EVALUATION OF POLYHYDROXYALKANOATE (PHA) FILMS CONTAINING STINGLESS BEES (*GENIOTRIGONA THORACICA*) PROPOLIS FOR BURN WOUND HEALING IN A STREPTOZOTOCIN-INDUCED DIABETIC RAT MODEL

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by

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LIST OF ABBREVIATIONS

ACTB	Beta Actin
AMDI	Advanced Medical and Dental Institute
CoA	Coenzyme A
COSHH	Control of Substances Hazardous to Health
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco’s Phosphate Buffered Saline
EDX	Energy Dispersive X-Ray Analyzer
FBS	Fetal Bovine Serum
HDF	Human Dermal Fibroblast
NKRA	National Key Results Area
NO	Nitric Oxide
PDGF	Platelet Derived Growth Factor
PHA	Polyhydroxyalkanoates
PVA	poly vinyl alcohol
SD rats	Sprague Dawley rats
SDG 3	Sustainable Developmental Goals
SEM	Scanning Electron Microscopy
STZ	Streptozotocin
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
VVC	Vulvovaginal Candidiasis

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PENILAIAN FILEM POLIHIDROKSIALKANOAT (PHA) YANG MENGANDUNGI PROPOLIS LEBAH KELULUT (*GENIOTRIGONA THORACICA*) UNTUK PENYEMBUHAN LUKA TERBAKAR PADA MODEL TIKUS DIABETES YANG TERARUH STREPTOZOTOSIN

ABSTRAK

Tisu kulit yang luka atau rosak akan digantikan dengan tisu baru yang terhasil semasa proses penyembuhan luka. Propolis mempamerkan pelbagai aktiviti biologi dan komposisi kimia yang berkhasiat dalam bidang perubatan dan kesihatan, termasuk potensi sebagai agen penyembuhan luka. PHA ialah bahan polimer sintetik yang bio-serasi dan berpotensi untuk pelbagai aplikasi perubatan. Dalam kajian ini, sifat dan ciri PHA dan propolis telah dikaji untuk menyediakan tampalan pembalut luka baharu yang berkos rendah, berkesan dan selamat. Tampalan yang berasaskan polimer yang mengandungi propolis merupakan strategi baru untuk merawat luka pada kulit. Propolis telah diekstrak dan dioptimumkan secara *in vitro* menggunakan sel fibroblas kulit manusia (HDF) untuk menunjukkan bio-serasi, migrasi sel dan ekspresi gen angiogenik. Viabiliti sel HDF telah diuji untuk menentukan ketoksikan paling rendah konsentrasi DMSO dalam media propolis. PHA telah disintesis daripada *C. necator*, dicirikan dan diuji untuk kompatibiliti sel. STZ pada dos optimum 65 mg/Kg telah mengaruh diabetes dalam tikus seperti yang disahkan dengan paras gula dalam darah. Propolis ditambah kepada tampalan sejurus sebelum digunakan pada luka. Luka pada kulit terhasil selepas sentuhan dengan menggunakan rod yang telah dipanaskan. Konsentrasi 0.1% (v/v) DMSO memberikan viabiliti sel HDF yang tertinggi. Pada pengoptimuman dilusi secara bersiri, kepekatan 10 µg/mL propolis dalam kultur HDF memberikan viabiliti tertinggi iaitu 100%. EC50 propolis ialah 47.0 µg/mL. Migrasi sel lebih tinggi dalam media

propolis berbanding dengan kawalan pada titik akhir 3 jam, 6 jam dan 9 jam, $P < 0.05$. Hemokompatibiliti adalah pada kadar 3.3% hemolisis yang menunjukkan PHA adalah selamat dari risiko lisis sel. Kapasiti penyerapan yang tinggi (1200%) ditunjukkan oleh tampalan selepas 2 jam dan stabil pada 3 jam. Analisis SEM menunjukkan kedua-dua sampel (PHA sahaja dan PHA dengan propolis) menunjukkan saiz liang maksimum 150 μm. Analisis graf EDX menunjukkan ketiadaan porogen (NaCl) sepenuhnya dalam kedua-dua sampel. Analisis sudut sentuhan air PHA ialah 79.9° menunjukkan permukaannya sukar basah dan kasar. Ekspresi gen VEGF dan b-FGF bagi HDF yang dikultur dalam sampel bahan yang berbeza menunjukkan ekspresi gen yang lebih tinggi ditunjukkan dalam kumpulan propolis dan propolis + PHA, berbanding dengan sampel kontrol, walaupun perbezaannya tidak signifikan secara statistik, $P > 0.05$. Untuk morfometri luka *in vivo* dan peratusan penyembuhan dan pembentukan kudis, sampel PHA + propolis memberikan hasil keseluruhan terbaik berdasarkan skor keradangan dan pembentukan kudis berbanding kumpulan lain. Sebagai bahan tampalan luka, PHA dan propolis memberi alternatif kepada bahan pembalut luka yang sedia ada. Kajian ini menunjukkan prestasi baik bahan pembalut luka tersebut dalam model tikus diabetes yang diaruh oleh STZ.

EVALUATION OF POLYHYDROXYALKANOATE (PHA) FILMS CONTAINING STINGLESS BEES (*GENIOTRIGONA THORACICA*) PROPOLIS FOR BURN WOUND HEALING IN A STREPTOZOTOCIN-INDUCED DIABETIC RAT MODEL

ABSTRACT

Wounded or damaged tissues on the skin are replaced with freshly created new tissues during the wound healing process. Propolis exhibit diverse array of bioactivities and chemical composition which possess nutritive, medicinal and health benefits, including the potential as a wound healing agent. PHA is a synthetic biocompatible polymer material which has potentials for a wide range of medical applications. In this study, the properties and characteristics of PHA and propolis were studied to provide a novel, low-cost, effective, and safe wound dressing patches. This polymer-based film containing propolis is a novel strategy for treatment of skin wounds. Propolis was extracted and optimised *in vitro* using the human dermal fibroblasts (HDF) cells to demonstrate biocompatibility, cellular migration and angiogenic gene expression. HDF cells viability was tested to determine the least toxicity of DMSO concentration in the propolis media. PHA was synthesized from *C. necator,* characterised and tested for its biocompatibility. STZ at an optimised dose of 65mg/kg had successfully induced diabetes in the rats as confirmed by blood sugar analysis. Patches were fabricated and propolis was loaded to the patch just before applying it to the wound created by using a self-made heated rod. Concentration of 0.1% (v/v) DMSO gave the highest HDF viability. In a serial dilution optimisation step, the concentration of 10 µg/mL of propolis gave the highest cell viability (100%). EC50 of propolis was 47.0 µg/mL of propolis concentration in HDF cultures. Cell migration was significant in propolis media in

comparison to control, at 3 hours, 6 hours and 9 hours endpoints, $P < 0.05$. Haemocompatibility was at 3.3% rate of haemolysis which justified the safety of PHA and low risk of cell lysis. High absorption capacity (1200 %) was shown by the dressing patch after 2 hours and stabilized at 3 hours**.** SEM analysis demonstrated both samples (PHA alone and PHA with propolis) showed maximum pore size of 150 μm. EDX graphs analysis showed complete absence of the porogen (NaCl) in both samples. Water contact angle analysis of PHA was 79.9° demonstrating poorly wettable surface with observable roughness. VEGF and b-FGF gene expressions in HDF cultivated on different surface materials showed higher gene expression was demonstrated on the propolis and propolis + PHA groups, compared to control, although the difference was not statistically significant, $P > 0.05$. For *in vivo* wound morphometry and percentage of healing and scab formation, wound treated by PHA + propolis gave the best overall results based on the inflammation and scab formation scores in comparison to other groups. PHA and stingless bee propolis as a wound dressing material provide an alternative to the existing wound dressing materials. This study demonstrated good performance of the wound dressing material in an STZ-induced diabetic rat's model.

CHAPTER 1

INTRODUCTION

1.1 Overview

Chronic wounds are a major health problem worldwide that creates a lot of demand for the healthcare system. The rise in traumatic wounds, diabetes, obesity, and ageing population contributes to a large increase in skin wounds. The challenges in wound treatment are associated with pre-existing medical conditions such as diabetes mellitus, inflammatory conditions, and obesity. Secondary bacterial infection is a confounding factor that often worsens the healing process, causing considerable delay in wound healing. The economy of wound treatment is multiplied by these factors. For example, diabetic foot ulcer affects around 9.1 to 26.1 million people worldwide (Armstrong et al., 2017). The percentage of diabetes mellitus patients thatcan develop diabetic ulcer in their lifetime are 15-25% of total patients (Mutluoglu etal., 2012).

There are many contemporary wound management strategies that involve wound dressing materials, skin substitutes and compound use of antibiotics or substances to accelerate the wound healing process. Many dressing materials have been developed from natural polymers like chitosan, gauze, films, hydrocolloids, foams, hydrogels, alginates, and hydrogel fibers, and polyhydroxyalkanoate. Tissue engineered skin substitutes is also an emerging technology to replace massive tissue loss in extended wounds (Han & Ceilley, 2017; Okur et al., 2020). To treat complicated wounds implicated in patients with diabetes, wound dressing which can control the exudation and provide a moist environment are extensively investigated. General health and metabolic control are important parameters to ensure good glycaemic

control and prevention of infection; as well as healthy blood flow and circulation in diabetic population (Everett & Mathioudakis, 2018).

Propolis is a natural sticky resinous substance derived from the collection of various bees from parts of plants, bark, and exudates (Marcucci, 1995). Propolis is used to construct and to seal hives; therefore, maintaining it from being decomposed by the invading creatures (Marcucci, 1995) and maintaining a low level of microorganisms sequestered in the hives (Choudhari et al., 2012). Propolis exhibits a diverse array of bioactivities and chemical composition which are dependent on geographical locations, bee species and seasonal variations (Kujumgiev et al., 1999; Marcucci, 1995). Diverse bioactivities of propolis have been observed such as anti-microbial (Kujumgiev et al., 1999; Marcucci, 1995) and anti- oxidant effects (Teixeira et al., 2010), anti-tumor activities (Teixeira et al., 2010; Umthong et al., 2009), anti-inflammatory and strong wound healing properties (Martinotti & Ranzato, 2015; Olczyk et al., 2013).

Indo-Malayan stingless bee or 'lebah kelulut' is one of the bee species found in Malaysia. It was claimed to produce propolis that is more potent than the one produced by honeybees (Ismail, 2016). The biomedical applications of propolis are many, including in the area of wound healing. So far, there is scant literature on the effects of 'kelulut' propolis on skin wound healing conducted in Malaysia. Due to its strong anti-inflammatory, anti-microbial and healing properties, 'kelulut' propolis has a high potential as a wound treatment agent.

Polyhydroxyalkanoates (PHA) has been used as a wound dressing material previously with encouraging results. PHA is advantageous for biomedical applications due to its biocompatibility, biodegradability, non-immunogenic and the versatility of

PHA design and fabrication due to the different types of its monomers' properties. The combined use of honey and propolis and use of polymeric scaffolds such as PHA had been shown to be effective as wound healing materials as formerly investigated (Stojko et al., 2021). The combined use of 'kelulut' propolis with PHA needs further *in vivo* exploration in a diabetic-induced wound healing model.

1.2 Problem statement and study rationale

Wound healing is a complicated process with many steps which need special care to accomplish complete healing. Good dressing materials will help in accelerating the rate of wound healing. A big concern is the selection and usage of dressing materials. Literature review showed that indications for use that have been cleared for certain wound dressings containing chemicals or drugs may not be clinically relevant or supported by available evidence. Thus, an extensive study to provide adequate evidence demonstrating substantially equivalent safety and effectiveness are highly necessary.

1.3 Research questions

i) Would polyhydroxyalkanoate (PHA)-based wound dressing patches containing stingless bees propolis improve the wound healing in STZ-induced diabetic rat model?

ii) Would PHA be biocompatible with the human dermal fibroblasts (skin cells) *in vitro*?

iii) Would the study's wound dressing material (PHA-propolis patch) be effective in STZ-induced diabetic rats' wound healing?

1.4 Research hypothesis

1.4.1 Null hypothesis (H_0)

PHA-based polymeric wound dressing patches containing stingless bees propolis do not improve wound healing in a streptozotocin (STZ)-induced diabetic rat model.

1.4.2 Alternate hypothesis (H_1)

PHA-based polymeric wound dressing patches containing stingless bees propolis improve wound healing in STZ-induced diabetic rat model.

1.5 Objectives

1.5.1 General objective

To evaluate the effects of polyhydroxyalkanoate (PHA)-based wound dressing patches containing stingless bees propolis on wound healing in STZ-induced diabetic rat model.

1.5.2 Specific objectives

i) To biosynthesize PHA using shake flask technique and fabricate PHA films using solvent-cast techniques.

ii) To isolate and optimize concentrations of stingless bees propolis.

iii) To fabricate PHA-based films containing stingless bees propolis as wound dressing material.

iv) To study the biocompatibility of PHA in human dermal fibroblast (HDF) and the vasculogenic gene expression of HDF on PHA *in vitro.*

v) To study the effectiveness of PHA-propolis patches in STZ-induced diabetic wound in rats.

1.6 Project framework

This project is summarized in Figure 1.1. It started by the extraction of propolis, then the extracted product was characterised and optimised by doing dose optimisation, EC50 and migration assay. PHA was biosynthesised, characterised and the PHA biocompatibility was tested by checking the cell viability on PHA. *In vivo* diabetic wound model using SD rats was established, wound healing rate was assessed by wound morphometry, hemolysis rate test was conducted and angiogenic genes expression in PHA was studied.

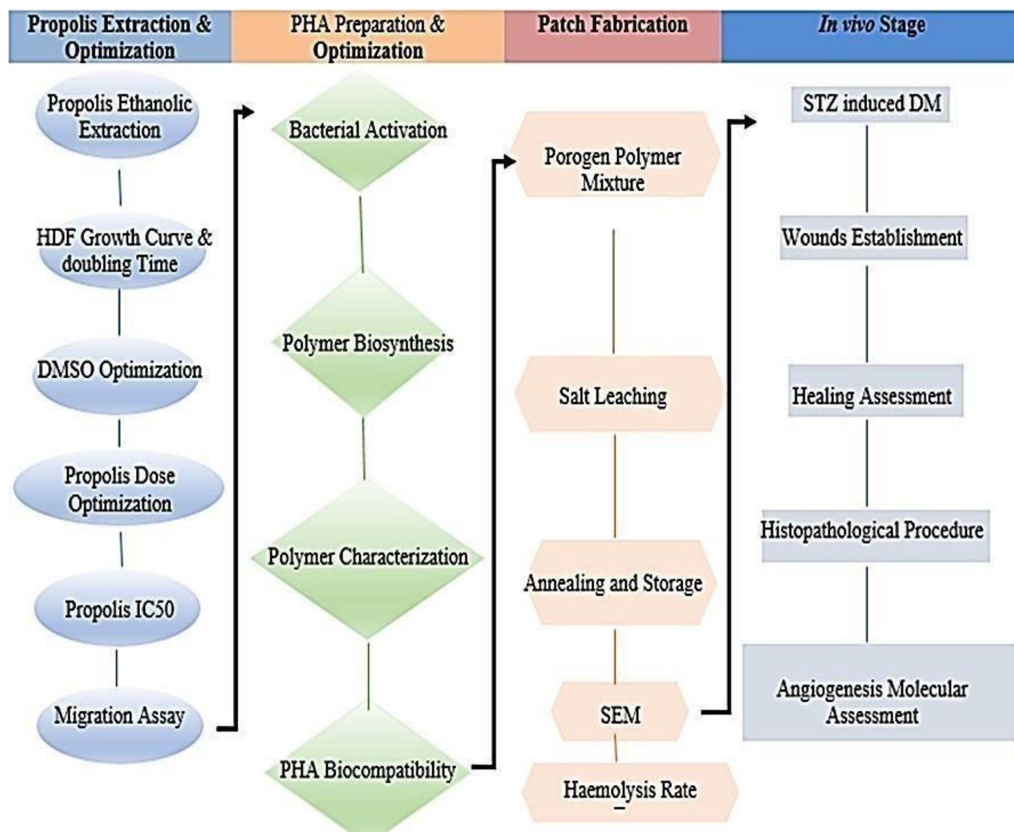

Figure 1.1 Project framework

CHAPTER 2

LITERATURE REVIEW

This chapter will review types of wounds, the basic concepts of wound healing physiology, wound dressing materials and the current literature on the use of propolis and PHA as wound healing and dressing materials.

2.1 Wound healing

Chronic wounds impose a heavy financial and economic burden, often characterized by delayed or impaired tissue repair, requiring specialized medical and surgical interventions and prolonged care. The cost of managing chronic wounds in 2009 was £2.5 billion to £3.1 billion annually in the UK (RM 13.7- 16.9 billion) (Vowden & Kathry, 2016). Closer to home, in a developing country like Singapore, the cost of diabetic foot ulcer management was reported to be about $1.68 million (RM 5.2 million) (Tan et al., 2016).

2.1.1 Introduction

Skin is the largest organ in human body covering an area of two m^2 surface area (Gallo, 2017). Skin functions as a barrier from physical, chemical, thermal, mechanical, and infective insult. The term ‘wound’ is defined as a disruption of normal anatomical structure and function, resulting from a pathological process which can begin internally or externally in the involved organ. Wound can be as simple as a break in skin epithelial integration or can be deeper as it can involve the subcutaneous tissues with damage in other body structures like muscles, tendons, nerves, vessels and bones (Alonso et al., 1996). Skin wound healing is a complex mechanism which is considered

as an evolutionary advantage for mammals. Skin wound healing ends with wound closure, so it is considered as an essential mechanism for survival.

The process of wound healing is a physiological process that involves many cell types and mediators interact in a sequence of events leading to wound closure (Sorg et al., 2017). Different cell types are involved in the process of wound healing (Reinke and Sorg, 2012). The most important role of wound healing is skin repair and restoration of its function as the physiological barrier (Landén et al., 2016; Landén et al., 2016).

2.1.2 Historical perspectives on wound healing

Old Egyptian papyrus described the process of wound care had been practiced as early as 3,200-300 BC. They described a significant wound dressing procedure implemented by Hippocrates involving pus drainage from the wound. Galen described the principles of wound healing by primary and secondary intentions. This information has been lost over time, then rediscovered by Brunchwig, Gersdorff and Paracelsus. The 19th century witnessed a great addition to this field; when Lister and Semmelweis introduced antisepsis for the first time, Koch discovered the pathogenic microorganisms and finally the great achievement of penicillin discovery by Felming and Sulfonamides by Domagk (Robson, 2001). Multiple investigators continued to work on strategies to enhance the wound healing process and explored the use of bio-compatible materials as wound healing agents.

2.1.3 Normal wound healing process

Wound healing process is divided into four main steps: bleeding and homeostasis, inflammation, proliferation, and wound remodeling with scar tissue

formation (Figure 2.1). Wounds may be acute or chronic; the acute wounds normally follow an orderly and timely reparative process which leads to restoration of anatomic and functional organ integrity. The wound is classified as chronic when there is a delay in restoration of anatomic and functional integrity in a timely process or when there is no successful anatomic and functional result (Lazarus et al., 1994).

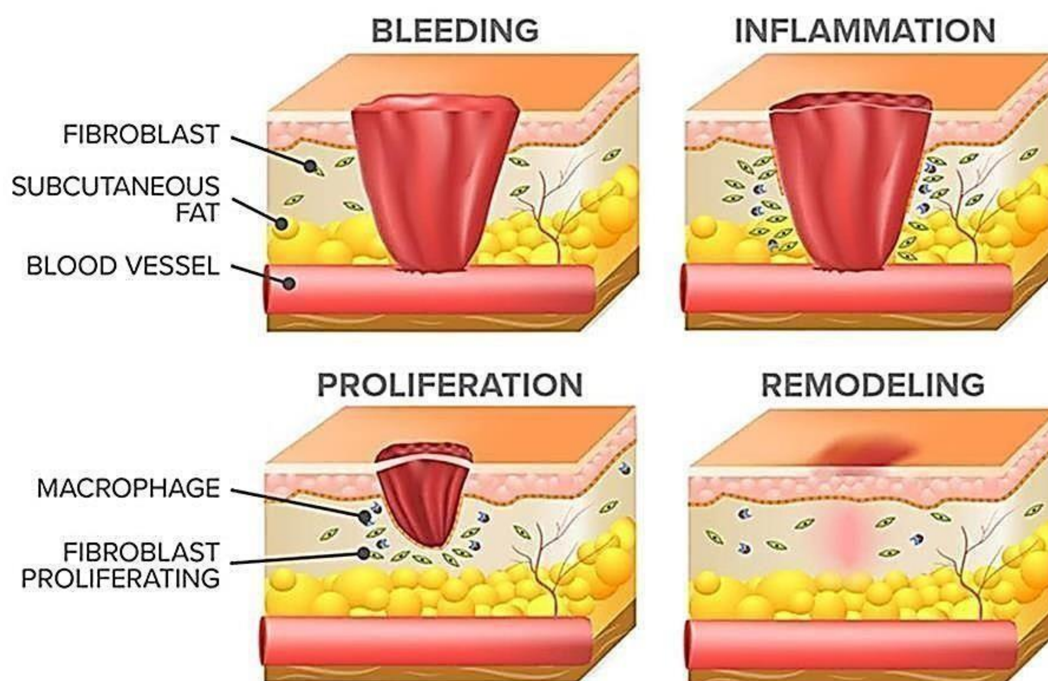

Figure 2.1 Stages of wound healing. The four stages of wound healing mechanism involve bleeding, inflammation, proliferation, and remodeling, happening in a sequence controlled by cellular factors released in response to the wound bed. (https://wasatchphotonics.com/application-tags/dermatology/).

2.1.4 Detailed process of wound healing

The bleeding and coagulation stage precedes the other events and often coincide with the inflammatory stage. The injury leads to vasodilation together with high vascular permeability and this is the first response from the body after injury occurs. There is a vascular inflammatory response which includes the lesioned blood vessels contraction followed by coagulation in order to control blood loss. This coagulation consists of fibrin mesh composed of thrombocytes and platelets (Gonzalez

et al., 2016). In skin wounds, blood leaks out of the damaged vessels, followed by fibrin clot formation. This serves as a wall or shield to protect the denuded tissues and provide a matrix for facilitating the cell migration during the repair process. Constituents of this fibrin clot are platelets in cross-linked fibres of fibrin, plasma fibronectin, vitronectin and thrombospondin.

During this process, activated platelets degranulate leading to the release of cytokines and growth factors which are reserved inside the clot. This master mix of growth factors is the beginning of wound closure mechanism. It plays a big role in attracting the inflammatory cells to the wound site by providing chemotactic cues, this is the initiation of inflammatory response. This will lead also to three important events: reepithelization initiation, contraction of connective tissues as well as stimulation of the angiogenic response of the wound (Eming et al., 2007; Gonzalez et al., 2016; Landén et al., 2016).

After clot formation, there is a generation of cellular signals, the result will be neutrophil response. Inflammatory mediators accumulate, prostaglandin elaborated, and blood vessels vasodilate to increase cellular traffic. Neutrophils are there, in the injured area, drawn by interleukin 1 (IL-1) tumour necrosis factor (TNF)-alpha, transforming growth factor (TGF)-β, and platelets factor 4 (PF4) (Gonzalez et al., 2016). Monocytes migrate and transform into macrophages after 48 to 96 hours from the injury time. Macrophage is very important in transition from this phase to the next phase (proliferative phase).

a) Activated macrophage plays a crucial role in:

b) Mediating angiogenesis, this will happen by producing vascular endothelial growth factor (VEGF), fibroblast growth factor and TNF alpha.

c) Mediating fibroplasia by producing TGF beta, platelet derived growth factor (PDGF), IL-1 and TNF alpha.

d) It synthesizes nitric oxide. The mechanism is as follow, IL-1 and TNF alpha activate inducible nitric oxide (NO) synthase which will lead to NO synthesis (Ellis et al., 2018).

Neutrophils then enter the site of injury and start extensive cleaning of cellular debris as well as invading bacteria by releasing proteolytic enzymes, these enzymes digest bacteria and non-viable tissues (Ellis et al., 2018). Interestingly, the matrix of un-wounded tissues is protected by an armour of protease inhibitors (McCarty and Percival, 2013). Neutrophils play another important role in sterilization of the wound by producing reactive oxygen radicals via myeloperoxidase pathway, reactive oxygen radicals together with chlorine can sterilise the wound mainly from bacteria (Wilgus et al., 2013).

Apoptotic neutrophils are engulfed by macrophages; this event provides a strong signal for resolution of inflammation. This allows the continuation of the wound healing process and movement of this process to other phases. The continuation of neutrophil recruitment as well as dysregulation of neutrophilic apoptosis, reduction of macrophage ability to clear apoptotic neutrophil will lead to prolongation of inflammatory condition, which may result in a chronic wound (Wilgus et al., 2013).

After neutrophilic apoptosis, macrophages continue killing the pathogens by producing NO. The stimulation occurs via TNF-α and IL-1, both react with peroxide ion oxygen radicals. In response to TNF alpha, macrophages together with fibroblasts and keratinocytes and monocytes expressed matrix metalloproteinase (MMP). Its function in this case is to clear and remove the damaged extracellular matrix, clear inflammatory debris as well as to enable and allow the migration of wound cells via the extracellular matrix (Keller & Sabino, 2015).

Then, to stop this inflammation phase, stop signals are required. Lipoxin together with aspirin triggered lipoxin are the stop signals for inflammation. Lipoxin produced by platelets and leukocytes, platelets alone cannot produce it unless it adheres to neutrophil. The mechanism is as follows: when platelets adhere to leukocytes, leukotriene A4 which is transferred to the platelets is produced. Platelets convert it to lipoxin A4 and B4 by platelets 12- lipoxygenase (Yasukawa et al., 2020).

This hemostasis and inflammation phase starts after injury and takes four to six days to complete (Ellis et al., 2018). During the inflammatory stage, influx of the leukocytes is the event by which we can characterise this stage. This is revealed by oedema and erythema at the lesion location. This cellular response is established within 24 hours, but sometimes it can extend up to 48 hours. Activation of the immunecells resides within the tissue may occur as well, like mastocytes and Langerhans cells leading to cytokines secretion together with chemokines. The role of inflammatory cells in wound healing is so important as they can contribute to lysosomal and reactive oxygen species release and for cleaning up the cell debris (Wilkinson and Hardman, 2020).

This proliferative phase involves epithelisation, angiogenesis and matrix formation. This is mainly for the process of tissue reconstruction. The number of cells in wound area started to increase due to migration and proliferation of endothelial cells and keratinocytes (Rodrigues et al., 2019). All three types of cells secrete mediators that stimulate and modulate the extracellular matrix biosynthesis, epithelisation and angiogenesis. The mediators release by fibroblasts are insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and epidermalgrowth factor(EGF). Endothelial cells produce vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and platelets-derived growth factor (PDGF), keratinocytes produce Transforming growth factorα (TGF-α), and TGF-β (Kurek- Górecka et al., 2020).

To stop fluid losses and avoid more bacterial attacks and invasions, skin epithelial cells begin to proliferate forming out projections for re-establishing the protective barrier. TGF-α and EGF are the stimuli for epithelial cells proliferation. The predominant cells proliferate in this phase is the fibroblasts and endothelial cells. IL-1 and tumour necrosis factor- α (TNF-α) upregulates the expression of keratinocyte growth factor (*KGF*) gene in fibroblast. Fibroblasts secrete KGF-1 and KGF-2 and IL-6. This will lead to keratinocytes stimulation and the cells then migrate, proliferate and differentiate (Deonarine et al., 2007). Fibroblasts and endothelial cells then start to form new capillary tubes by the effect of VEGF.

Hypoxia in wounds is due to disturbance in the vasculature around the wounded area. This will lead to an impairment in the oxygen delivery process and there will also be an influx of the inflammatory cells and these cells are in high demandto oxygen (Hong et al., 2014). Due to hypoxia, endothelial cells produce NO, this event

stimulates more production of VEGF. NO in high concentrations can also protect the newly made tissues from the ischaemia effect which is toxic to the tissues, it will lead to endothelial vasodilatation as well (Dulak et al., 2000).

Platelets and macrophages produced PDGF and EGF, both served as signals for fibroblasts to migrate into the site of injury. Platelets release PDGF and EGF via its alpha granules (Kanikarla Marie et al., 2021) . When fibroblasts become activated, they start to synthesize collagen (Tracy et al., 2016). The migration started in 2-3 days from the injury moment (Olczyk et al., 2014). The cells attracted to the site of injury due to chemotactic effect of PDGF, EGF and IGF-1. Cells then start to proliferate as well. Wound fibroblasts are already reside in the wound site (Diller & Tabor, 2022), these cells synthesise collagen and transform into myofibroblasts for wound contraction (Desjardins-Park et al., 2018).

The next event in the proliferative state is the synthesis of ECM and formation of granulation tissues. The newly formed connective tissues look granular because the tissues are intertwined with many capillaries (Maquart & Monboisse, 2014). Granulation tissues are generated by collagen, elastin, and proteoglycans, and glycosaminoglycans. Collagen type I and III stimulate collagenase expression and elastin regulates the accumulation of ECM components (Olczyk et al., 2014). The early granulation tissue matrix will be *in situ* for up to three days after injury. It contains high amounts of hyaluronic acid and fibronectin. Hyaluronic acid has the ability to swell, creating a woven structure which eases the cells to penetrate the wound area, while fibronectin facilitates the collagen fibrogenesis (Neuman et al., 2015). From the third day and forward, collagen replaces glycosaminoglycan, and hyaluronic acid concentration becomes reduced. The granulation tissues replace the dermis

temporarily, and matures to scar when it comes to the remodelling phase (Olczyk et al., 2014).

The tissue reconstruction starts with epithelisation. The main player is the epithelia cells participating in wound surface closure. It originates from either the wound edge or epithelial extensions like hair follicles and sweat glands (Pastar et al., 2014). The cells migrate, proliferate, and differentiate. Proliferation and migration are stimulated by EGF, KGF and TGF-α. When epithelial layers connected and formed a layer, the migration stopped. TGF- β is very important because it accelerates the maturation of epithelial cell layers (Kahata, Dadras, 2018). Keratinocytes separated from its basement membrane with the help of the matrix metalloproteinase MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B), both degrade collagen type IV and collagen type VII. Epithelization is the sign of healing (Rohani & Parks, 2015).

Endothelial cells migrate to the matrix to start tissue angiogenesis or vascularisation. The formation of new blood vessels is very important because it will lead to restoration of blood circulation at the place of injury. It stimulates the repair process and prevents ischemic necrosis development. The last phase of wound healing process is remodeling which features the deposition of collagen in an organized network. The wound surface becomes contracted. The granulation tissue develops, matures and forms a scar. Bigger blood vessels aggregate together, and this leads to reduction in the capillary. This will lead to reduction of proteoglycans and glycosaminoglycans (Qin, 2016).

During this phase, the cell density and the metabolic activity in the tissues becomes low. The amount of collagen type I increases in comparison with collagen type III, resulting in increased total collagen and tissue tensile strength. The number

of fibroblasts decreases. Then the scar tissues are being replaced by matrix, resembling the dermis in which collagen fibres construct a newly formed tissue (Cao et al., 2017).

Burn wounds are severe injuries that can be associated with a range of complications, thus pose a major healthcare problem. The number of people who are suffering from burn wounds was approximately 11 million, which is life threatening, as 180,000 people had died from burn wounds. There are multiple mechanisms of burn injuries such as intense heat, exposure to chemicals, injury due to electrical shock, or radiation. In these conditions, the damage can extend beyond the superficial skin layers, affecting the underlying tissues and organs. Complications associated with burn wounds can arise due to the extent and depth of the burn, as well as the presence of infection due to the loss of the skin's protective barrier. Burn wounds are vulnerable to bacterial invasion, resulting in cellulitis, abscess formation, sepsis, and fatal toxic shock syndrome (Markiewicz-Gospodarek et al., 2022). Another complication is impaired wound healing, where the damaged tissues struggle to regenerate and repair. This can result in the formation of hypertrophic scars, contractures, and keloids, which can restrict mobility and affect the functional and cosmetic outcome (Berman et al., 2017).

There are multiple steps involved in burn wounds management. Fluid resuscitation is a critical approach which is often lifesaving in most cases of burns. Intravenous fluid is instituted for rapid fluid replacement to avoid hypovolemia and organ failure. Adequate nutrition for wound healing is also important for recovery. This process must be controlled to avoid any complications. For example, giving excess carbohydrates can lead to hyperglycemia. Also, any infections in the wound should be controlled like giving antibacterial agents to burn wounds patients. Surgical

approaches are also a strategy either by wound dressings or surgical treatment of the wound (Kim et al., 2022).

2.1.5 Challenges in diabetic wound management

The patient factor is key to successful wound management. In the case of diabetic patients, good control of hyperglycaemia and keeping other medical conditions in optimum control are crucial for wound healing. Keeping the wound free of infections is major hurdle in diabetic wound care (Spampinato et al., 2020). Finding a wound dressing suitable to overcome these challenges is a critical hurdle for the scientists and researchers, as well as the clinicians working by the bedside for optimum wound care management in the patients.

Poor wound management results in complications, which may happen immediately or delayed. Some of these could lead to become a reason for morbidity and mortality (Sen, 2021). Wound complications include loss of wounded organ function, limited range of movement, loss of sensation, infections like cellulitis, gangrene, and devastating complication like lower-extremity amputation (Järbrink et al., 2016).

Diabetic wounds tend to heal more slowly than normal wounds because of the deficiency in insulin production (Spampinato et al., 2020). Insulin is crucial for regulating blood sugar levels and promoting the uptake of glucose by cells for energy production. In diabetic patients, the inadequate production or impaired functioning of insulin leads to hyperglycemia.

High glucose levels impede the normal healing process by several mechanisms. Firstly, hyperglycemia can cause microvascular complications that lead to local ischaemia, reducing blood flow to the wound site and compromising the delivery of oxygen and essential nutrients necessary for tissue repair. Secondly, elevated glucose levels can impair the immune system, by reducing neutrophils and macrophages functions, impairment of migration of the fibroblasts and also defect in the production of growth factors that will help in wound healing mechanism (Burgess et al., 2021).

Additionally, high glucose can directly affect the function of cells involved in wound healing, such as fibroblasts and keratinocytes, leading to slower collagen production and delayed epithelialization. Therefore, addressing both insulin deficiency and hyperglycemia is crucial in managing diabetes-related delayed wound healing, ensuring optimal healing conditions and minimizing the risk of complications. The main complications are infections, limitations in function, abscess, gangrene, and septicaemia (Patel et al., 2019).

It is challenging to manage diabetic wounds and to prevent the wound from getting infected. Some of the important preventive steps from wound infection is to maintain a good wound hygiene by using appropriate wound dressing materials, wound debridement (Singh & Gupta, 2017) and proper use of antimicrobials. Wound dressings serve many purposes like providing a suitable environment for the wound and maintaining it, for example, providing a moist environment. The moist environment facilitates wound healing process by improving the epithelization rate and improve the inflammatory phase via providing low oxygen tension in the woundedarea. Wound dressings materials play an important role in wound healing mechanismto promote humidity in the wounded area. The latter is to prevent contamination by micro-organism and bacterial growth (Jones et al., 2006).

Traditional wound dressings consist of inert materials such as cotton gauze and bandages. The cost is considerably low, and it is easy to make. There are some disadvantages associated with these materials; the fact that these dressings can adhere to the granulation site, and it is difficult for traditional dressings to keep the wound bed moist (Moore & Webster, 2018). The other type is modern dressings; which can maintain a moist environment, biodegradable and biocompatible (Brumberg et al., 2021). Modern wound dressings are made from synthetic polymers, and it contains three types: bioactive, interactive, and advanced interactive. Bactigras is a modern wound dressing usually used for treatment of wounds in burn patients (Dhivya et al., 2015). Melolin is also a commercialised wound dressing material made of polyester fibre pad and cotton which has a hydrophobic backing layer bonded with heat on the side of the film.

Studies are still ongoing to make more commercialised dressing materials. Currently, more wound dressing materials are made commercially and composed of biocompatible and biodegradable biomaterials and combined with drugs and antibiotics to improve the process of wound healing. As an example, chitosan, which has an antimicrobial property, has been used as a wound dressing material (Matica et al., 2019). Chitosan was shown to stimulate the mechanism of natural wound healing. It has been tested with mammalian cells and it was biocompatible. Chitosan hydrolyses glucosamine, which is natural in the body (Matica et al., 2019).

Selenium and silver had been loaded into a chitosan scaffold to test its antimicrobial effect and to be used as a wound dressing material. This strategy showed improvement in wound healing. Scaffolds of chitosan with selenium and silver were also shown to have anti-microbial effects, but not toxic to the fibroblasts (Biswas et

al., 2018). This biomaterial scaffold needed to be further characterized and warrants more *in vivo* studies.

Collagen is a protein produced by fibroblasts, it has a role in wound healing process, mainly in the improvement of the newly made tissues and in cell migration. Collagen wound dressings are very good for wound healing because it has the ability to stimulate the deposition of recently formed collagen. It also has a chemotactic effect on fibroblasts, thus, it can promote the wound healing mechanism and make a faster recovery (Mathew-Steiner et al., 2021). Clinical trials on different types of collagens are needed to compare and contrast its performance to be potential treatment agents.

Polymers, both natural and synthetic, are a major player in regenerative medicine and tissue engineering. Polymers have been used as wound dressing materials in its natural and synthetic forms. Hydrogels are an example of natural polymers. The most popular one from this group is the dextrans which is used for wounds and burn dressings, cellulose for chronic wound dressings, chitosan, and heparin (Alven & Aderibigbe, 2020). This will also give more options and varieties in the market.

Synthetic polymers have also been used for wound dressings. The synthesis methods are many but the most famous one is the electrospinning process. Synthetic polymers could be mixed with some agents that can enhance its performance like antibiotics. Polyurethane with dextrans nanofiber mats loaded with ciprofloxacin (a fluoroquinolone antibiotic) showed a good wound dressing biomaterial and it is a clear example of combining biomaterials with drugs to improve the performance. It showed a bactericidal activity with gram negative and gram positive bacteria and it was safe to cells (Unnithan et al., 2012). It also needs to be tested with different antibiotics and need

to be characterised further. The use of synthetic polymer is also good for wound dressing, but the availability and low cost of natural polymers are factors that encourage the use of natural biopolymers for wound dressing.

2.1.6 Diabetes induction by streptozotocin (STZ)

STZ is an antibiotic which acts by destroying the pancreatic β-cells. It is used in the establishment of diabetic models. STZ had been isolated from *Streptomyces achromogenes* in 1960 (Furman, 2021). STZ is a good model for both type I and type II diabetic induction in rats which has been widely used and well-studied in diabetic research (Wang-Fischer & Garyantes, 2018).

There are other methods to induce diabetes, using chemicals instead of STZ. For example, dithizone permeates the membranes, allowing zinc to enter inside the liposomes and this will enhance the diabetogenicity. In addition, repeated administration of growth hormones can induce diabetes in adult dogs and cats. Diabetes induction by STZ in animal models have been evaluated for the role of insulin resistance, obesity, and the inflammatory effects in diabetes (Kottaisamy et al., 2021).

The dose of STZ in diabetic induction varies between high and low concentrations. High STZ dose leads to severe hyperglycemia in mice, resembling a type 1 diabetic effect. In case of giving low concentration at successive doses, this will induce mild hyperglycemia in mice (Furman, 2021). Alloxan has also been used in diabetic induction in rats, rabbits, and monkeys. Single or multiple doses could be given either intraperitoneal or intravenous routes (Macdonald & Mohammed, 2018).

2.2 Propolis

Propolis is produced by honeybees. Honeybees collect materials like buds, exudates, some plants, and use it in the formation of propolis (Wagh, 2013a). The word 'propolis' is a Greek word meaning 'at the gate of the city' (Ahangari et al., 2018). Propolis has a role in the conservation and development of beehives. Honeybees use propolis in wall smoothing and close the holes in the honeycomb.

Propolis is a resinous substance also named bee glue. Honeybees use propolis for the building and maintenance of hives due to its nature and also the mechanical properties, it has a waxy nature.

Propolis from different sources have different compositions, but generally there is a common structure for different propolis types (GHANIA et al., 2018) as shown in Table 2.1. The structure of propolis is affected by the season when the propolis is harvested, the geographical area of collection, and the plant source that is used for propolis production (Anjum et al., 2018).

Table 2.1 General structure of propolis

Substance	**Percentage**
Resin	50%
Wax	30%
Essential and aromatic oils	10%
Pollen	5%
Various substances	5%

Propolis also acts as a guard against invaders from outside like lizards and snakes. It also has a role in the protection against natural circumstances like the wind and rain (Wagh, 2013). The role of propolis in human health had been well demonstrated. It had been used by ancient Egyptians for cadavers mummify. It was also

used by the Romans and Greeks in the ancient times for mouth disinfection and for treating mucosal and cutaneous wounds.

Propolis is produced by many species of bees like *Trigona* and *Apis* species. In Malaysia, the two main species that produced propolis are *Genitrigona thoracia* and *Heterotrigona itama* lebah Kelulut is the name of the propolis that produced by the stingless bee (*Trigona thoracica*) in Malaysia (Ibrahim et al., 2016; Mohd Izwan Zainol et al., 2013).

The propolis produced by the stingless bees is more potent than the one produced by honeybees. The stingless bees produced propolis more than honey. Also, the biological activity and composition of the propolis is widely affected by the species that produced it (Ibrahim et al., 2016).

2.2.1 Physical characteristics

Propolis has different physical properties, and it is variable based on the collection area and sources and the geographical region (Anjum et al., 2018; Sulaeman et al., 2019) (Table 2.2).

Table 2.2 Physical criteria of propolis

Melting temperature	60-100 °C
Color	Different according to the area of collection and the plant source
Low temperature	Hard
Appearance at high temperature	Soft

The applications of propolis are many and it has been used in many different areas in medicine, health, and medical research due to its characteristics as antibacterial, antifungal, and its role in wound healing.

(a) Antibacterial

Propolis can act directly on the microbes, and it can stimulate the host immune system as well. The immune system triggers its defense mechanism naturally (Magnavacca et al., 2022). Ethanolic extracted propolis has been tested for its antibacterial activity. It has been tested with different bacterial strains isolated from humans and the bacteria were tested with propolis collected in different seasons to study the seasonal effect and whether there is a different in the activity ofthe propolis collected in the four seasons. Gram positive bacteria were inhibited by propolis, while Gram negative bacteria have low susceptibility to the propolis. The seasonal differences have no much effects on the activity of the propolis (Przybyłek & Karpiński, 2019).

The propolis works *in vivo* by activating the immune system, while the mechanism of action *in vitro* is by direct attacking of the microorganism. The difference in the effect of propolis between Gram positive and Gram negative bacteria is due to the fact that the membrane of Gram negative bacteria produce enzymes with hydrolysis activity that halt the propolis effective components (Sforcin, 2016). The composition of the propolis has a major role in its antibacterial activity through the phenolic compounds like flavonoids (Przybyłek & Karpiński, 2019).

Propolis has previously tested with poly vinyl alcohol (PVA) polymer to study the antibacterial effect. A blend from polymer and propolis extract was prepared and it showed a good antibacterial effect against Gram positive bacteria. It also had good healing effect after Gram positive bacterial infected-wound (Arıkan & Solak, 2017). To increase and boost the biological activity, Al-Waili and colleagues mixed two different propolis types and studied its effect in wound healing and microbial growth (Al-Waili, 2018). They tested two types of propolis collected from different

geographical areas in Iraq with *E. coli, S. aureus, and C. albicans* and they used rabbits in an *in vivo* study. Their results demonstrated that the mixing of different types of propolis is good for wound healing expressed better antimicrobial activity (Al-Waili, 2018).

(b) Antifungal

Propolis also has antifungal activity against 80 strains of *Candida* yeasts. The highest antifungal activity was observed with *C. albicans, C. tropicali, C. Krusei,* and C. *guilliermondii*. It worked well on fungal-infected dentures, when the number of *Candida* had significantly decreased after using hydroalcoholic propolis as mouth rinse. Also, saliva-isolated *Candida* strain were shown to be sensitive to propolis (Rivera-Yañez et al., 2022).

Another study had been intended to assess the antifungal activity of the Brazilian propolis on patients with vulvovaginal candidiasis (VVC). Ethanolic extract of propolis was used together with propolis microparticles and the results showed that the propolis have an inhibitory effect on yeast (Bruschi et al., 2011).

2.2.2 Propolis as a wound healing agent

Propolis is beneficial for wound healing enhancement. The influence of the topical application of propolis in the enhancement of the wound healing process has been tested in an *in vivo* studyusing streptozotocin-induced type I diabetic mouse model. Topical propolis application improved wound healing and closure. There is a reduction in the level of IL-1β, IL-6, matrix metallopeptidase 9 (MMP9), and TNF-α. There is also an increase in collagen production in the mice treated with propolis and this happened via the TGF-β1/Smad 2,3 signaling pathways (Garraud et al., 2017).