al.*, 2009). Polymorphonuclear leucocytes (PMN) are the first blood leucocytes to enter the site of injury (Majumdar, 2005). Their main functions appear to be phagocytosis of the bacteria and foreign substance that enter the wound during injury. Without infection, PMN have a relatively short life span in the wound. Their numbers decrease rapidly after third day of injury (Majumdar, 2005; Velnar *et al.*, 2009).

Macrophages are another cellular immune component that introduced into the wound (Majumdar, 2005; Velnar *et al.*, 2009). Macrophages have longer life span compared to PMN. It remain in the wound until healing process is complete (Majumdar, 2005). Macrophages just like neutrophils phagocytose and digest pathological organisms and tissue debris and secrete collagenase and elastases which break down injured tissue and release cytokinins. Macrophages release number of active substances and growth factor which are needed for the initiation and propagation of granulation tissue formation (Marcandetti & Cohen, 2002; Romo & McLaughlin, 2003).



Note: By the first day following injury, neutrophils attach to endothelial cells in the vessel walls surrounding the wound (margination), then change shape to move through the cell junctions (diapedesis) and migrate to the wound site (chemotaxis). This is the beginning of the inflammatory phase

Source: Diegelmann and Evans (2004)

Figure 2.4: Inflammatory Phase

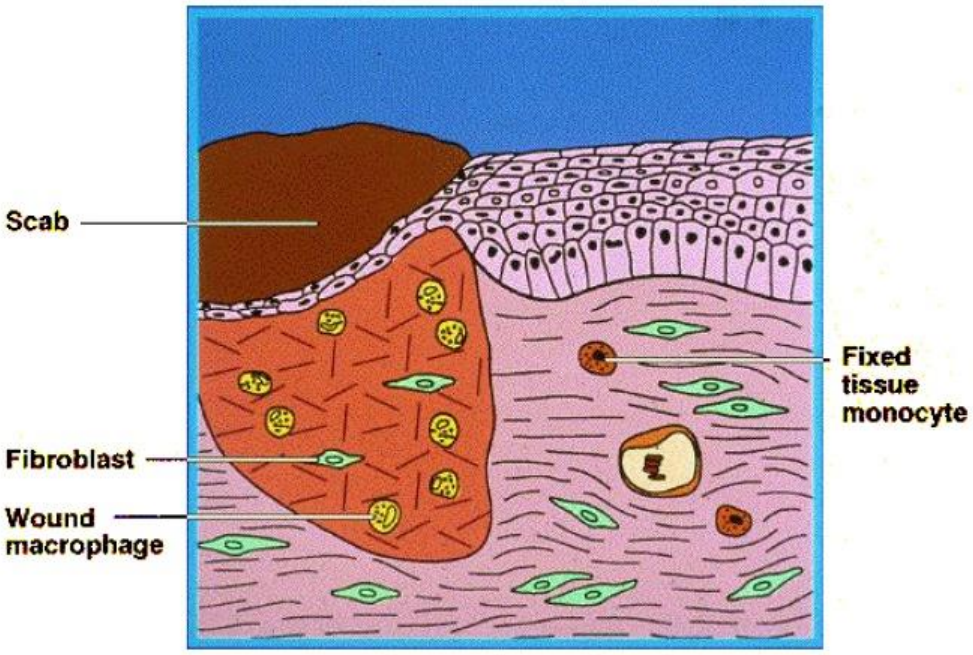
2.3.3 Proliferative phase

The proliferative phase is illustrated at Figure 2.5. In the absence of significant infection, the inflammatory stages process is shortened. After the wound has been cleared from foreign material, it gives way to the proliferative stage of healing. Granulation tissue consists of cellular elements including fibroblasts and inflammatory cells. Fibroblasts accumulate in high number on the third day after injury and reach peak numbers on the seventh day (Romo & McLaughlin, 2003) .

This rapid accumulation in the fibroblast population at the wound site occurs via a combination of proliferation and migration. Fibroblasts responsible for the production of the structural proteins used during tissue reconstruction. It produce large quantities of collagen which are responsible for instructing tensile strength to the scar (Romo & McLaughlin, 2003).

While these mechanism taking place deep in the wound, restoration of epithelial integrity is proceeding at the wound surface. Epithelization is complete in less than 48 hours in the case of approximated incised wounds and take longer in the case of high tissue defect (Romo & McLaughlin, 2003).

The stimuli for epithelization is still unknown but it seems that the process is mediated by a combination of loss of contact inhibition, exposure of constituents of the extra cellular matrix. However, it is believed that platelet-derived growth factor (PDGF) and insulin like growth factor is responsible to promote epithelization process (Marcandetti & Cohen, 2002).



Note: The inflammatory phase continues as fixed tissue macrophages become active and move into the site of injury and transform into very active wound macrophages. These highly phagocytic cells also release PDGF and TGF-ß to recruit fibroblasts to the site and thus begin the proliferative phase

Source: Diegelmann and Evans (2004)

Figure 2.5: Proliferative Phase.

2.3.4 Remodeling phase with scar tissue formation

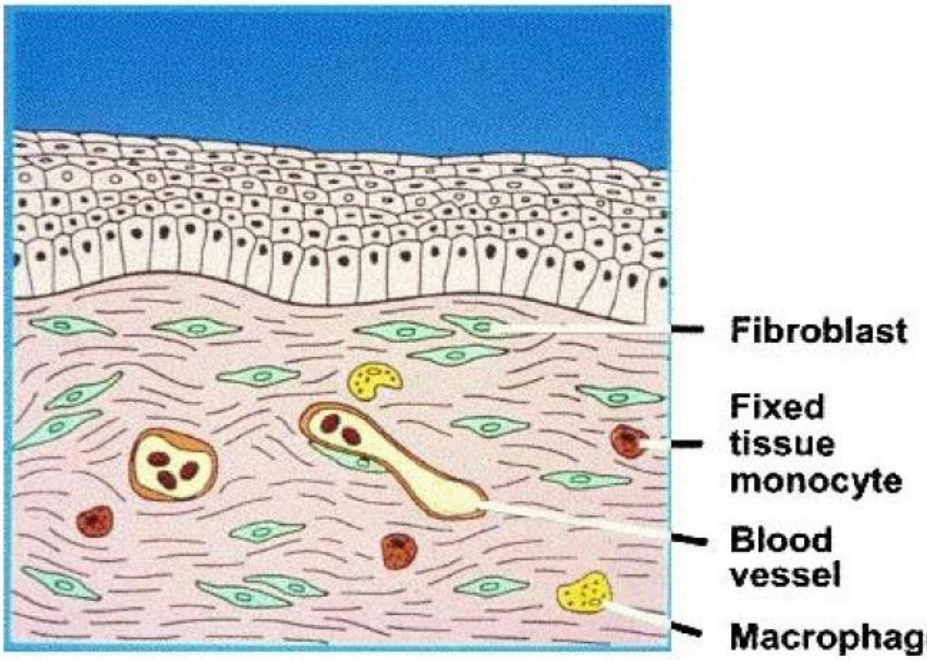
The phase is illustrated at Figure 2.6. Collagen remodelling during the maturation phase depend on collagen synthesis continuation by stimulation of growth factor. Some of the growth factors also modulate the synthesis and activation of metalloproteinases. They are enzymes that responsible to degrade epithelial cell migration (ECM) component (Majumdar, 2005). Collagen rapidly becomes the predominant constituent of the matrix. The collagen fibres become cross-linked and aggregated into fibrillary bundles which gradually provide the healing tissue with increasing stiffness and tensile strength (Majumdar, 2005).

Approximately after a five days of injury there is a rapid increase in wound breaking strength due to collagen fibrogenesis. The high rate of collagen synthesis and remodelling of the scar continues for six months up to 1 year after injury (Majumdar, 2005). Wound healing time can be different as some wounds may take more than a year to heal completely (Velnar *et al.*, 2009). The final product of the healing process is a scar. Delays of the healing process due to certain condition may lead to abnormal scar formation (Majumdar, 2005).

2.4 Sea Cucumber (Gamat)

2.4.1 Biology

Sea cucumber is scientifically known as holothuroid and holothurian. In Malaysia, sea cucumber is locally recognized as timun laut, bat, balat, brunok and gamat (Kamarudin *et al.*, 2010). Sea cucumber is a traditional remedy that been used to treat numerous ailments for over 300 years (Barathi *et al.*, 2013) as well as for healing various internal and external wounds (Fredalina *et al.*, 1999). Anecdotes claims that people have used sea cucumber as a medicine after childbirth and



Note: It is characterized by continued synthesis and degradation of the extracellular matrix components trying to establish a new equilibrium.

Source: Diegelmann & Evans, (2004)

Figure 2.6: The remodeling phase

surgery, wound healing, ulcer, chest pain, pneumonia, (Barathi *et al*., 2013) and have widely been utilized as a traditional remedy for hypertension, rheumatism, sinus, cuts, burns (Fredalina,1999) and asthma (Barathi *et al*., 2013; Fredalina,1999).

Morphologically, as illustrated in Figure 2.7 and 2.8 the taxonomy grouping of sea cucumber to species level is usually based on the shapes of calcareous skin ossicle or spicule as shown in Table 2.1 (e.g. cup, table, button, perforated plate, wheel, anchor and plate and basket), types of tentacle (e.g. pinnate, peltate, dendritic and digitate) and types of calcareous ring. (Kamarudin *et al.*, 2010). Similar finding shows that identifications are often based solely on external features such as the colouration and appearance of the body wall and the shape of tentacles. These outer features may indeed be valuable in determining the identity of species, but the examination of ossicles and other internal features such as the calcareous ring can be crucial in distinguishing closely related species (Ong & Wong, 2015).

Sea cucumber from a nutritional point of view is an ideal tonic food with high nutritional value. It contains a high level of protein and a low level of fat (Chen, 2004; Ridzwan *et al.*, 2003). Its body wall consists of insoluble collagen has been utilized as a nutrient supplement of hemotogenesis (Ridzwan *et al.*, 2003). Sea cucumber protein is rich in lysine, arginine, and tryptophan. The gelatin from sea cucumber is believed to be more valuable than other gelatins due to its characteristic of amino acid composition particularly the essential amino acids (Chen, 2004).