THE EFFECT OF ORAL *CHANNA STRIATUS* EXTRACT ADMINISTRATION ON TOTAL ANTIOXIDANT STATUS AND ITS RELATIONSHIP WITH HIGH SENSITIVE C-REACTIVE PROTEIN (HSCRP) DURING WOUND HEALING IN POST LOWER SEGMENT CAESAREAN SECTION WOMEN

By

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AA	:	Arachidonic acid
ABTSS	:	Azinobis 3-ethylbenzothiazoline-6-sulfonate
ADP	:	Adenosine diphosphate
ALT	:	Alanine transaminase
AST	:	Aspartate transaminase
bFGF	:	Basic fibroblast growth factor
BUSE	:	Blood urea serum electrolyte
CRP	:	C-reactive protein
CTGF	:	Connective tissue growth factor
DHA	:	Docosahexaeonic
DNA	:	Deoxyribonucleic acid
ECM	:	Extracellular matrix
EDTA	:	Ethylenediaminetetraacetic acid
EGF	:	Epidermal growth factor
EPA	:	Eicosapentaenoic acids
ESR	:	Erythrocyte sedimentation rate
FBC	:	Full blood count
FGF	:	Fibroblast growth factor
G6PD	:	Glucose-6-phosphate dehydrogenase
GM-CSF	:	Granulocyte macrophage colony stimulating factor
GSH	:	Glutathione
H_2O_2	:	Hydrogen peroxidase
HCLO	:	Hypochlorous acid

HsCRP	:	High sensitive C-Reactive Protein
ICT	:	Integrated chip technology
IGF-I	:	Insulin-like growth factor-I
IL	:	Interleukin
LDH	:	Lactate dehydrogenase
LFT	:	Liver function test
LSCS	:	Lower Segment Caesarean Section
MMPs	:	Matrix metalloproteinases
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NSAIDs	:	Nonsteroidal anti-inflammatory drugs
O2-	:	Superoxide anion
.OH	:	Hydroxyl radical
PDGF	:	Platelet Derived Growth Factor
PG	:	Prostaglandin
PMNs	:	Polymorphonuclear neutrophils
ROS	:	Reactive oxygen species
RFT	:	Renal function test
TAS	:	Total antioxidant status
TNFα	:	Tumour necrosis factor α
TGF-β	:	Transforming growth factor-beta
WBC	:	White blood cells

ABSTRACT

THE EFFECT OF ORAL *CHANNA STRIATUS* EXTRACT ADMINISTRATION ON TOTAL ANTIOXIDANT STATUS AND ITS RELATIONSHIP WITH HIGH SENSITIVE C-REACTIVE PROTEIN (HSCRP) DURING WOUND HEALING IN POST LOWER SEGMENT CAESAREAN SECTION (LSCS) WOMEN

Introduction: *Channa striatus* (Haruan) is widely consumed in Malaysia to promote wound healing which involves three overlapping phases; inflammation, new tissue formation and tissue remodelling. During inflammatory phase, large amount of reactive oxygen species (ROS) are produced resulting in severe cell damage which in turn delays wound healing. CRP has been shown to significantly increase in response to local inflammation. High sensitive C-reactive protein (hsCRP) detects the same CRP molecule but its lower limit of detection is lower. *C.striatus* has been proposed to have antioxidant and anti-inflammatory properties for better healing of the wound.

Objectives: This study was done to determine the level of Total Antioxidant Status (TAS) in subject receiving *C.striatus* extract and placebo (maltodextrin) in post Lower Segment Caesarean Section(LSCS) women. The specific objectives were to compare the TAS level in *C.striatus* extract group and placebo group and to study the relationship between the level of TAS and hsCRP during wound healing.

Methods: This was a randomized; double blinded, placebo-controlled study conducted in HUSM. The treatment group consumed 500mg of freeze dried *C.striatus* extract daily while the placebo group consumed 500mg of maltodextrin daily for 6 weeks. Venous bloods were taken from each subject

postoperatively at day 1, day 3, week 2, week 4 and week 6 and were analyzed for TAS and hsCRP using Selectra E machine. Data analysis was done using SPSS Version 20.

Result: A total of 73 patients were studied, 39 patients consumed *C.striatus* and 34 consumed maltodextrin. The result for TAS, when compared between both groups showed no significant differences in all the period studied. Within groups analysis showed that the TAS levels in patients who consumed *C.striatus* were all significant (p-value < 0.05) between Day 1 till Week 6 and for Day 3 with Week 2 and Week 6. The results of hsCRP within groups showed a significant level between all the periods studied in *C.striatus* group. The hsCRP level was highest on day 1 and showed a reducing trend with time. When compared between the two groups, the hsCRP level showed no significant difference except for week 6. There was only weak relationship between TAS and hsCRP on week 4 for *C.striatus* group.

Conclusion: There was no significant difference in the level of TAS and hsCRP between *C.striatus* and placebo group. However, the TAS level showed increment within group from week 2 onwards which might involve in the enhancement of wound healing. For hsCRP, there was significant decrease in these parameters in *C.striatus* group at week 6 compared to the placebo which indicates the beneficial effect of *C.striatus* administration during wound healing of post LSCS Women. This study showed weak relationship between TAS and hsCRP only on week 4.

ABSTRAK

KESAN EKSTRAK ORAL *CHANNA STRIATUS* KE ATAS STATUS ANTIOKSIDA TOTAL DAN HUBUNGANNYA DENGAN HSCRP SEMASA PENYEMBUHAN LUKA DALAM WANITA SELEPAS PEMBEDAHAN CAESAREAN

Pengenalan: *Channa striatus* (Haruan) digunakan secara meluas di Malaysia untuk meransang penyembuhan luka yang melibatkan tiga fasa yang saling bertindih; inflamasi, pembentukan tisu baru dan pembentukan semula tisu. Semasa fasa inflamasi, banyak spesis reaktif oksigen (ROS) terhasil menyebabkan kerosakan sel teruk yang melambatkan proses penyembuhan luka. *C-Reactive Protein* telah terbukti meningkat secara signifikan terhadap inflamasi setempat. *High sensitive C-reactive protein* (hsCRP) mengesan molekul yang sama dengan CRP tetapi tahap pengesanannya adalah lebih rendah. *C.striatus* telah dicadangkan mengandungi antioksida dan antiinflamasi untuk penyembuhan luka yang lebih baik.

Objektif: Kajian ini dijalankan untuk mengetahui tahap status antioksida total di dalam subjek yang menerima ekstrak *C.striatus* dan plasebo (maltodextrin) semasa proses penyembuhan luka dalam wanita selepas pembedahan caesarean. Objektif spesifik adalah untuk membandingkan tahap status antioksida total di dalam kumpulan *C.striatus* ekstrak dan plasebo dan mengkaji hubungan di antara tahap status antioksida total dan hsCRP semasa penyembuhan luka.

Kaedah: Kajian ini adalah secara rawak rabun dua pihak plasebo terkawaldijalankan di HUSM. Kumpulan yang dirawat mengambil 500mg ekstrak *C.striatus* sekali sehari sementara kumpulan plasebo mengambil 500mg maltodextrin sekali sehari selama 6 minggu. Darah vena diambil dari setiap subjek pada hari pertama, ketiga, minggu kedua, keempat dan keenam selepas pembedahan dan dianalisa untuk status antioksida total dan hsCRP dengan menggunakan mesin Selectra E. Analisa data dijalankan menggunakan SPSS versi 20.

Keputusan: Jumlah pesakit yang dikaji adalah 73 orang, 39 pesakit mengambil *C.striatus*dan 34 mengambil maltodextrin. Keputusan untuk status antioksida total, apabila dibandingkan di antara dua kumpulan menunjukkan perbezaan yang signifikan di dalam semua masa yang dikaji. Tetapi apabila dibandingkan di dalam kumpulan, tahap status antioksida total di dalam pesakit yang mengambil *C.striatus* adalah semua signifikan (p-value < 0.05) di antara hari pertama hingga minggu keenam dan di antara hari ketiga dengan minggu kedua dan keenam. Untuk hsCRP, apabila dibandingkan di dalam kedua-dua kumpulan, menunjukkan tahap signifikan di dalam semua masa yang dikaji dalam kumpulan *C.striatus*. Tahap hsCRP adalah paling tinggi pada hari pertama dan menunjukkan trendyang semakin rendah dengan peningkatan masa selepas pembedahan.Apabila dibandingkan di antara dua kumpulan, tahap hsCRP menunjukkan tiada perbezaan signifikan kecuali pada minggu keenam.Keputusan untuk hubungan di antara status antioksida total dan hsCRPmenunjukkan hubungan yang lemah pada minggu ke-empat untuk kumpulan *C.striatus*.

Konklusi: Tiada perbezaan signifikan didapati di antara tahap status antioksida total dan hsCRP di antara kumpulan *C.striatus* dan plasebo. Walaubagaimanapun, tahap status antioksida total menunjukkan peningkatan bermula daripada minggu kedua yang menunjukkan *C.striatus* mungkin terlibat dalam meningkatkan proses penyembuhan. Untuk hsCRP, walaupun menunjukkan tiada perbezaan signifikan di antara kedua-dua kumpulan pada hari pertama, ketiga, minggu kedua dan keempat tetapi pada minggu keenam, menunjukkan penurunan signifikan pada semua parameter berbanding kumpulan plasebo. Ini menunjukkan pengambilan *C.striatus* memberi kesan yang berfaedah semasa penyembuhan luka selepas pembedahan caesarean. Kajian ini juga menunjukkan hubungan yang lemah di antara status antioksida total dan hsCRP hanya pada minggu keempat.

CHAPTER 1: INTRODUCTION

Channa striatus (Haruan) is a tropical, fresh water, air breathing fish species that belong to the family channidae. It is widely distributed within Malaysia and commonly consumed as a food fish and by mothers recuperating from normal or Caesarean delivery or patients recovering from surgical operations. *Channa striatus* is popular as a therapeutic agent due to the folk belief in its efficacy in treating wounds, relieving pain and boosting energy in the sick and elderly (Mohd and Abdul Manan, 2012). It was reported to enhance dermal wound healing by influencing the different phases of wound healing.Other pharmacological activities include anti-microbial, anti-inflammatory, cells proliferation, induction of platelet aggregation and anti-norciceptive as well as its instrinsic nutritional values contributed to the synergy and better healing of the wound (Jais, 2007).

Channa striatus proved to have antioxidative property(Haniffa *et al.*, 2014), high levels of anti-oxidant activities (Daud and Dahlan, 2011) which are most likely to be lipophilic antioxidants that represent powerful defence tools particularly against omega-3 oxidation (Mohd and Abdul Manan, 2012).All the pharmacological properties of *C.striatus* are due to the biochemical components of the fish. It contains all the essential amino acids for wound healing particularly glycine as well as high contents of arachidonic acid (AA) and polyunsaturated fatty acids that can promote prostaglandin synthesis (Baie and Sheikh, 2000).These high level of specific amino acids, fatty acids and AA are effective in wound healing by promoting the initiation of a series of reactions

which is remodelling of collagen, re-epithelialisation of wound and induction of wound contraction (Mohd and Abdul Manan, 2012). These amino acids and fatty acids content of *C.striatus* also contribute to its antioxidative property (Haniffa *et al.*, 2014).

Wounding and wound healing take place in all tissues and organs of the body. A wound is defined as damage or disruption to the normal anatomical structure and function. It can range from a simple break in the epithelial integrity of the skin or it can be deeper into subcutaneous tissue with damage to other structures such as tendons, muscles, vessels, nerves, parenchymal organs and even bone. Wound healing involves multiple cell populations, the extracellular matrix and the action of soluble mediators such as growth factors and cytokines. It is a dynamic and complex process that involved a series of co-ordinated events which include bleeding, coagulation, initiation of an acute inflammatory response to the initial injury, regeneration, migration and proliferation of connective tissue and parenchyma cells, as well as synthesis of extracellular matrix proteins, remodelling of new parenchyma and connective tissue and collagen deposition (Velnar *et al.*, 2009). It can be summarized as involved three distinct but partly overlapping phases which is inflammation, new tissue formation and tissue remodeling (Eming *et al.*, 2007; Schäfer and Werner, 2008).

The acute inflammatory phase is characterized by neutrophils migration to the wound, followed by monocytes which differentiate into macrophages that predominate after 24 hours. The neutrophils secrete more pro-inflammatory

cytokines (tumour necrosis factor and interleukins), engulf and destroy bacteria, and release proteases (elastase and collagenase) that remove damaged and denatured extracellular matrix components (Mast and Schultz, 1996). At this time, they also produce and secrete large amounts of reactive oxygen species (ROS) that are essential to protect the organism against invading bacteria and other microorganisms (Schäfer and Werner, 2008).

The inflammatory phase begins to decline by about three days as neutrophils disappear from the wound probably by undergoing apoptosis. The growth factors secreted locally in the wound by macrophages continue to stimulate migration of fibroblasts, epithelial cells and vascular endothelial cells into the wound for the next phase of wound repair (Mast and Schultz, 1996). It occurs two to ten days after injury and is characterized by cellular proliferation and migration of different cell types(Gurtner *et al.*, 2008). The endothelial cells, fibroblasts and keratinocytes that migrate into the wound will proliferate and there will be formation of granulation tissues that are responsible for wound contraction and for the production of collagen and other extracellular matrix proteins. The granulation tissue is continuously replenished by new blood vessels (Schäfer and Werner, 2008).

The last stage of wound repair is remodelling that begins two to three weeks after injury and lasts for a year or more (Schäfer and Werner, 2008). The resolution phase of wound repair is essential for restoration of full functionality and a ‘normal’ appearance to the injured tissue. During wound resolution, many changes occur in the dermis. The blood vessels within the scar are refined and

mature to form a functional network (Adams and Alitalo, 2007). During this stage, there is formation of collagen, other extracellular matrix protein and wound contraction as the fibroblasts differentiate to myofibroblasts (Schäfer and Werner, 2008). The dense extracellular matrix that was haphazardly deposited early in repair is remodelled by a delicate balance of collagen synthesis, bundling and degradation that aims to restore normal architecture to the dermis (Shaw and Martin, 2009).

A series of recent studies revealed that ROS are crucial regulators of wound healing. All stages of the wound healing process which is migration, adhesion, proliferation, neovascularization, remodelling and apoptosis may involve, or at least modulated, by ROS (Wlaschek and Scharffetter-Kochanek, 2005). ROS traditionally regarded as toxic by product of aerobic metabolism is term that includes both oxygen radicals for example superoxide anion (O2-), hypochlorous acid (HCLO), hydrogen peroxide (H_2O_2) and free radical such as hydroxyl radical (.OH). Besides being produced as antimicrobial reactions by the neutrophils and macrophages during inflammation, they are also being produced during normal metabolism and involved in enzymatic reactions, mitochondria electron transport, signal transduction and gene expression (Bayr, 2005).

Excessive production of ROS or insufficient detoxification of ROS that lead to the oxidative stress results in damage of all cellular components including proteins, lipids and nucleic acids which in turn delay wound healing (Cerutti and Trump, 1991; Rosenfeldt *et al.*, 2013). Large amounts of ROS that

was produced in wounded and inflamed tissue are through the enzyme Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme complex, which is expressed at high levels by inflammatory cells (Schäfer and Werner, 2008). NADPH oxidase catalyzes the production of ROS by cells has been extensively investigated in phagocytes (neutrophilic, eosinophilic granulocytes, monocytes, and macrophages) (Sen, 2003).

Many facets of wound healing under redox control require a delicate balance between oxidative stress and antioxidants. An antioxidant is any substance that when present at low concentrations as compared with those of an oxidizable substrate, significantly delays or prevents the oxidation of that substrate (Halliwell and Poulsen, 2006). An antioxidant defense system involves reduction (scavenging) and/or dismutation of O_2– and/or HO_2– and their protonated forms and when this defense system fails to eliminate the oxidants, the alteration in homeostasis leads to oxidative stress (Soneja *et al.*, 2005a).It can be classified into two major groups which is enzymatic and non-enzymatic antioxidants. Some of these antioxidants are endogenously produced which include enzymes, low molecular weight molecules and enzyme cofactors. Among non-enzymatic antioxidants, many are obtained from dietary source (Ratnam *et al.*, 2006).

Antioxidant plays a significant role as health-benefiting factors which protect the body from oxidative stress (Chalamaiah *et al.*, 2012). These antioxidants are postulated to help control wound oxidative stress and thereby accelerate wound healing (Fitzmaurice *et al.*, 2011). Insufficient levels of

antioxidants or inhibition of the antioxidant enzymes cause oxidative stress and may damage or kill cells. *Channa striatus* extract were proven to have high antioxidant activities (Daud and Dahlan, 2011).

Many markers are involved in wound healing. It is regulated by a complex signalling network that involved numerous growth factors, cytokines and chemokines. The importance regulators involved are the epidermal growth factor (EGF) family, transforming growth factor beta (TGF-β) family, fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), platelet derived growth factor (PDGF), connective tissue growth factor (CTGF), interleukin (IL) family and tumour necrosis factor-α (TNF-α) family (Barrientos *et al.*, 2008). Platelets are also involved in wound healing by initiation the inflammatory stage with the processes of blood clotting and platelet degranulation that release all the contents which is growth factors that is important for healing (Tarnuzzer and Schultz, 1996; Young and McNaught, 2011).

Another wound healing markers that are also being utilized is C-reactive protein (CRP). It was discovered by Tillet and Francis over 70 years ago in the blood of patients with *Streptococcus pneumoniae* infection (Tillett and Francis, 1930). It is a member of the family of proteins known as pentraxins that is composed of five identical 21,500 molecular weight subunits which the gene is located on chromosome 1 at q21–q23. CRP is primarily synthesized in the liver andknown as Type I acute phase proteins (Kluft and de Maat, 2002).The C reactive substance appeared very early during the course of infections and

plays a role in both innate responses and as an adaptor to the adaptive immune system. CRP reaches peak levels in approximately 50 hours with a long half-life of 18 hours and falls once the inflammatory stimulus has been removed (Blake and Ridker, 2003). It is an important constituent of the first line of innate host defense as it can recognize pathogens and mediate their elimination by recruiting the complement system and phagocytic cells. Furthermore, the protein appears to play a role in the clearance of apoptotic and necrotic host cells, that contribute to the restoration of normal structure and function of injured tissues (Volanakis, 2001).

Recently, high-sensitivity C-reactive protein (hsCRP) or also known as Ultra-sensitive CRP has been developed. This new hsCRP assay detects the same CRP molecule as older CRP tests but its lower limit of detection is lower. Therefore, it can detect lower levels of inflammation (Wilson *et al.*, 2006). Its values have shown to significantly increase in response to local infection and are a hallmark of TNF-α, lL-6 and IL-1 mediated immune response (Rothenburger *et al.*, 1999; Jeandrot *et al.*, 2008). hsCRP has also been found to be a marker of inflammation that predict the incident of a heart attack, stroke, sudden cardiac death and peripheral arterial disease, even when cholesterol levels are within an acceptable range (Bassuk *et al.*, 2004).

This study is to assess the possible role of *Channa striatus* in wound healing by analysing the total antioxidant status (TAS) in post lower segment caesarean section (LSCS) women on *C.striatus* extract and its relationship with hsCRP. The study was conducted in Department of Obstetric and Gynaecology, Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, Kelantan.

CHAPTER 2: LITERATURE REVIEW

2.1 *Channa striatus* (Haruan)

Channa striatus (Haruan) is an indigenous fresh water, carnivorous air breathing fish species that is widely distributed in Malaysia. It is a snakehead fish, belongs to the family Channidae, known as ikan haruan locally and widely consumed in Malaysia as it is believed to promote wound healing. It's therapeutic agent is believed to be effective in treating wounds, relieving pain and boosting energy in the sick and elderly as mothers recuperating from normal or Caesarean delivery and patients recovering from surgical operations are routinely advised to eat meals containing *Channa striatus*(Mohd and Abdul Manan, 2012). Other pharmacological activities include anti-microbial, anti-inflammatory, cells proliferation, induction of platelet aggregation, anti-norciceptive as well as its instrinsic nutritional values contributed to the synergy and better healing of the wound (Jais, 2007). The traditional drug that is widely used is the boneless meat, however, the mucus and the roe are also medicinal.

Haruan contains protein, lipids (phospholipid, partial glyceride, cholesterol, fatty alcohol, triglyceride and cholesterol ester), vitamin A and has a good profile of dietary minerals (Jais, 2007). The medicinal effects of *Channa striatus* are attributed to two major components; the amino acids and the fatty acids. The content of these acids differ between the meat (fillet), roe (gonad of fish in pre spawning seasons) and the mucus. The meat is a good source of protein, low content of lipid, high content of arachidonic acid (AA) and

docosahexaeonic (DHA) and also has dietary minerals such as magnesium, copper, calcium, manganese, iron and zinc (Jais, 2007). The mucus which has antinociceptive activity consists of 95% water, glycoprotein and fatty acids (Jais, 2007). The roe has good composition of fatty acids with higher proportion of unsaturated compared with saturated fatty acids, and it's a good alternative as a potential health food products (Jais *et al.*, 1998).

Researchs have shown that *C.striatus* has good properties for wound healing. Fourteen amino acids were detected in *C.striatus*. They are leucine, isoleucine, methione, tryptophane, lycine, histidine, alanine, oxyproline, tyrosine, theonine, glycine, serine, aspartic and glutamic acid, which are the basis element for wound healing (Jais *et al.*, 1994; Jais *et al.*, 1998). Mat Jais *et al*. (1994) also revealed that *C.striatus* has relatively high content in Vitamin A, an essential factor for wound healing.The major amino acids content in Haruan especially glutamic acid, glycine, leusine, aspartic acid and some dominant fatty acids which are palmitic acid, myristoleic acid, oleic acid, linoleic acid and arachidonic acid are suggested to play an important roles in healing (Dahlan-Daud, 2011). Studies on animals reported that *C.striatus* enhance dermal wound healing by influencing the different phases of wound healing (Jais, 2007). A fatty acid compositional study of the flesh of Haruan revealed unusually high AA but almost no Eicosapentaenoic Acids (EPA), which were hypothesized to be actively involved in initiating tissue wound repair (Jais *et al.*, 1998).

Studied done by Baie and Sheikh in 2000 revealed that *C.striatus* contains all the essential amino acids for wound healing particularly glycine as well as high contents of AA (precursor of prostaglandin) and polyunsaturated fatty acids that can promote prostaglandin synthesis during wound healing. These high level of specific amino acids, fatty acids and AA are effective in wound healing by promoting the initiation of a series of reactions which is remodeling of collagen, re-epithelialisation of wound and induction of wound contraction (Mohd and Abdul Manan, 2012). It is also found, wound that have been treated with Haruan had increase in the tensile strengths of the wounds. This may be attributed to the polypeptide formation by the combination of glycine with aspartic and glutamic acid in the presence of leucine, methionine, alanine and arginine in which it may be inferred that ‘‘it increases the number of cells and thereby the amount of collagen’’ that may lead to the enhancement of the wound healing process (Baie and Sheikh, 2000) .

Channa striatus extract were proven to have high antioxidant activities (Daud and Dahlan, 2011). The amino acids content of Haruan are known to have significant antioxidant properties as synergists or primary antioxidants and are believed to be important metal chelators with significant potential in linoleic acid and methyl esters of linoleic acid system (Haniffa *et al.*, 2014). The antioxidants present in this fish are most likely to be lipophilic antioxidants that represent powerful defence tools particularly against omega-3 oxidation (Mohd and Abdul Manan, 2012).

2.2 Wound healing

Wound healing is a dynamic process and involves complex interaction of extracellular matrix molecules, soluble mediators and various immune cells (Eming *et al.*, 2007). It involved three distinct but partly overlapping phases :

i) Inflammation
ii) New tissue formation
iii) Tissue remodeling (Eming *et al.*, 2007; Schäfer and Werner, 2008) (Figure 2.1).

The first stage of wound repair which is inflammation phase occurs immediately after tissue damage. The components of the coagulation cascade, inflammatory pathways and immune system are needed to prevent on-going blood and fluid losses, to remove dead and devitalized (dying) tissues and to prevent infection (Gurtner *et al.*, 2008). Immediately after wounding, vascular injury initiates the formation of platelet plug and a blood clot that result in temporary seal of the wound (Eming *et al.*, 2007). This clot also provide provisional matrix for cells migration and this initiates the inflammatory phase. The first cell to arrive is neutrophils due to their abundance, followed by monocytes (after two to three days), which differentiate into mature tissue macrophages. These innate immune cells secrete proteolytic enzymes (matrix metalloproteinase or MMPs), growth factors such as Vascular endothelial growth factor (VEGF), transforming growth factor (TGF) and inflammatory cytokines (IL-6, IL-10, TNFα and etc). They also produce and secrete large amount of reactive oxygen species (ROS), which are essential to protect the

organism against invading bacteria and other microorganisms (Schäfer and Werner, 2008).

The second stage of wound repair which is new tissue formation occurs two to ten days after injury and is characterized by cellular proliferation and migration of different cell types. The first event is the migration of keratinocytes over the injured dermis and formation of the new blood vessels (Gurtner *et al.*, 2008). In the transition between inflammation and repair, there is decrease level of inflammatory cytokines and immune cells as an effect by the release of anti inflammatory mediators IL-10 and TGF-β1 by macrophages. Other cells such as the endothelial cells, fibroblasts and keratinocytes start migrating into the wound and proliferate. Granulation tissue which consists of fibroblasts, endothelial cells and inflammatory cells is the new tissue that initially replaces the lost dermis. In the granulation tissue, fibroblasts differentiate to myofibroblasts that are responsible for wound contraction and for the production of collagen and other extracellular matrix proteins. To support the new tissue with oxygen and nutrients, sprouting of blood vessels at the wound edge occurs and the granulation tissue is continuously replenished by new blood vessels (Schäfer and Werner, 2008).

The third stage of wound repair which is remodelling stage begins two to three weeks after injury and lasts for a year or more. During this stage, all of the processes wind down and cease. Most of the endothelial cells, macrophages and myofibroblasts undergo apoptosis or exit from the wound, leaving a mass consists of mostly collagen, few cells and other extracellular-matrix proteins

(Gurtner *et al.*, 2008). The fibroblasts differentiate to myofibroblasts, which are responsible for formation of collagen and other extracellular matrix protein and also for wound contraction (Schäfer and Werner, 2008). The skin will regain a structure similar to that seen in unwounded tissue (type three collagen will replace type one collagen) but the wounds never achieve the same level of tissue strength with on average reaching 50% of the original tensile strength by three months and only 80% long-term (Young and McNaught, 2011).

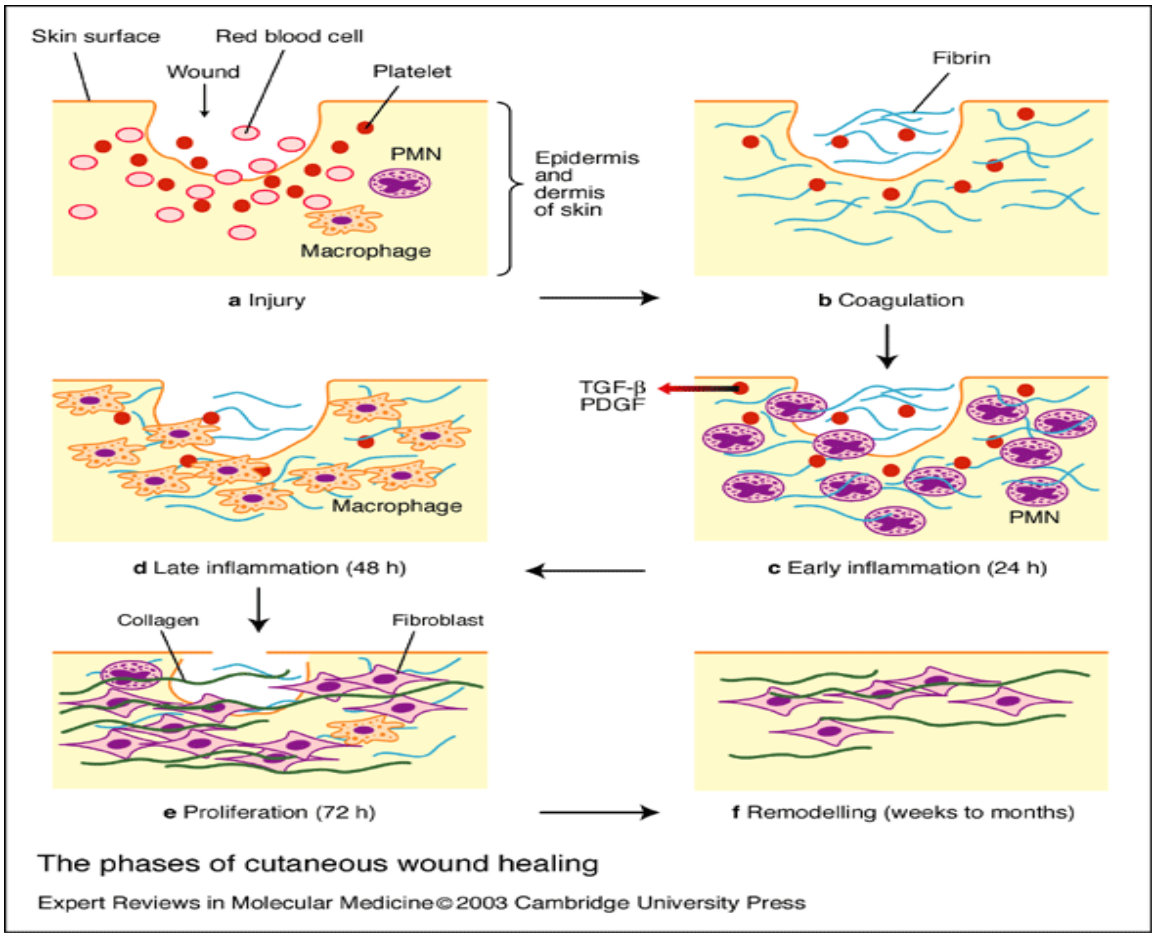

Figure 2.1: The phases of cutaneous wound healing.

Adapted from: Expert Reviews in Molecular Medicine © 2003, Cambridge University Press(Beanes *et al.*, 2003).

(a) Immediately following cutaneous injury, blood elements and vasoactive amines extravasate from locally damaged blood vessels within the dermis. Vascular permeability is temporarily increased to allow neutrophils [polymorphonuclear neutrophils (PMNs)], platelets and plasma proteins to infiltrate the wound. Vasoconstriction follows, in response to factors released by these cells. (b) Coagulation then occurs as platelets aggregate with fibrin, which is deposited in the wound following its conversion from fibrinogen. (c) Platelets release several factors, including platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β), which attract PMNs to the wound, signalling the beginning of inflammation. (d) After 48 hours, macrophages replace PMNs as the principal inflammatory cell. Together, PMNs and macrophages remove debris from the wound, release growth factors, and begin to reorganise the extracellular matrix. (e) The proliferation phase begins at about 72 hours as fibroblasts, recruited to the wound by growth factors released by inflammatory cells, begin to synthesize collagen. (f) Although the rate of collagen synthesis slows down after about three weeks, collagen crosslinking and reorganization occur for months after injury in the remodelling phase of repair.

2.3 Wound healing and reactive oxygen species (ROS)

Oxidation means loss of electrons by a species, gain of oxygen, or loss of hydrogen whereas oxidative stress initially defined by Sies (1985, 1986) as a serious imbalance between oxidation and antioxidants which is “a disturbance in the prooxidant–antioxidant balance in favor of the former, leading to potential damage.” (Halliwell and Poulsen, 2006). Oxidative stress in the body represents an imbalance between the production of reactive oxygen species (ROS) and the ability of the antioxidant defence mechanisms to detoxify the reactive intermediates (Rosenfeldt *et al.*, 2013). This imbalance can occur as a result of either heightened ROS generation, impaired antioxidant system or a combination of both (Vaziri, 2008). An excess of ROS can damage all cellular components including proteins, lipids and nucleic acids. The greater the oxidative stress, the more severe the resulting cellular damage because moderate oxidation can trigger apoptosis, more intense stresses may cause necrosis (Rosenfeldt *et al.*, 2013). Oxidative stress can be quantified by measuring different biomarkers that can be done by direct measurement of free radicals, the end-products of free radical damage or the levels of individual total antioxidants and yet, there is not a single biomarker that can truly represent oxidative stress (Arsalani-Zadeh *et al.*, 2011).

ROS have been traditionally regarded as toxic products of aerobic metabolism. However, ROS can be beneficial. Its’ beneficial effects occur at low or moderate concentrations and involve in physiological roles in cellular responses to noxia, for example in defence against infectious agents and in the

function of a number of intracellular signalling molecules in vascular cells (Irani, 2000; Valko *et al.*, 2007). The other beneficial example is the induction of a mitogenic response (Valko *et al.*, 2007). On daily basis, humans use about 250gm of oxygen out of which 2–5% is converted to reactive oxygen species (Soneja *et al.*, 2005a). The primary sources of ROS include mitochondrial electron transport system and various oxidase enzymes including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, cycloxygenase, lipoxygenase, P450 enzymes, glucose oxidase and uncoupled nitric oxide synthases among others (Vaziri, 2008).

Under normal conditions, significant amounts of ROS such as superoxide anion (O2-), hypochlorous acid (HCLO), hydrogen peroxide (H_2O_2) and free radical such as hydroxyl radical (.OH) are produced in the course of oxygen metabolism(Vaziri, 2008). Superoxide anion, arising either through metabolic processes or following oxygen activation by physical irradiation, is considered the primary ROS and can further interact with other molecules to generate secondary ROS, either directly or prevalently through enzyme or metal-catalysed processes (Valko *et al.*, 2007). This primary ROS is a short-lived, highly reactive and potentially cytotoxic molecule which can attack, denature or modify adjacent molecules that is formed during normal respiration in mitochondria and by autoxidation reactions (Evans and Halliwell, 2001). Normally, superoxide is converted to H_2O_2 by a family of enzymes known as superoxide dismutase (Vaziri, 2008).

The hydroxyl radical, •OH, is the neutral form of the hydroxide ion. It has a high reactivity that makes it a very dangerous radical with a very short in vivo half-life (Valko *et al.*, 2007). It has the highest rate constants for the reaction with target molecules and its reactions are diffusion limited as they take place practically at the site of generation (Sies, 1997). Hydroxyl radical attacks and denatures the adjacent molecules such as lipids, proteins, carbohydrates, and nucleic acids for example DNA (Vaziri, 2008). H_2O_2 is another ROS formed during normal metabolism which much is produced in the brain during the catalytic degradation of neurotransmitters such as dopamine (Evans and Halliwell, 2001). It is a weak oxidant and a weak reducing agent that is relatively stable in the absence of transition metal ions. It readily mixes with water and rapidly diffusing across cell membranes. Hypochlorous acid(HCLO) is a powerful oxidant that is formed in the body by activated neutrophils. The heme-containing enzyme myeloperoxidase in the phagocyte cytoplasm can catalyze the formation of HCLO from H_20_2 and chloride ions (Gutteridge, 1995).

Although ROS can target any component of the cell, they generally react with the first structure they encounter, frequently the lipid components of cell or organelle membranes, although proteins and DNA are often targets, particularly in mitochondria where it is in close physical proximity to sites of ROS production. However, proteins are vulnerable to free radical-mediated denaturation, which results in the subsequent loss of important cellular functions, such as growth, division and repair (Rosenfeldt *et al.*, 2013). Because ROS are short-lived and need expensive equipment for their direct measurement, ROS activity is usually assessed indirectly by measuring stable metabolites of ROS or by assessing the various end products derived from the

interaction of these radicals with cellular components such as lipids, amino acids and DNA (Rosenfeldt *et al.*, 2013). Modification of oxidative stress, by administration of drugs with antioxidative actions or supplementation of naturally occurring antioxidants in pharmacological concentrations, has been shown to reduce morbidity and mortality in various patient populations (Kücükakin *et al.*, 2009).

ROS are involved in all stages of the wound healing process. During the inflammatory phase of wound-healing which occur within one to three hours, neutrophils and macrophages that invade the wound produce large amounts of superoxide radical anions in which a phenomenon often described as ‘‘respiratory burst’’ (auf dem Keller *et al.*, 2006). Activated neutrophils and macrophages produce large amounts of superoxide and its derivatives via the phagocytic isoform of NADPH oxidases (Soneja *et al.*, 2005b). Furthermore, other cells such as fibroblasts can be stimulated by pro-inflammatory cytokines (IL-1 or TNF) to produce ROS (Meier *et al.*, 1989). The production of large amounts of ROS, which is part of the innate immune system are essential to protect the organism against invading bacteria and other microorganisms (Schäfer and Werner, 2008). The proliferating and migrating cells in the wound tissue that exposed to large amounts of ROS during the respiratory burst are also the strategies to protect themselves against harmful insults (auf dem Keller *et al.*, 2006) (Figure 2.2).

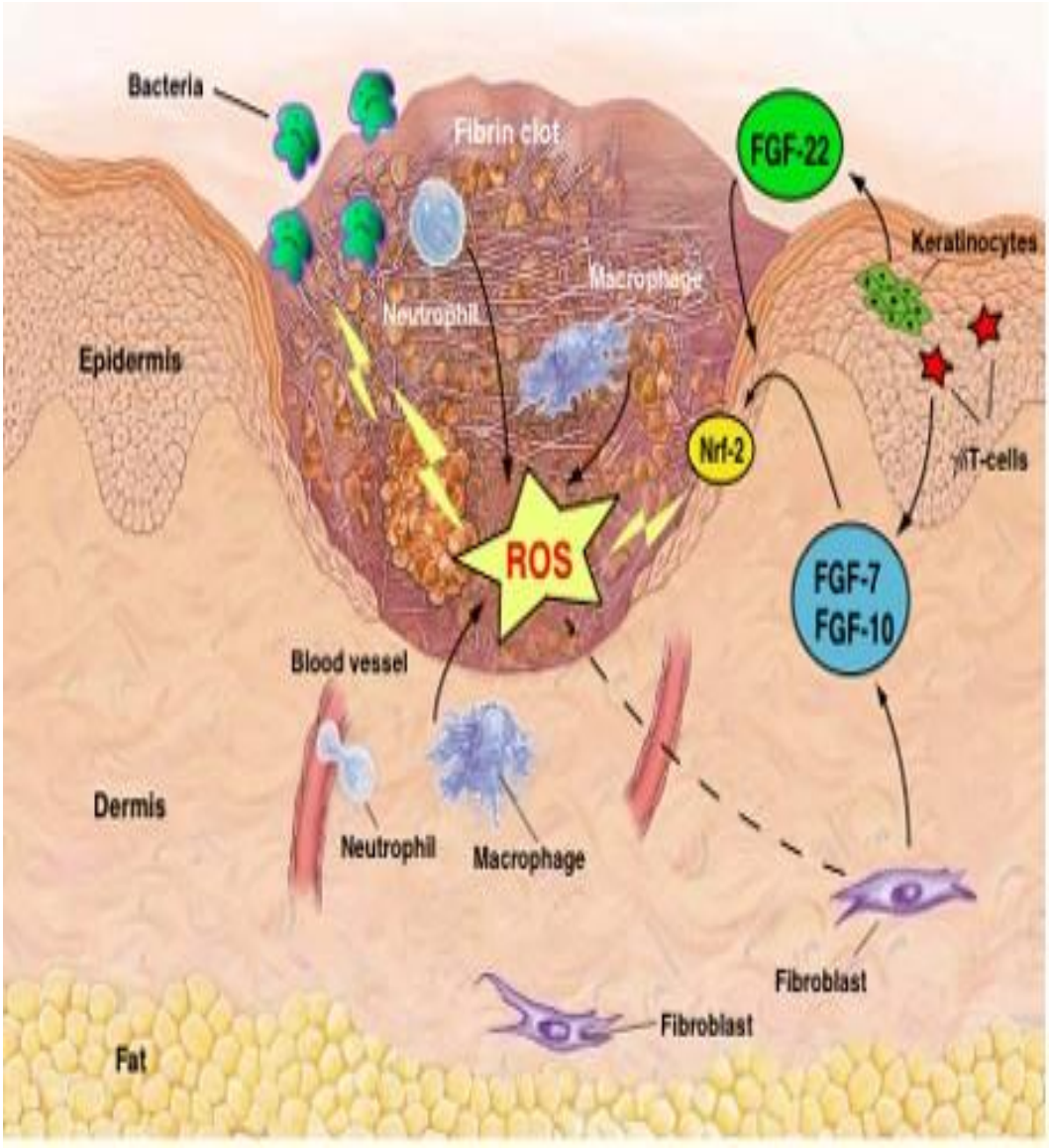

Figure 2.2: ROS defense in the healing skin wound.

Source: (auf dem Keller *et al.*, 2006).

As a defense against invading bacteria, inflammatory cells produce ROS via nicotinamide adenine dinucleotide phosphate (reduced form) oxidase. To protect themselves against these harmful molecules, cells at the wound site, in particular keratinocytes, express various ROS-detoxifying enzymes and other antioxidant proteins. The expression of some of these genes is regulated by the Nuclear erythroid 2-related factor 2 (Nrf2) transcription factor, which is also up-regulated after skin injury.

Superoxide that was formed is rapidly converted to membrane permeable form which is H_2O_2 by superoxide dismutase activity or even spontaneously (Sen *et al.*, 2002). The combined activities of NADPH oxidase and myeloperoxidase in phagocytes lead to the production of HCLO which is one of the strongest physiological oxidants and a powerful antimicrobial agent. Importantly, physiologically relevant ROS concentrations can also modulate redox-sensitive signal cascades and enhance immunological functions of lymphocytes (Dröge, 2002). Endothelial cells can also produce high concentrations of O2–, H_2O_2 and $OH^{\cdot}$ under ischemic conditions in wounds (Soneja *et al.*, 2005b). Thrombin, PDGF and TNF-α stimulate release of superoxide from endothelial cells whereas IL-1, TNF-α and platelet activation factor stimulate superoxide release from fibroblasts (Dröge, 2002).

In wound healing process, ROS also helps in the re-epithelization of wounds by activating collagenase expression and mediate epidermal growth factor signalling.H_2O_2 induces collagenase (MMP-1) expression and it is also responsible for mediating signalling of epidermal growth factor that promotes proliferation of keratinocytes. Furthermore, ROS also mediate conversion of fibroblasts to myofibroblasts thus aiding in wound contraction (Soneja *et al.*, 2005a). It was proved in murine models of dermal wound healing that shown that oxygen and ROS may trigger the differentiation of fibroblasts to myofibroblasts which is a key mediator of the contractile process in the early phase of healing (Roy *et al.*, 2003). Other than that, ROS not only support angiogenesis but also stimulate collagen production (Bartosz, 2009).

The normal physiology of wound healing depends on low levels of reactive oxygen species and oxidative stress which in turn an overexposure to oxidative stress leads to impaired wound healing (Fitzmaurice *et al.*, 2011). As a consequence of the underlying signalling and damage pathways, oxidative stress could also result in disturbed wound healing (Wlaschek and Scharffetter-Kochanek, 2005). If detoxification of ROS is insufficient or if ROS are produced in excessive amount, it results in severe cell damage which in turn delay wound healing. Furthermore, at high levels ROS can lead to severe tissue damage and even neoplastic transformation (Cerutti and Trump, 1991). The functional importance of these antioxidant in wound healing is suggested by their depletion in healing skin wound as proven by an acute wound in rodent, where there is 60-70% reduction of antioxidant level as compared to normal skin (Schäfer and Werner, 2008). There is a growing awareness that oxidative stress plays a role in various clinical conditions for example malignant diseases, diabetes, atherosclerosis, chronic inflammation, human immunodeficiency virus infection, ischemia, reperfusion injury and sleep apnea (Dröge, 2002).

2.4 Antioxidants

The body possesses a number of mechanisms both to control the production of ROS and to limit or repair the damaged tissues. An antioxidant is defined as any substance that considerably delays or inhibits the oxidation of a substance (Chalamaiah *et al.*, 2012). In healthy individuals, the antioxidant system defends tissue against free radical attack. Antioxidants are important mediators in regulating the damage that is potentially incurred by biological molecules such as DNA, protein, lipids and body tissue in the presence of reactive species (Fitzmaurice *et al.*, 2011). It plays a significant role as health-benefiting factors which protect the body from oxidative stress (Chalamaiah *et al.*, 2012).

The antioxidant defense system is a highly complex biochemical organization that consists of numerous enzymes and a large number of scavenger molecules that neutralized and converted the ROS and the byproducts of their reaction to harmless molecules. The body's pool of antioxidant molecules is derived from endogenous and exogenous sources (Vaziri, 2008). The endogenous sources are: a) first line enzymes (catalase, superoxide dismutase, glutathione peroxidase, peroxiredoxin, epoxide hydrolase); b) second line enzymes that provide co-substrate molecules (glutathione and NADPH) for first line enzymes (glutathione reductase, glucose-6-phosphate dehydrogenase (G6PD), glutathione-S-transferase); c) small molecule antioxidants (uric acid, glutathione, α-tocopherol, ascorbic acid, β-carotene, coenzyme Q10, dihydrolipoic acid, melatonin, bilirubin); d) large

molecule antioxidants (albumin, transferrin, ceruloplasmin); e) metal chelators (lactoferrin). The exogenous sources are from dietary plants (polyphenolic compounds including flavonoids and hydroxycinnamic acids) and synthetic antioxidant drugs(various vitamins and phytochemicals) (Firuzi *et al.*, 2011).

In general, there are two major strategies of antioxidation which is non-enzymatic and enzymatic (auf dem Keller *et al.*, 2006). Enzymatic antioxidant defences includes superoxide dismutase, glutathione peroxidase, catalase whereby non-enzymatic antioxidants are ascorbic acid (Vitamin C), tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids and other antioxidants. Normally, there is a balance between both the activities and the intracellular levels of these antioxidants (Valko *et al.*, 2007). Antioxidants are capable of neutralizing free radicals or their actions by acting at different stages or levels of prevention, interception (scavenger) and repair (Devasagayam *et al.*, 2004). Three classes of antioxidants have been identified:

i) Primary (Preventive) antioxidants

Primary antioxidants inhibit or prevent the formation of new free radicals. The examples are caeruloplasmin, transferrin, ferritin, albumin, myoglobin and metallothionine. Protection of cells from incident radiation may occur through specialized pigments for example the melanins for ultraviolet radiation or the carotenoids for electronically excited states such as singlet oxygen. However, these and other strategies are not completely preventative (Sies, 1997).