THE EFFECTS OF CHANNA STRIATUS EXTRACT ADMINISTRATION ON WOUND HEALING MARKERS (IL-6, MMP-9 AND VEGF) IN POST LOWER SEGMENT CAESAREAN SECTION (LSCS) WOMEN

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by

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"Jazzakallah khairan kathira"

Ahmad Ezam Zainan

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BUSE	Blood Urea Serum Electrolyte
CNTF	Ciliary Inhibitory Factor
CT-1	Cardiotropin-1
DHA	Decosahexaenoic Acid
DNA	Deoxyribonucleic Acid
ECL	Electrochemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylene-di-amine-tetra-acetic acid
ELISA	Enzyme Link Immunosorbent Assay
EPA	Eicosapentaenoic Acid
EV-T	Extravillous Trophoblast
FBC	Full Blood Count
FGF	Fibroblast Growth Factor
GMP	Good Manufacturing Practice
HRP	Horseradish Peroxidase
HRPZII	Hospital Raja Perempuan Zainab II
HUSM	Hospital Universiti Sains Malaysia
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-11	Interleukin-11

LIST OF ABBREVIATIONS (Continued)

IL-27	Interleukin-27
IL-30	Interleukin-30
IL-6	Interleukin-6
ITT	Intention-To-Treat
LIF	Leukemia Inhibitory Factor
LOCF	Last Observation Carried Forward
LSCS	Lower Segment Caesarean Section
MMP-9	Matrix Metalloproteinase-9
mRNA	Messenger Ribonucleic Acid
NAGly	N-arachidonoylglycine
NIBSC	National Institute for Biological Standards and Control
NNT-1	Neurotrophin-1
NPN	Neuropoietin
NSAIDs	Non-Steroidal Anti-Inflammatory Drug
O&G	Obstetrics and Gynaecology
OSM	Oncostatin M
PDGF	Platelet-Derived Growth Factor
PIGF	Placental Growth Factor
PMNs	Polymorphonuclear Leukocytes
PP	Per Protocol
TGF-β1	Transforming Growth Factor Beta 1
TIMPs	Tissue Inhibitors of Metalloproteinase
TNF-α	Tumor Necrosis Factor-α

LIST OF ABBREVIATIONS (Continued)

TPA	Trypropylamine
VEGF	Vascular Endothelial Growth Factor

KESAN PENGAMBILAN EKSTRAK CHANNA STRIATUS KE ATAS PENANDA-PENANDA PENYEMBUHAN LUKA (IL-6, MMP-9 DAN VEGF) DI KALANGAN WANITA SELEPAS PEMBEDAHAN CAESAREAN

ABSTRAK

Channa Striatus (Haruan) digunakan secara meluas di Malaysia untuk menggalakkan penyembuhan luka. Sejak turun temurun, *C.striatus* telah dipercayai mempunyai sifat anti-radang, anti-oksida dan anti sakit untuk penyembuhan luka yang lebih baik. Penyembuhan luka adalah satu proses dinamik yang melibatkan empat fasa yang berbeza namun berlaku secara serentak dan bertindih iaitu hemostasis, keradangan, percambahan dan pembentukan semula tisu. Proses yang sangat rumit ini bermula dengan serta-merta selepas kecederaan dan melibatkan tindakbalas yang tersusun antara pelbagai jenis tisu-tisu dan sel-sel. Di dalam proses penyembuhan ini, leukosit, makrophaj dan sel endothelial akan mengeluarkan pelbagai faktor-faktor pertumbuhan dan sitokin seperti *Interleukin 6 (IL-6), Matrix Metalloproteinase-9 (MMP-9)* dan *Vascular Endothelial Growth Factor (VEGF).* IL-6 mengawal penghasilan leukosit ke kawasan inflammasi. Fungsi MMP-9 adalah mengeluarkan extracellular matrik yang telah rosak, pemindahan sel-sel epidermal dan penghasilan semula tisu baru. VEGF pula merangsang pembentukan saluran darah baru melalui proses yang dipanggil angiogenesis. Kajian ini dijalankan untuk membandingkan paras dan trend IL-6, MMP-9 dan VEGF di dalam kalangan pesakit-pesakit mengambil ekstrak *C.striatus* dan placebo (maltodextrin) dalam proses penyembuhan luka untuk wanita yang menjalani pembedahan caesarean (LSCS). Kajian ini telah dijalankan secara rawak, *double blinded* dan kawalan plasebo di Hospital USM dan Hospital Raja Perempuan Zainab II. Bagi tempoh setiap hari selama 6 minggu, kumpulan yang *C.striatus* diberikan dos 500 mg serbuk

kering ekstrak *C.striatus* manakala kumpulan plasebo pula diberikan 500 mg maltodextrin. Sampel darah vena diambil dari setiap pesakit selepas pembedahan pada hari 1, hari ke-3, minggu ke-2, minggu ke-4 dan minggu ke-6 dan telah dianalisa untuk penanda ini. Analisis data dilakukan menggunakan perisian SPSS versi 22. Sejumlah 73 pesakit telah dikaji, 39 pesakit diberikan ekstrak *C.striatus* dan 34 pesakit lagi diberikan maltodextrin. Daripada kajian ini, didapati bahawa perbandingan antara kumpulan ekstrak *C.striatus* dan kumpulan plasebo (maltodextrin) untuk IL-6 dan MMP-9 adalah signifikan pada minggu ke-4 ($p < 0.001$, $p < 0.05$) dan minggu ke-6 ($p < 0.001$, $p < 0.05$) masing-masing manakala VEGF pula menunjukkan signifikan pada hari 1 ($p < 0.05$), hari 3 ($p < 0.05$), minggu ke-4 ($p < 0.05$) dan minggu 6 ($p < 0.001$). Sebaliknya, perbandingan antara kumpulan menunjukkan perbezaan yang tidak signifikan untuk IL-6 dan MMP-9 pada hari 1 ($p = 0.475$, $p = 0.104$), 3 hari ($p = 0.131$, $p = 0.315$) dan minggu 2 ($p = 0.052$, $p = 0.397$) masing-masing manakala VEGF pula pada minggu 2 ($p = 0.738$). Kajian ini juga menunjukkan bahawa penanda-penanda ini menunjukkan tren yang berkaitan terus dengan proses penyembuhan luka serta membuktikan bahawa pengambilan ekstrak *C.striatus* terlibat dalam membantu meningkatkan proses penyembuhan luka.

THE EFFECTS OF CHANNA STRIATUS EXTRACT ADMINISTRATION ON WOUND HEALING MARKERS (IL-6, MMP-9 AND VEGF) IN POST LOWER SEGMENT CAESAREAN SECTION (LSCS) WOMEN

ABSTRACT

Channa striatus (ikan haruan) is widely consumed in Malaysia to promote wound healing. For centuries, *C.striatus* has been proposed to have anti-inflammatory, anti-oxidant and anti-nociceptive properties for better healing of the wound. Wound healing is a dynamic process that involves four disticnt yet an overlapping phases; haemostasis, inflammation, proliferation and tissue remodeling. It starts immediately after injury and proceeds with a complicated but well-organized interaction among various types of tissues and cells. During wound healing process, leukocytes, macrophages and endothelial cell release various growth factors and cytokines such as interleukin 6 (IL-6), matrix metalloproteinase 9 (MMP-9) and vascular endothelial growth factor (VEGF). IL-6 regulates leukocytes recruitment to the inflammatory sites. MMP-9 involves in removal of damaged extracellular matrix, migration of epidermal cells and tissue remodelling. VEGF stimulates the new blood vessels formation through a process called angiogenesis. This study was done to compare the level and trends of IL-6, MMP-9 and VEGF in patients receiving *C.striatus* extract and placebo (maltodextrin) during wound healing of post lower segment caesarean section (LSCS). This was a randomized; double blinded, placebo-controlled study conducted in Hospital USM and Hospital Raja Perempuan Zainab II. The treatment group was administered orally with 500 mg of freeze dried *C.striatus* extract daily while the placebo group with 500 mg of maltodextrin daily for 6 weeks. Venous blood samples were taken from each subject post-operatively at

day 1, day 3, week 2, week 4 and week 6 and analysed for healing markers (IL-6, MMP-9 VEGF) and safety profile test (FBC, BUSE, Creatinine, ALT and AST). Statistical analysis were done using SPSS Version 22. A total of 73 patients were studied, 39 patients consumed *C.striatus* and 34 consumed maltodextrin. From this study, the level of wound healing markers (IL-6, MMP-9 and VEGF) was higher in *C.striatus* extract group compared to placebo. Comparison between *C.striatus* extract group and placebo (maltodextrin) group for IL-6 and MMP-9 were significantly different at week 4 ($p < 0.001$, $p < 0.05$) and week 6 ($p < 0.001$, $p < 0.05$) respectively whereas VEGF show significant different at day 1 ($p < 0.05$), day 3 ($p < 0.05$), week 4 ($p < 0.05$) and week 6 ($p < 0.001$). In contrast, comparison between groups shows no significant different for IL-6 and MMP-9 at day 1 ($p = 0.475$, $p = 0.104$), day 3($p = 0.131$, $p = 0.315$) and week 2 ($p = 0.052$, $p = 0.397$) respectively, except for VEGF which not significant at week 2 ($p = 0.738$).This study also shows some trends in conjunction with healing phase and shows that the indication of *C.striatus* extract was involved in enhancing the wound healing process.

CHAPTER 1

INTRODUCTION

1.1 *Channa striatus*

Channa striatus (Haruan) or snakehead fish, known locally to Malays as haruan, is freshwater, air-breather and carnivorous fish indigenous to many tropical and subtropical countries including Malaysia which are a valuable source of protein throughout the Asia Pacific region (Mat Jais, 2007). For centuries, it has been used as traditional medicine for treatment of illness, wound healing supplement and as a pharmaceutical among Malaysian populations especially Malays.

C. striatus has been studied extensively for its commonly believed on wound healing properties. It is used by patients in the post-operative period to enhance the wound healing processes and to reduce pain (Mat Jais *et al*., 1997).

C.striatus is found to contain all the essential amino acid for wound healing ,particularly glycine which is the most important component of human skin collagen (Mat Jais *et al*., 1998). Several studies have found that, *C. striatus* also rich in fatty acid. Among them, arachidonic acid is most important since it plays an important role as a precursor of prostaglandin to initiate blood clotting and be responsible for growth. The high reputation of *C.striatus* in traditional medicine is due to its exceptionally properties such as anti-nociceptive, energy booster, anti-oxidant, anti-inflammation, wound healing, anti-microbial as well as cardivascular and neurological effects (Haniffa *et al*., 2014).

1.2 Wound healing phase

Wound healing is a dynamic process and involves complex interaction of extracellular matrix molecules, solubles mediators and various immune cells (Eming *et al*., 2007). It is best understood as an organism's global response to injury, regardless of whether the location is skin, liver, or heart (Gurtner, 2007) and well described at the histological levels. Classically, wound healing phase involved three distinct and overlapping phases : inflammation, new tissue formation (proliferation) and tissue remodelling (Schafer and Werner , 2008; Eming *et al*., 2007). However in some literature, wound healing phases are divided into four overlapping processes; haemostasis, inflammation, new tissue formation (proliferation) and tissue remodelling (DiPietro and Luisa , 1995).

In the moment where tissue is injured, the normal healing response begins with a set of complex biochemical events take place in a closely intergrated cascade to repair the damage (Diegelmann *et al.*, 2004). As the blood components spill into the site of injury, the platelets come into contact with exposed collagen and other elements of the extracellular matrix , resulting in temporary seal of wound (Eming *et al.*, 2007). The activated platelets together with fibrin close off the lymphatic channels in the injured area (Myers, 2012). This plug clot also provide provisional matrix for cells migration and this initiates the inflammatory phase thus triggers the platelets to release various chemotactic agents and chemical mediators, including growth factors (such as vascular endothelial growth factor (VEGF), platelet-derived growth factors (PDGF) and fibroblast growth factor (FGF) and cell signalling chemical known as cytokines (such as interleukin-1(IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Gurtner, 2007).

In the transition between inflammation and repair, there is a decrease level of inflammatory cytokines and immune cells and effect by the release of anti inflammatory mediators interleukin-10 (IL-10) and TGF-β1 by macrophages. Other cells such as the endothelial cells, fibroblasts and keratinocytes start migrating into the wound and proliferate for initiation of second phase of wound healing, the phase of new tissue formation. Granulation tissue is the new tissue that initially replaces the lost dermis. It consists of fibroblasts, endothelial cells and inflammatory cells (Gurtner, 2007). The granulation tissue is continuously replenished by new blood vessels in order to support the new tissues with oxygen and nutrients (Schafer and Werner, 2008). Termination of wound healing process is by a long remodelling phase (Schafer and Werner, 2008) whereby, the fibroblasts differentiate to myofibroblasts, which are responsible for formation of collagen and other extracellular matrix protein and also for wound contraction (Schafer and Werner , 2008). Among the mediators involved in wound healing, interleukin-6 (IL-6), matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) is chosen in this study to represent different type of markers ; cytokine, enzyme and growth factor respectively.

1.3 Wound healing markers

Interleukin-6 is a proinflammatory cytokines that has long been thought to play an important role in wound repair (Eming *et al*., 2007). The role of interleukin-6 (IL-6) is well known as a proinflammatory cytokines which is crucial for kick-starting of the healing response and was shown to be up regulated in inflammatory phase of healing (Ringsdorf and Cheraskin,1982). There are diffrent types of cells involve in stimulation of IL-6 secretion such as fibroblast, macrophages, endothelial cells and keratinocytes in response to multiple stimuli together with other cytokines

such as interleukin-1, tumor necrosis factor and platelet-derived growth factor (Grossman *et al.*, 1989). IL-6 could help initiate the cellular factors in the local inflammatory infiltrate such as T cells, macrophages and polymorphonuclear neutrophils (PMNs), give rise to an intensification of the local wound (Grossman *et al.*, 1989). The coordinated expression of this cytokine is important for normal repair, since reduced expression seen after wounding of healing-impaired glucocorticoid-treated mice (Ringsdorf and Cheraskin,1982, Werner and Grose,2003) and prolonged in genetically diabetic mice (Werner and Grose,2003). A study on IL-6 knock-out mice, showed that, it took up three times longer for the wound to heal than those of wild type control (Galluci *et al*.,2000;Werner and Grose, 2003). These abnormalities were rescued by administration of recombinant murine IL-6 protein one hour before wounding. Thus it appears that IL-6 is crucial for kick-starting the healing response (Eming *et al*., 2007). A complete lack of it causes impaired healing and excessive level of IL-6 have been associated with cutaneous scarring (Werner and Grose,2003) and also in epidermal hyperplasia as shown in psoriasis patients (Grossman *et al*., 1989).

Matrix metalloproteinases (MMP-9) known as gelatinase B are family of structurally related, zink-dependent endopeptidases and play an important role in the proteolytic remodeling by degrading most of the structural components of extracellular matrix (ECM) in various physiologic condition including tissue repair (Iba Y *et al*., 2004). MMP-9 is produced during inflammation phase by inflammatory cells including the PMNs, monocytes, macrophages and keratinocytes and their concentration levels are gradually decreases after the initial burst and also coordinated by hormones, growth factors, and cytokines, and also involved in ovarian bioactivity. A sustaintial elevated level of MMP-9 is found in chronic wound

cases while low levels are found in excessive scar formation. MMP-9 enzymes are secreted as zymogens, which in turn treated by other proteolytic enzymes (such as serine proteases, furin, plasmin and others) to provoke the active forms (Verma and Hansch, 2007).

Vascular endothelial growth factor (VEGF) is sub-family of growth factor, specifically platelet derived growth factor (PDGF) family of cystine-knot growth factors. They are important signaling proteins involved in both vasculogenesis and angiogenesis. The important role of VEGF for the healing process is supported in several studies where reduced expression of VEGF or its accelerated degradation were found to be associated with wound healing defect. Furthermore, treatment of wound with VEGF accelerated healing process. Recently one study has shown, application of neutralizing antibodies to VEGF caused a striking reduction in wound angiogenesis, fluid accumulation and granulation tissue formation in pig model (Howdieshell *et al*., 2001). In another study in human wound fluid, the angiogenic activity was strongly inhibited by VEGF neutralization (Werner and Grose , 2003).

1.4 Rationale of study

Although there are many extensive studies have been done on the effect of *C. striatus* in wound healing, the clinical trial on human is rarely done. The true and specific biological effects of this fish in the improvement of wound healing remain unclear because of the lack of study in human subjects.

This study was done to assess the effect of orally administered *C.striatus* extract that may possible have a role in wound healing. This study would observe at the selected markers to substantiate the claims. Subjects among post LSCS women

were chosen in this study because this group is the commonest group that consumes *C.striatus* to promote wound healing after delivery.

CHAPTER 2

OBJECTIVE AND HYPOTHESIS

2.1 Objective of the research

2.1.1 The general objective of this study was to evaluate the effects of *C.striatus* extract consumptions on wound healing markers among post Lower Segment Caesarean Section (LSCS) women.

2.1.2 The specific objectives were :

a. To compare the level of wound healing markers (IL-6, MMP-9 and VEGF) between *C. striatus* (ikan haruan) extract group and Placebo (maltodextrin) group among post lower segment caesarean section (LSCS) women

b. To observe the trend of the wound healing markers within groups (*C.striatus* group as well as placebo group).

c. To observe the trend of the wound healing markers between groups (*C.striatus* group and placebo group).

2.2 Benefit of the study

Since this fish is widely consumed by the population for the medicinal value and with the believe that it fasten the wound healing process, this study will provide scientific evidence on the biomedical potential of *C.striatus* (Haruan) as claimed. This study would also promote and commercialise *C.striatus* product and unravelled the biomedical potential of the fish. It is also set as research platform to higher value-capture in agricultural sector thus generate ideas for further studies regarding the medical use of this fish.

These initiatives can help expedite higher value-capture in agricultural sector thus generate greater economic activities and lay foundation for a sustainable agro-based industry driven by agro-biotechnology development.

2.3 Research hypothesis

There are differences in the level and trend of wound healing markers (IL-6, MMP-9 and VEGF) in post LSCS women who consumed *C.striatus* extract compared to placebo.

CHAPTER 3

LITERATURE REVIEW

3.1 Wound Healing

Wound can be defined as a break in the epithelial integrity which could range from simple to severe. Severe deep wound causes multiple destructions throughout all tissue structures. It involves cell layers and lineages from both sides (inside and outside) including epidermal keratinocyte layer (outmost skin layer), epidermal appendages (such as sweat glands and hair follicle), basement membrane (epidermis and dermis) which also involves complex cellular structure such as fibroblast, nerves, extracellular matrix and blood vessel (Shaw *et al.*, 2009). Depending on the depth of the wound, it can even involve other body structures such as nerves, muscles, tendons, parenchymal organs and bone as well (Velnar *et al*., 2009).

Wound healing is a dynamic and interactive continual biological activity in living cell which start in split second following tissue injury (Zi-Qing Lin *et al*., 2003). Its process encompasses of complicated well-organized biological process involving many biomarkers, enzymes and cell-signalling events (Diegelmann *et al*., 2004) to recover and stabilize the physiology normal function and activity of tissue/organ after the physical disruption (Gurtner, 2007). All of the reactions in the healing process dependent on energy and nutrition such as micronutrients, protein, carbohydrates, fat and amino acids (Arnold and Barbul, 2006). Wound healing process is divided into four sequential, distinct and overlapping phase as shown in Figure 3.1. The four phases are haemostasis phase, inflammatory phase, proliferation phase and tissue remodelling phase. In some literature, only three phases of wound healing were elaborated (Gurtner *et al*., 2008). Haemostasis phase and inflammation

phase are considered as one as both occurs concurrently. This phase is called as the inflammation phase.

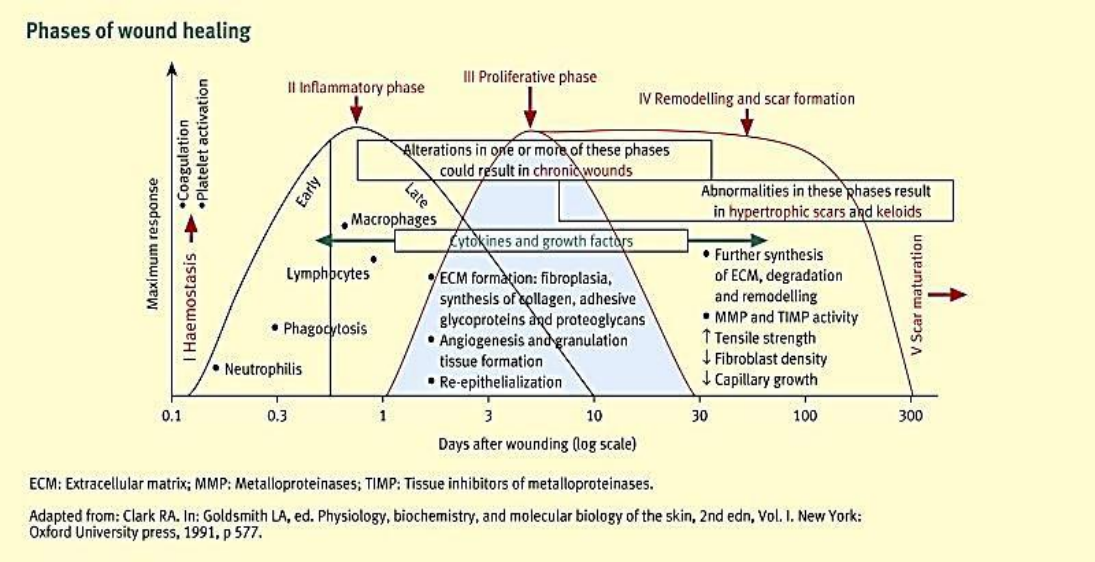

Figure 3.1 Wound healing phases (Adapted from : Brenner K., 2010).

Many factors can affect wound healing. It can be divided into local and systemic factors as shown in Table 3.1 (Guo and DiPietro, 2010). According to Guo and DiPietro (2010), local factors are mainly cause by the symptom arise from the wound itself, whereas a systemic factors referred to an individual health status that could affect the healing mechanism such as chronic diseases.

Hypertrophic scars or non-healing chronic wounds (ulcers) occur as a result of improper wound healing. Chronic wounds are presenting the most prevalent wound healing problems in people which also associated with a small number of well defined, clinical entities, in particular venous insufficiency, diabetes mellitus, pressure necrosis, and vasculitis (Eming *et al.* 2007).

Table 3.1 Factors affected wound healing mechanism (Guo and DiPietro,2010)

Local factors	Systemic factors
Oxygenation	Age and gender
Infection	Sex hormones
Foreign body	Stress
Venous sufficiency	Ischemia
	Diseases: diabetes, keloids, fibrosis, hereditary healing disorders, jaundice, uremia
	Obesity
	Medications: glucocorticoid steroids, non-steroidal anti-inflammatory drugs, chemotherapy
	Alcoholism and smoking
	Immunocompromised conditions: cancer, radiation therapy, AIDS
	Nutrition

All the above factors could result in non-healing wounds as it interferes with the normal physiological process of wound healing.

3.1.1 Haemostasis phase

In split second after tissue injury, coagulation and haemostasis phase begin followed by inflammatory phase. This phase lasts for 24 to 48 hours (Gurtner, 2007) and requires the combination activity of vascular, platelet, and plasma factors. During this phase, platelets play a critical function by forming an immediate supportive web-like matrix so call a fibrin clot or blood clot to prevent blood loss and enhance the collagen accumulation at the wound area as shown in Figure 3.2. Initial platelet adhesion factor secreted by endothelial cells into the sub endothelium. The clot also acts as a transitional matrix to which growth factors bind and set up the movement of a cell space (Shaw *et al.*, 2009). In the meantime, platelets also stimulate the next healing phase by producing various cytokines and growth factors

which is responsible for promoting various mechanisms such as angiogenesis, inflammation and attraction of keratinocytes and fibroblast into the wound area (Shaw *et al*., 2009). Approximately over 300 signalling molecules are segregated from activated platelets (Young *et al*., 2001). Once formation of platelet plugs and fibrin clot are stable, next phase of wound healing which is inflammation phase begins. Abnormalities in this haemostasis phase can lead to excessive bleeding or thrombosis.

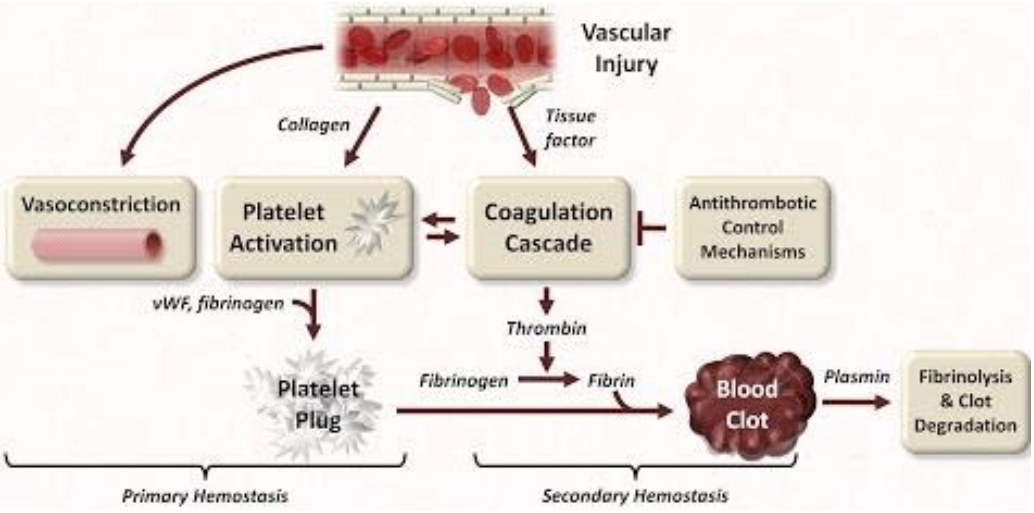

Figure 3.2 Haemostasis phase pathway diagram (Adapted from : Digplanet : Hemostasis, 2009)

3.1.2 Inflammation phase

This phase begins immediately with haemostasis and leads to inflammation. Inflammation is a key phase of wound healing that is required to protect against infection. There are 4 types of key cells involve directly in inflammatory phase as shown in Table 3.2. Platelets that form the clotting or thrombus release several growth factors that promote the chemotaxis and proliferation of inflammatory cells (Gurtner, 2007). Resident immune cells such as mast cells, T cells and Langerhans cells are rapidly activated to release chemokines and cytokines (Shaw *et al.*, 2009).

Platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) are two types of important cytokine release by platelet (Gurtner, 2007). They are important chemo attractant which promote the migration of macrophage, smooth muscle, fibroblast and polymorphonuclear neutrophils (PMNs) to the wound. Within hours after injury residential mast cells degranulation and become less plausible. About 48 hours later, mast cells activity become normal, and rises in number as tissue repair progresses (Eming *et al*., 2007).

Table 3.2 Type of cells involve in inflammatory phase and its function (Myers, 2012)

Cell	Functions
Platelet	1. Forming fibrin clot to control and stop the bleeding 2. Secretes growth factor and chemotactic agents (cytokines)
Polymorphonuclear neutrophils(PMNs)	1. First cell arrive to wound area 2. Act as scavenger or hunter 3. Kill bacteria 4. Cleans wound area 5. Produce inflammatory mediators and MMPs
Macrophages	1. Manage and promotes healing process 2. Kill bacteria (phagocytosis) and cleaning wound area 3. Produce growth factors and MMPs
Mast cell	1. Release enzyme to destroy damage cells. 2. Secretes inflammatory mediators

Within one hour, PMNs are among the first inflammatory cells arrived into wound area and become predominant cells after 24 hours. PMNs migrate towards the wounded area by amoeboid-like movements known as diapedesis (Myers, 2012). They work by eliminating all debris and bacteria by mechanism called phagocytosis.

They also clear death and damaged tissue by secreting an enzyme called protease that breakdown unwanted tissue from wounded area. By this mechanism, wound areas are clean and ready for formation of collagen through proliferation phase stimulated by fibroblast (Young *et al*., 2001).

Within a few days, PMNs activity slows down and is replaced by macrophage and lymphocytes. Once all the contaminating bacteria have been eliminated, PMNs are eliminated from the wound after completing their task prior to breakthrough to the next phase of healing. The cell remnants and apoptotic bodies are then digested by macrophages (Velnar *et al*., 2009). Macrophage is believed to be the predominant cells two days after injury and involve in promoting angiogenesis.

Macrophage is the primary source of pro-inflammatory cytokine (Interleukin-6), growth factor (such as vascular endothelial growth Ffactor (VEGF)) and proteolysis enzymes (matrix metalloproteinase (MMP's). Other roles of macrophages are killing pathogens by releasing nitrous oxide and bactericidal enzymes (Myers, 2012). At the end of this phase, formation of extracellular matrix and re-epithelialization begins together with angiogenesis and formation of tissue granulation (Turabelidze *et al.*, 2012). Once the fibrin complexes are cleared by fibrinolysis enzymes, the activated keratinocytes migrate along the interface between the clot and the underlying healthy dermis to aid the re-epithelialization of new epithelial cells (Hoeben *et al*., 2004). Signs of inflammation at this phase are pain, swelling, redness, warmth and decreased function and commonly known in Latin words;" *tumor, rubor, calor, dolor and functio laesa"* (Myers, 2012).

3.1.3 Proliferation phase

In the proliferation or known as tissue repairing phase, TGF-β stimulates the macrophages to secrete fibroblast growth factor (FGF), PDGF, tumour necrosis factor alfa (TNF-α) and interleukin (Diegelmann *et al*., 2004) to initiate new tissue formation. Table 3.3 list four main cells involve in proliferation phase and its specific function. There is an extensive growth of epithelial cells under the scab that bridges the wound.

Table 3.3 Main cells involved in proliferation phases and its function (Myers, 2012)

Cell	Function
Angioblast	Forming new blood vessel
Fibroblast	Builds granulation tissue and collagen
Myofibroblast	Causes wound contraction
Keratinocyte	Re-epithelializes wound surface

Fibroblast forming a network of collagen in wound bed which gives strength to tissue matrix and this process depends on zinc, oxygen and ascorbic acid. The proteolytic matrix metalloproteinases (MMPs) facilitate the movement of fibroblast within the provisional matrix (Shah *et al*., 2012). TGF-β stimulates the secretion of proteoglycans and fibronectin in the proliferative phase to induce the protein matrix formation and promoting a new connective tissue formation to fill in wound (Diegelmann *et al*., 2004). Meanwhile, anti-inflammatory cytokines such as IL-10 and TGF-β produced by macrophages inhibit and reduce the inflammatory cytokine. More oxygen and nutrients are needed to support this high metabolic activity. New blood vessels are produced by a process known as angiogenesis or neovascularization in order to transport oxygen and nutrients to the wound area at the same time removing the end product and toxin from the area (Nieves *et al*., 2009).

Angiogenesis mechanism led by angioblasts is regulated and induced by vascular endothelial growth factor (VEGF). VEGF is secreted by epidermal cell, fibroblast, macrophage and vascular endothelial cell (Diegelmann *et al.*, 2004). Angiogenesis is an example of a process that occurs during wound healing that requires adhesion of cells to the ECM in order for them to respond to growth factors. Once the angiogenic switch is induced, the formation of new blood vessels, a complex and highly regulated sequence of events takes place. Multiple proteases are activated and this pathway promotes the degradation of the basement membrane surrounding the existing vessel. This is followed by increased proliferation of endothelial cells and in turn the formation of a lumen as well as a new basement membrane. Finally, the newly formed vessels are fused together. With adequate oxygen supply into wound area, the proliferation phase and cellular functions are maintained (Youn, 2001).

Later, some of fibroblast within the healing site are transformed into myofibroblast and act as smooth muscles pulling the wound edge together. These mechanisms are called wound contraction and are affected by the shape, depth and wound size. Wound contraction is an important part of wound healing as it means that the body does not have to make as much granulation tissue to fill in the wound cavity. The tensile strength of the wound is increased during this stage of the healing process and this process continues into the next phase, the maturation and tissue remodelling phase.

3.1.4 Tissue remodelling phase

The remodelling phase is triggered when wound has filled in and re-surfaced. This phase begins at about 2 to 3 weeks and could last from 21 days to a year (Gurtner, 2007). At this time, the production of collagen and tissue degradation have reached equilibrium phase. Tissue remodelling and scar formation are mainly influenced and promoted by the effects of TGF-β on fibroblasts. TGF-β induces the synthesis of collagens and glycosaminoglycans thus stimulate the fibroblast function to reorganize and cross-links the collagen. Collagen remodelling is also regulated by matrix metalloproteinase (MMP's) that are produced by macrophage, fibroblast and endothelial cells. There are many types of MMPs, however MMP-9 are involved directly in the contraction and remodelling of scar tissue in the extracellular matrix by removing and replacing with new and highly organised tissue structure (Gibson *et al*., 2009). Fibroblast in contracting wound increases actin microfilaments and the area is designated as myofibroblast which then pull collagen fibres together. In turn, this normal process closes wound from the external environment with new cell barriers. Most myofibroblast diminished themself through programmed suicide process known as apoptosis (Shaw *et al*., 2009). After 3 months, tensile strength of the wound gradually increased and reaches about 50% of original tensile strength (Young et al. 2001). The recovered tensile strength in a wounded area reduced about 20% compared to the original tissue strength (Diegelmann *et al.*, 2004). In cases where the wound recurs in the same area, the maximum tensile strength will achieve approximately 64% out of 80% of original tissue strength (Myers, 2012).

3.2 Wound healing markers

Wound healing markers have been studied extensively. As shown in Table 3.4, there are examples of some wound healing markers and that have been reported and studied nowadays. Many endless and comprehensive studies done by researchers around the globe have identified many biochemical pathways and molecules that correlate with wound healing activity. Mostly, platelets, neutrophils, macrophages and fibroblast commit important role in the wound healing process by secretion of various cytokines and growth factors (Shah *et al.*, 2012).

Table 3.4 List of some cytokines, growth factors, and enzymes involved in the wound healing process (Shah *et al.*, 2012)

Name	*Abbreviation*	*Class*	*Produced by*	*Action*
Interleukins 1, 6, 8	IL1, IL6, IL8	Cytokines	Macrophages, keratinocytes	Proinflammatory; recruit fibroblasts and keratinocytes
Interleukin 2	IL2	Cytokine	T lymphocytes	Recruits fibroblasts
Interleukin 4	IL4	Cytokine	T lymphocytes	Inhibits TNF, IL1, IL6, inhibits fibroblast proliferation
Tumor necrosis factor alpha	TNF-α	Cytokine	Macrophages	Proinflammatory; helps collagen synthesis
Epidermal growth factor	EGF	Growth factor	Platelets, macrophages, keratinocytes	Promotes keratinocyte and fibroblast proliferation, keratinocyte migration, and granulation tissue formation
Fibroblast growth factors acidic and basic	FGF-a and b	Growth factors	Endothelial cells, fibroblasts, macrophages, T lymphocytes	Cause angiogenesis, fibroblast chemotaxis and proliferation
Keratinocyte growth factors 1 and 2	KGF	Growth factors	Fibroblasts	Stimulate keratinocyte division and differentiation
Platelet derived growth factors (PDGF exists in several forms: AA, BB, AB, others)	PDGF	Growth factor	Platelets, macrophages; also fibroblasts, endothelial cells	Cause neutrophil and fibroblast chemotaxis; fibroblast proliferation, and synthesis of matrix proteins, metalloproteinases, stimulates angiogenesis
Transforming growth factors (alpha and beta)	TGF–α,β	Growth factors	Platelets, macrophages, fibroblasts, keratinocytes, T lymphocytes	Cause fibroblast and keratinocyte chemotaxis, angiogenesis; upregulates TIMP; inhibits production of MMPs and keratinocyte proliferation, induces TGFb production
Vascular endothelial growth factors (a family of peptides)	VEGF	Growth factors	Endothelial cells, keratinocytes, platelets, macrophages, fibroblasts	Cause angiogenesis (mitogenic for endothelial cells). Expression increased in the presence of hypoxia
Tissue inhibitor of metalloproteinase	TIMP	Enzyme	Most mesenchymal cells	Inhibits MMPs
Matrix metalloproteinases	MMPs	Enzymes	Monocytes, macrophages, endothelial cells	Degrade the extracellular matrix

The biological activity of many cytokines and growth factors is mediated by activation of a signal transducer and activator of transcription which expose typical functional pleiotropic and redundancy characteristic (Scheller *et al.*, 2011).

Clinically, some of wound healing markers are useful in diagnosis and treatment of several diseases. VEGF for instance, are clinically used as therapeutic agent in coronary artery disease, lymphedema, lymphangiogenesis, rheumatoid arthritis, atherosclerosis, intimal hyperplasia, diabetic retinopathy, psoriasis and tumour angiogenesis (Roy *et al*., 2006).

In this study three wound healing markers were selected. Each healing markers studied represent the different phases and function in the wound healing process. Interleukin-6 (IL-6) represents haemostasis and inflammation phase. Matrix metalloproteinase-9 (MMP-9) represents the tissue remodelling phases. Vascular endothelial growth factor (VEGF) represents angiogenesis process. These markers have also been studied by most researchers regarding their role and management in certain diseases especially in wound healing process.

3.2.1 Interleukin-6 (IL-6)

IL-6 a cytokine that plays a very important role in nearly all aspect of inflammation and innate immunity. Interleukins are types of cytokines that were first seen to be expressed by leukocytes. Family of this cytokines include IL-6, IL-11, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary inhibitory factor (CNTF), cardiotropin-1 (CT-1), cardiotrophin-like related cytokine and stimulating neurotrophin-1/B-cell stimulating factor 3 (NNT-1), neuropoietin (NPN), IL-27, and IL-31 (Heinrich *et al*., 2003). Also known as B-cell stimulatory factor 2, IL-6 has both pro-inflammatory and anti-inflammatory properties (Jurgen Scheller *et al*., 2006). It serves as multifunctional cytokine that regulates cell growth and differentiation of various tissues. It is also known particularly for its role in host defence due to its wide range of immune and hematopoietic activities and its potent ability to induce the acute phase response.

IL-6 produces inflammatory effects by inducing the transcription of factors in multiple pathways of inflammation. IL-6 is a molecule with multiple forms and functions, depends on where it is secreted. IL-6 is secreted by various type of cells such as macrophage, keratinocytes, endothelial cells and fibroblast (Zi-Qing Lin *et al.*, 2003) and it affects various processes including the immune response, reproduction, bone metabolism and aging as illustrated in Figure 3.3. As a response to inflammation and infection, IL-6 regulates leukocytes infiltration and recruitment, angiogenesis and collagen accumulation into wound area (Scheller *et al*., 2011).

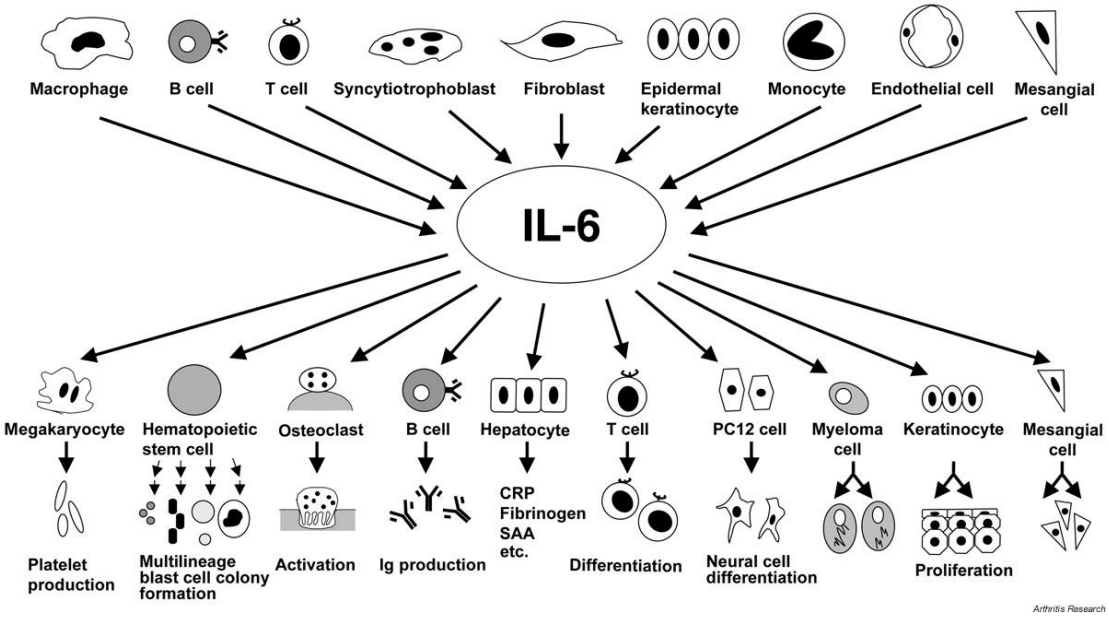

Figure 3.3 Biological activities of IL-6 in human body (Adapted from : Ramandip Singh *et al*., 2005)

A study done by Zi-Qing Lin *et al.* (2003) on IL-6-deficient mice suggests that there would be an interruption on all wound healing phase due to lack of IL-6. They also found that IL-6 promotes angiogenesis through induction of VEGF production. Over production of IL-6 together with TGF-β1 in some pathological cases will cause excessive formation of collagen which will lead into the formation

of keloids (Turabelidze *et al*. 2012). Over production of IL-6 has also been involved in the pathology of a number of diseases in conjunction with multiple myeloma, rheumatoid arthritis, Castleman's disease, psoriasis, and post-menopausal osteoporosis (Simpson *et al.*, 1997). Clinically, IL-6 is also used as immune response stimulator in chemotherapy for cancer patients (Thomas, 2015).

3.2.2 Matrix metalloproteinase-9 (MMP-9)

Matrix metalloproteinases (MMP's) also called matrixins (Nagase and Woessner , 1999) are referred to a large family of endopeptidases or protease enzyme that consist of 25 members out of which 24 of them are found in mammals. They utilize Zn^{2+} or Ca^{2+} ion in their active site (Gill and Parks,2008), which are responsible for the regulation of tissue remodelling and degradation of the extracellular matrix (ECM), in conjunction with collagens, elastins, gelatines, matrix glycoproteins and proteoglycan (Verma *et al*., 2007) . Inflammatory cells are well known to produce MMP's. Epithelial and stromal cells in wounded tissue have also been demonstrated to secrete multiple MMP's (Gill and Parks, 2008)

There are many types of MMP's that have been associated with wound repair, and these include MMP-1, 3, 7, 9, 10, 14, and 28. They are divided into collagenases, gelatinases, stromelysins, and matrilysins depends on the physical foundation of their specificity (Verma *et al.*, 2007).

MMP's trigger the proliferation and migration of endothelial cells by increasing the biological opportunity of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and transforming growth factor β (TGF-β) but reversely produce fragments that are

angiogenesis inhibitors. MMP's therefore represent as both inducers and inhibitors of angiogenesis (Di Carlo, 2012).

MMP-9 also known as gelatinase-B or type IV collagenase, is one of 23 human MMP's that have been widely and extensively studied by researchers exclusively related to wound healing processes (Gibson *et al*., 2009). Since angiogenesis involves migration/invasion of endothelial cells into surrounding tissues, the degradation of ECM by these proteolytic enzymes would allow the invasion of endothelial cells into wound area. However, the complete regulations of MMP-9 in angiogenesis pathway are still unclear (Di Carlo, 2012).

Among all cell types, macrophages secreted more MMP-9 compared to neutrophils, lymphocytes and stromal cells. MMP-9 are neutral proteinases that are involved in the breakdown and remodelling of the extracellular matrix (ECM) under a variety of physiological and pathological conditions, such as morphogenesis, differentiation, angiogenesis, as well as pathological processes including inflammation, arthritis, cardiovascular diseases, pulmonary diseases and tumour invasion (Gam *et al.,* 2006). They degrade ECM with subsequent activation of major pro-angiogenic factors such as vascular endothelial growth factor and fibroblast growth factor. The level of MMP-9 is regulated by endogenous inhibitors so called tissue inhibitors of metalloproteinase (TIMPs). In addition, there are other substances and mechanisms that inhibit metalloproteinase activity such as α2-macroglobulin, a primary inhibitor of metalloproteinases in bodily fluid.

Modification reactions by reactive oxygen species also have been exhibited to cease the bioactivity of MMP-9 (Gill and Parks, 2008). High levels of MMP-9 causes

improper deposition of protein matrix on wound area (Shah *et al*., 2012) and tissue damage if untreated with anti-oxidant (Gibson *et al*., 2009).

3.2.3 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor was found in 1983 is a secretory protein which consist of seven family member, VEGF-A, B, C, D, E, F and placental growth factor (PIGF). It has the capability to aid an expansion of vascular permeability in endothelium cells (Nieves *et al*., 2009) and serve as main stimulator of angiogenesis (Di Carlo,2012). VEGF has been identified as one of the most potent and predominant among the many factors implicated in angiogenesis. There are many publications about VEGF that have been published involving diagnostic and therapeutic drug monitoring (Jelkman,2001). VEGF can induce growth of pre-existing (angiogenesis) or de novo vessels (vasculogenesis). This action of VEGF ensures that it is critical within embryonic development as well as vessel repair during wound healing process. VEGF plays an important role in both normal and pathological angiogenesis and being used as an indicator in cardiovascular disease, malignancies and inflammatory disease (Jelkman,2001). VEGF also serve as an endothelial cell mitogen and chemotactic or chemo attractant agent (Bao *et al*., 2009). Recently, numerous studies have also emphasized the role of VEGF in pathological conditions involving the formation of new blood vessels, cancer, rheumatoid arthritis and age-related macular degeneration. An increased serum VEGF levels is useful for the prognosis of increased tumour growth, recurrence, or metastatic spread in individual patients (Hoeben *et al*., 2004). Majority of the studies showed circulating serum VEGF had positive correlation with tumour stage or patient survival, by that supporting the clinical relevance of VEGF in cancer patients (Hoeben *et al*., 2004).

All VEGF members have common homology territory and are encoded by multiple exons that can give rise to different isoforms after alternative slicing. The gene expressions of VEGF are stimulated by several pro-inflammatory cytokines such as IL-6 and TNF-α (Jelkman, 2001). Specifically, VEGF-A is characterized by seven isoforms with 121, 145, 148, 165, 183, 189, or 206 amino acids, while VEGF-B presents two isoforms, with 167 and 186 amino acids. Although these isoforms behave identically in solution, they differ in their ability to bind heparin and the extracellular matrix. Amongst the family members, VEGF-A is the most dominant inducer of blood vessel growth known to date. VEGF-A is a dimeric, disulphide-bound glycoprotein and exists in at least seven homodimeric isoforms. Normally the highest levels of VEGF- A mRNA are found in adult lung, kidney, heart, and adrenal gland (Hoeben et al. 2004). By binding to VEGFR-2 receptor, VEGF increased cell permeability and also activate the production of guanylyl cyclase, cGMP through the present of nitric oxide (Roy *et al*., 2006). VEGF-A also involve in enhancing the transportation of oxygen and nutrition into wounded area (Hoeben *et al*., 2004).

VEGF-B was discovered since 1995 and abundantly found in the adult myocardium, skeletal muscle and pancreas. Role of VEGF-B remains unclear even though several studies have been done (Scheller *et al*., 2011). Roy *et al*. found that VEGF-B plays an important role in inflammatory angiogenesis (Roy *et al*., 2006) with 88% amino acid sequence homology to VEGF-A.

VEGF-C is produced mostly in heart, placenta, ovary, small intestine and the thyroid gland, whereas in embryonic tissue, the production occurs where lymphatic vessels undergo sprouting from embryonic veins, such as the perimetanephric, axillary and jugular areas (Hoeben *et al.*, 2004). The main role of VEGF-C is as lymphangiogenic growth factor and regulated by VEGFR-3 receptor (Scheller *et al.*,