CHAPTER-1

Introduction
1.1 Historical Aspect of Wound Healing

The history of wound healing is as old as the history of medicine and probably as mankind itself. In the evolutionary process, humans traded the slow biological complexity of regeneration, seen in many less complex organisms, for the relatively simple and more rapid healing of wounds.

1.1.1 Medicine at Ancient India

The earliest documents of Indian medicine are found in the Vedas, the oldest sacred books of the Hindu religion. Vedas are considered to be the first record of the ancient knowledge and civilization in the world. These were compiled in Sanskrit, between 3000 and 1000 B.C. This era is referred to as the Vedic period, during which the four Vedas, namely the Rigveda, the Samaveda, the Yajurveda, and the Atharvaveda were compiled. Most of the early Vedic medicine was compiled in Atharvaveda, which was an amalgam of religion, magic and empiric elements.

Sushruta, one of the earliest surgeons of the recorded history (800 B.C.) and recognized as the “The Father of Surgery” in the world [Figure-1.1 & 1.2]. The Sushruta Samhita [Figure 1.3] was written in the holy city of Kashi (Varanasi). The holy book has two separate chapters dealing with healing of wounds, he was first to explains the sign and symptoms of burn wound, and describes more than 100 plants for treatment of wounds both singly and in combination (1). Sushruta has mentioned not only procedures and drugs to obtain a clean wound (Vrana Shodan) followed by healing (Vrana Ropan) but also medicines to help treatment of keloids (2). He clarified that blood clots, foreign materials like stones, hair, nails, fragment of fractured bone, etc., should be removed and wound should be thoroughly cleaned before applying sutures. If these materials were not removed, the wound will proceed to Pakavastha i.e., suppuration and will increase pain over affected part. Sushruta also defined the suturing procedure as a process of tying two ends of thread for union of wound edges with the help of needle and appropriate suturing material. Four methods of suturing techniques are described in ancient texts as Seevan karma- Riju granthi - straight, interrupted type, Vellitaka - continuous type, Tunnasevani - zigzag type or subcuticular and Gopahanika - interlocking or blanket type suturing.

Plants have served as healing agents of wounds since ages and Ayurvedic texts of Sushruta, Jivika, Charaka and Vagabhatta are a testament to this fact. Several drugs of plant, mineral, and animal origin are described in the Ayurveda for their wound-healing properties under the term Vranaropaka.
Some of these plants have been screened scientifically for the evaluation of their wound-healing activity in different pharmacological models and patients, but the potential of most remains unexplored. In a few cases, active chemical constituents were identified. Some
Ayurvedic medicinal plants, namely, *Ficus bengalensis*, *Cynodon dactylon*, *Symlocos racemosa*, *Rubia cordifolia*, *Pterocarpus santalinus*, *Ficus racemosa*, *Glycyrrhiza glabra*, *Berberis aristata*, *Curcuma longa*, *Centella asiatica*, *Euphorbia nerifolia*, and *Aloe vera*, were found to be effective in experimental models and have long been incorporated in modern medicine (3).

1.1.2 Medicine at Mesopotamian Era

The world’s first written historical record and oldest medical manuscript was found on a Sumerian clay tablet of 2100 BC [Figure 1.4]. Washing the wound, applying dressings/plasters, and band-aging the wound were the “three healing gestures” described in this tablet. These constitute the basic principles of wound treatment today.

![Figure 1.4: Cuneiform medical clay tablet.](image)

Guido Majno in his book ‘The Healing Hand: Man and Wound within the Ancient World’, states that there have been fifteen prescriptions recorded on the pill, while not indication of the diseases that they were meant. Twelve of the fifteen were for external use, eight being plastered, indicating that they’ll are used for native diseases. Majno presents many samples of prescriptions, such as: “Pound together: dried wine dregs, juniper and prunes, pour brewage on the mixture. Then rub (the morbid part) with oil, and bind on (as a plaster)”. One of the earliest acknowledged wound care merchandise was beer! that was wide utilized in Sumerian treatments and it's seemingly that, it contains antiseptic ingredients, it did have some useful result within the treatment of wounds and skin lesions (4).
1.1.2 Ancient Egypt

Medication information in ancient Egypt relies on the Smith and Ebers papyri, qualitative analysis from around 1650 BC and 1550 BC, respectively [Figure 1.5]. The ancient Egyptians use the mixtures prepared with substances like honey, grease, and lint (made up of vegetable fibers for absorption of exudate from the wound’s surface) for topical application to wounds. The Egyptian science of dressing wounds was similar to that of dressing the dead throughout the method of mummification. Previous to dressing, the materials were lordotic in varied preparations, together with flavouring extracts, gums, and resins. Gum applied to bandage strips was conjointly accustomed draw and to approximate wound margins (5, 6).

Figure 1.5: A piece of the Edwin Smith Papyrus.

1.1.3 Inflammation, Infection and the Approach to Purulent Discharge in the Past

Sumerian and ancient Egyptian documents embrace the terms ummu and shememet, which were understood nowadays as indicating the presence of inflammation. The Egyptians distinguished between two type of wounds: ‘Good wounds’ were treated with topical preparation and medical aid and dressing. On the other hand, ‘bad wounds’ were tormented by a ‘whirl of inflammation,’ known by touching the wound edge and by their tendency to secrete pus. These wounds were left open. Aulus Cornelius Celsus (42? BC–37 AD) set down ‘four cardinal signs of inflammation’ namely, inflammation (redness), tumor (swelling), calor (heat) and grief (pain) (7) [Figure 1.6]. UN agency wrote a comprehensive eight-volume compendium of medication (De re Medicina). This book was supported the Hippocratic Canon and alternative classical sources. American state re Medicina was forgotten some years when its writing, solely to be rediscovered when an extended amount, in 1426. it had been
Introduction

one amongst the primary medical books to be written, showing in 1478. Thereafter, it enjoyed nice success; new editions were revealed even within the nineteenth century (8).

Figure 1.6: Signs of Inflammation

The Egyptians recognised that a wound that pus should be drained (9). Later, anatomist indicated that once infection was localised in wound, the discharge of pus will be followed by healing. This observation was misinterpreted throughout the subsequent 1500 years (10, 11). During this era, pus secreted by a surgical wound was thought-about to be useful in cases wherever the number of secretions step by step decreased and therefore the patient recuperated. The presence of septic discharge was thought about to be auspicious; the ancient expression pus bonum et laudabile reflects this conception. Contrary to this, in cases of brown, thin, and putrid discharge, patients typically died. This sort of discharge was, most likely, a manifestation of invasive infection. Several topical preparations were introduced into wounds with the target of encouraging suppuration, a mistaken treatment that would really increase the chance of spreading infection with resulting mortality (10, 11). It took till the nineteenth century to understand the presence of pus in wound was undesirable. Not till the break through discoveries of Semmelweis, Lister, and others was it possible to prevent the development of pus in surgical wounds with any degree of efficiency. These principles played a significant role in the treatment of wounds and cutaneous ulcers.

1.1.4 Renaissance Era

Theodoric of Bologna (1205 - 1296), believed that the assembly of pus in a very wound was actually unfavorable to healing and promoted an identical method of wound healing including cleaning the wound with carboxylic acid, removing dead tissue by debridement, closing the
wound with stitches, and applying a bandage sadly this practise was abandoned when the death of his scholar, the French physician Henri American state Mondeville (12).

Ambroise Paré (1509–1590) was one in every of the best physicians of the renaissance and within the entire history of medication. His extensive data of medication and surgery, from his unique skills and plenty of years of service within the French army as a military physician, resulted in vital changes in the medical conceptions of those times.

Paré was chief physician to four kings of France (12): Henry II (1547–1559), Francis II (1559–1560), King of France (1560–1574), and Henry III (1574–1589). As a military physician he saved the lives of thousands of troopers. He wrote 2 books which were translated into many languages from the French: Treatment of gun fire Wounds and therefore the methodology of treating Wounds created by Arquebuses, in which he summarised the surgical techniques of his era and introduced those he had developed [Figure 1.7]. During a military expedition to Turin led by King Francis I (1536–1537), Paré gained important experience. His unique contribution to the field of wound healing prevented the suffering of many a wounded soldier. At that time, gunshot wounds were considered to be ‘poisoned wounds’ due to their direct contact with gunpowder. The accepted approach was to treat these wounds by cauterizing them with a red-hot iron or with boiling oil [Figure 1.8].

Figure 1.7: The cover picture of Paré’s book

In one of the battles, the oil he used to treat gunshot wounds ran out. He had no option but to improvise a mixture that included egg yolks, oil of roses, and turpentine. When he changed their dressings on the following day, he was surprised to see that the wounds treated with the
improvised mixture were greatly improved, compared with those treated with the usual boiling oil.

![Figure 1.8: Paré in the battlefield: A wood engraving of that period](image)

The recovery of those not cauterized with the oil was faster and with fewer complications, later he published his experience. Of this discovery, Paré wrote (12):

“I slept badly that night, as I greatly feared that, when I would come to examine the wounded the following morning, I should find that those whose wounds I had failed to treat with boiling oil will have died from poisoning. I arose at a very early hour, and was much surprised to discover that the wounds to which I had applied the egg and turpentine mixture were doing well: they were quite free from swelling and from all evidence of inflammatory action: and the patients themselves, who showed no signs of feverishness, said that they had experienced little or no pain and had slept quite well. On the other hand, the men to whom I had applied the boiling oil, said that they had experienced during the night, and were still suffering from, much pain at the seat of the injury; and I found that they were feverish and that their wounds were inflamed and swollen. After thinking the matter over carefully, I made up my mind that thenceforward I would abstain wholly from the painful practice of treating gunshot wounds with boiling oil.”

Paré was responsible for two further significant contributions: The first was the use of a ligature to stop bleeding, rather than cauterisation. The second was the development of an artificial hand, the prosthesis. Paré may therefore be viewed as the father of medical rehabilitation. His most memorable statement reflects his modesty: “Je le pansay, Dieu le quarit”: “I dressed him, God healed him.”

Cle de Villars in 1740, showed that wounds left open to the environment healed less effectively than wounds that were closed. This resulted in the birth of occlusive dressings. John Hunter (1728 - 1793), the 'Father of Modern Surgery', was one of the dominant
personalities in medicine. He noted that wounds were able to heal under a scab when left undressed (13). He was the first surgeon to advocate the laying open of narrow wounds and sinus tracts, which subsequently healed by secondary intention. He distinguished 'adhesive' inflammation which was amenable to surgical intervention, from 'suppurative' inflammation, which delayed healing, demonstrating that this was related to infection (11). In the nineteenth century major advances in wound healing were developed. The cause of infection and its prevention was recognised. Progress was made towards the greater understanding of the cellular processes which contributed to the inflammatory course of wound healing. Joseph Lister (1827 - 1912) realised that bacteria were the cause of wound sepsis. However he realised that Louis Pasteur's (1822 - 1895) method of destroying bacteria by heating could not be applied to living tissue. In his search to find an antiseptic he tried many chemicals and discovered that carbolic acid or phenol were most effective (14).

The results were dramatic, and within a few years, the antiseptic techniques of soaking the surgical instruments, sutures and lint dressings, and spraying the operating theatre with carbolic spray became standard procedure. Other pioneers in the evolution of asepsis were Ernst von Berman (1836 - 1907), who developed the method of steam sterilisation of instruments, and William Stewart Halsted (1852 - 1922) who actively promoted the use of rubber gloves in surgery to improve wound hygiene (15).

Understanding the biology of wound healing began with Virchow when he published a book in 1860 'Cellular pathology as based on physiological and pathological history' and Elie Metchnikoff's 'The identification of phagocytic cells in the process of wound healing', in 1890. With the invention of the electron microscope in 1930, the cellular and humoral immunology of wound healing grew rapidly.

The first researcher to study the biology of wound healing in humans was Shattuck W Hartwell in 1926, who presented his thesis, entitled 'The mechanism of healing in human wounds' to the University of Minnesota (16). Hartwell suggested that human wound healing differed from studies performed on other animal species.

He looked at 850 sections from a variety of wounds in experimental animals such as rabbits, dogs, pigs, pigeons and human. He found that although epithelialisation was similar in the human and animal wounds, the fibrous healing differed consistently in several ways:

- There were more mitoses apparent in the animal wounds.

- In the human, fat was considered the main locus of healing, but the pattern of subcutaneous fat in animals was different.
Introduction

- Animal wounds had larger vessels than human.
- The morphology of cells identified by Hartwell as macrophages was different.

He thus concluded that *the conception of healing in human tissue should rightly be built up from observations of human tissues. The fact that the histological findings are different in animals from those in human wounds merely emphasizes this fact. The differences serve to explain why the description of wound healing in human surgical wound does not correspond more closely to the descriptions of healing given in text books which are based on experimental wounds in animals.* (17, 18).

1.1.5 Recent Developments

The last few years have seen a number of interesting discoveries in the understanding of the healing process, mostly based on animal wound models. Efforts have also been made to stimulate healing in recalcitrant wounds by the application of topical growth factors such as Platelet Derived Growth Factor (PDGF) (19, 20), autografts in the form of a split thickness skin graft, and the use of tissue engineered products (21). From being merely scavenger cells to `mop up' debris in wounded tissue, Negative pressure wound therapy have been shown to reduces oedema and increases blood flow to the area. This improves the reduction of exudate and bacterial load, simplifies tissue granulation and accelerates wound healing (22, 23). Now it is most likely become the gold standard for treatment of hard-to-heal wounds especially those with increased amounts of exudation.

1.2 Human Skin

1.2.1 Structure and Function

Skin is the largest organ of human’s body. The integumentary system not only protects the body from dehydration and the underlying muscles and bone from environmental damage (24), but wards off multiple other injuries from the body, including infectious organisms, UV light, thermal, mechanical and chemical agents.
The skin is subdivided into three distinct anatomical compartments: the epidermis, the superficial epithelial skin layer which serves as the biological, chemical and physical barrier between the body and its environment; the dermis, which provides crucial structural support, perfusion, innervation, access to the general immune system and multiple secreted signals to the epidermis and holds most skin appendages; and the subcutis (hypodermis), the well-perfused and innervated adipose layer of skin [Figure 1.9 and 1.10].
1.2.2 Epidermis

The epidermis is a stratified, normally composed by four layers (from the superficial to the deepest) [Figure 1.10a and 1.10b]: stratum corneum (horny layer [stratum corneum]); stratum granulosum (granular cell layer); stratum spinosum (spinous or prickle cell layer); stratum basale (basal or germinativum cell layer) which also contains epidermal stem cells, including some cytokeratin 15 (CK15)-positive epithelial progenitor cells (25-27), while the epidermis is constituted mainly by keratinocytes, there are several other cell populations such as - melanocytes [Figure 1.11a], Langerhans cells [Figure 1.11b], Merkel cells [Figure 1.11c], and intraepithelial T cells. It is densely innervated without blood and lymphatic vessels, (27-30). The stratum basale is a continuous, innermost layer of undifferentiated, proliferating keratinocytes which lies next to the dermis comprise as a single cell layer that are attached to the basement membrane by hemidesmosomes (31). Located directly above the stratum basale, the keratinocytes of the stratum spinosum switch-on a program of terminal differentiation whose product, intracellular keratohyalin granules, are most prominently visible in the next layer, the stratum granulosum (Figure 1.10). Finally, in the stratum corneum, the outermost cornified layer of skin, keratinocytes have extruded their nuclei and contains densely packed intermediate keratin filaments that are bundled together in a manner that makes these non-viable cells highly resistant to environmental stressors, for example, temperature, pH, chemical insults and enzymatic digestion (32, 33).

![Figure 1.11: Immunohistochemical staining of human skin](image)

Melanocytes (a, Melan-A stain), dendritic cells (b, S100 stain, Langerhans cells), and Merkel cells (c, Cytokeratin-20 stain) in basal layer

1.2.3 Dermis

The dermis is mainly composed by extracellular matrix (ECM)-embedded fibroblasts and consists of two layers, the papillary and reticular dermis. These are interspersed by blood and lymphatic vessels by autonomic and sensory nerve fibers of different type and as well as by mast cells [Figure 1.12 c], macrophages and other dendritic cells, and a few lymphocytes (28,
Introduction

29) [Figure 1.12 b]. The fibroblasts, which also may become a contractile cell (myofibroblast) during wound contraction, play a key role in wound healing, e.g. by the production of ECM such as collagen I and IV [Figure 1.12 a].

Collagen is a tough, long-lived, water-absorbing fibrous protein, and the collagen fibers are mainly responsible for the mechanical strength and extensibility of the dermis [Figure 1.12 a-d] (34). Interspersed elastin fibers are responsible for elastic and recoil properties of the skin [Figure 1.12 a] (35). The dermis also contains the bulk of skin appendages, such as hair follicles, eccrine and apocrine glands (sweat glands and sebaceous glands), mechanoreceptors (provide the sense of touch, vibration and heat) [Figure 1.12d].

1.2.4 Hypodermis

The hypodermis (subcutis), which mainly consists of adipocytes and endothelial cells, is a loose connective tissue layer with major regional differences in the amount and arrangement of adipose tissue [Figure 1.12d] with multiple physiological functions, which range from energy storage, thermoregulation via hormone, neuropeptide synthesis and metabolism, to the regulation of food uptake via leptin (36). Subcutaneous adipocytes also operate as niche cells that provide important paracrine growth-regulatory signals, notably to the hair follicle and may promote wound healing by the release of leptin (36, 37).

1.2.5 Skin appendages

Skin appendages—hair, nails and glands—originate from the stratum basale and grow downward into the dermis and subcutis. The hair follicle (HF) [Figure 1.13] is a characteristic feature of mammals and the only organ that shows a lifelong cyclic remodelling activity. In its cycle, the HF undergoes autonomous, cyclic transformations from a stage of growth (anagen), via regression (catagen) to relative quiescence (telogen), which have also been demonstrated that HF itself and its cycling exerted wound healing promoting effects (38-39).

In the context of wound healing, the HF is of special interest in that its activities directly impact on the efficiency and quality of wound healing as well as on intracutaneous angiogenesis, both of which are significantly increased in murine skin with terminal HFs in anagen, compared to catagen or telogen skin (40, 41). The sweat glands of human skin may also play a more prominent role in human skin wound healing than previously thought, as their stroma is rich in nestin and stem cells, which can be utilized to promote wound healing in murine skin in vivo (42) and wounded organ cultured human skin.
Figure 1.12: Structure of skin
Dermis (a) Gieson & elastin staining of thick skin. (b) Hematoxylin and eosin staining (H&E) of dermis. A: Epidermis; B: Fibroblasts; C: Collagen fibre bundles; D: Lymphocytes; E: Blood vessels. (c) Visualisation of mast cells in human dermis by Leder esterase histochemistry (black arrow, red cells). (d) Hematoxylin and eosin staining (H&E). "Hypodermis" = subcutis.

Figure 1.13. Histomorphology of human scalp hair follicle
(a) H&E section showing infundibulum, isthmus and anagen-associated (suprabulbar and bulbar area) components of the hair follicle. (b) High magnification image of the isthmus. The dashed square indicates the approximate location of the bulge; (c) High magnification image of the bulb. (d) Melanin granules. (BM: basal membrane; APM: arrector pili muscle; CTS: connective tissue sheath; DP: dermal papilla; M: matrix; HS: hair shaft, IRS: inner root sheath; ORS: outer root sheath; SG: sebaceous gland).
Introduction

The HF consists of mesenchymal part the dermal papilla (DP) and the connective tissue sheath (CTS) that surrounds the entire HF and forms the infundibulum, isthmus, bulge and hair bulb (35, 43) [Figure 1.13]. HFs display a very dense innervation system, especially of the bulge and isthmus region. The perifollicular neural plexus release neurotransmitters, neuropeptides and neurotrophins and may thus fulfill important trophic and regulatory functions in hair biology (44).

1.3 Overview of Wound Healing

Wound healing involves different processes (cellular, physiological, biochemical and molecular) which result ultimately in connective tissue repair and fibrous scar formation (45). This is a dynamic process coordinated by an array of growth factors and cytokines. It involves a complex interaction between epidermal and dermal cells, the extracellular matrix (ECM), highly regulated angiogenesis, and plasma-derived-proteins. Wound healing is divided into three overlapping phases –inflammation, proliferation and remodelling [Figure-1.14] (46). The inflammatory phase begins immediately after injury. It is characterised by pain, heat, redness, swelling and loss of function at the site of the wound (47). The initial response to the disruption of blood vessels is bleeding and the homeostatic response to this is clot formation to stop haemorrhage. Collagen and basement membrane proteins exposed by the injury activate Hageman factor XII, which is responsible for activation of the healing cascade (48). There are several effector systems within the healing cascade such as the plasminogen cascade, the complement cascade, the kinin cascade and the clotting cascade. They release complement C5a, fibrin degradation factors, platelet activity factors and chemical mediators- serotonin and histamine, which interlink to control infection and regenerate tissue (49).

1.3.1 The Inflammatory Phase

This stage is characterised by erythema, swelling and warmth associated with pain, the classic “rubor et tumor cum calore et dolore”. This stage usually lasts up to 4 days post injury. At this stage blood vessels become leaky releasing plasma which contains neutrophils or PMN’s (polymorphonucleocytes) into the surrounding tissue. Neutrophils provide the first line of defense against infection by phagocytising debris and microorganisms. Macrophages are able to phagocytise bacteria and provide a second line of defense. They also secrete a variety of chemotactic and growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-β and interleukin-1 (IL-1) (50).
1.3.1.1 Blood Coagulation

Damage to the blood vessels causes extravasation of blood constituents into the injured tissue, which stimulates clotting. Blood clotting results from the surface activation of Hageman factor (Coagulation factor XII), tissue pro-coagulant factor from damaged endothelial cells, surface membrane coagulation factors and phospholipids expressed on activated platelets and endothelial cells (50). The critical event in all cases is the availability of a surface of damaged blood vessel wall, with its exposed collagen, to promote adsorption and activation of coagulation pro-enzymes such as fibrinogen and prothrombin. The coagulation cascade ceases with the production of prostacyclin, which inhibits platelet aggregation, antithrombin III binding to thrombin and thus inhibiting its activity, protein carbonic anhydrase is a potent
enzyme that degrades coagulation factors V and VIII, and the release of plasminogen activator which initiates clot lysis by converting plasminogen to plasmin (51, 52). Fibrin rich clot also provides a matrix scaffold for the recruitment of cells to the injured area and the fibrin acts as a provisional matrix for the influx of monocytes (53) and fibroblasts (54).

1.3.1.2 Platelets

Platelets are found in the intravascular space in an inert form. The platelets adhere to the damaged interstitial connective tissue and then aggregate. During aggregation, they become activated secreting multiple mediators, such as fibrinogen, fibronectin, thrombospondin and von Willebrand factor VIII, and others. Fibrinogen, fibronectin, and thrombospondin act as ligands for platelet aggregation and Fibrinogen is also converted to insoluble fibrin which adds to the fibrin clot (55). Platelets also release chemotactic factors for blood leukocytes, (56) growth factors such as platelet-derived growth factor (PDGF), (57) and transforming growth factor-alpha and beta (TGF α and β) (58, 59) which regulates the formation of new tissue.

1.3.1.3 Neutrophils

Neutrophils and monocytes enter the wounded tissue at the same time, but because of the greater number of neutrophils in the general circulation, they tend to be the predominant cell type in the early phase of inflammation. They are chemotactically attracted to the injured tissue by fibrin degradation products, such as fibrinopeptides cleaved from fibringen, C5a from the activated classical or alternative complement cascades; activated neutrophils release leukotriene B4, formyl methionyl peptides from bacterial proteins and PDGF from platelets (60). Activated neutrophils also release elastase and collagenase, which facilitate cell penetration through basement membranes of vessels (61). At the wound site they destroy contaminating bacteria by phagocytosis and subsequent enzymatic and oxygen radical mechanism (62). If wound contamination is minimal, neutrophil infiltration ceases within a few days. Early stage macrophages are also armed with collagenases which facilitate the debridement of non-viable tissue (63).

1.3.1.4 Monocytes

The accumulation of monocytes at the site of injury persists by selective monocyte chemoattractants such as fragments of collagen (64), elastin (65), fibronectin (66), enzymatically active thrombin (67), and TGF-β (68).
1.3.2 The Proliferative Phase

This phase commences at about the second day of injury and lasts about four weeks in normal healing wounds. During this phase, there is a marked influx of macrophages and fibroblasts into the wound along the fibrin scaffold laying down a variety of substances including hyaluronic acid, chonditin-4-sulphate, dermatan sulphate, heparan sulphate constituting the amorphous ground substance within the wound. There is also an increased synthesis of collagen. This increases the tensile strength of the wound.

1.3.2.1 Granulation Tissue

Approximately four to five days after injury at early phase of wound repair, new stroma, called granulation tissue, begins to form which consists of a dense population of fibroblasts, inflammatory cells, and new blood vessels embedded in a loose matrix of collagen, fibronectin and hyaluronic acid. 

In this phase the formation of new blood vessels -angiogenesis, and the laying down of the extracellular matrix and collagen-fibroplasia are the major events. The large number of new capillary loops on the surface of the granulation tissue creates a granular appearance, hence its name. The granulation tissue restores the soft tissue defect by creating new connective tissue and blood vessels, which forms a scaffolding for the migration of epithelial cells (69, 70). In healthy wound granulation tissue appears as a firm, pink tissue. It is the prerequisite to re-epithelialisation. Overgranulation observed in patients with chronic wounds is thought to be due to defective signaling or a result of infection, thus preventing re-epithelialisation.

1.3.2.2 Angiogenesis

Hertig in 1935 used the term angiogenesis to describe new blood vessel formation in the placenta, and Folkman and Shing has been extensively studied it in chick chorioallantoic membrane and the cornea (71). Angiogenesis in the healing wound usually starts at the same time as fibroplasia and is an essential part of wound healing. The process begins with the disruption of the basement membrane of the parent vessel by specific proteases released by endothelial cells. These proteases also breakdown the adjacent extracellular matrix (72). This creates tissue defects that facilitate the formation of capillary sprouts that form the basis of capillary plexuses (73). Cellular proliferation occurs in the endothelial cells remaining within the parent vessel, which then migrate in continuity with the lead cells. The capillary sprouts link up to form loops through which blood begins to flow. The process of budding repeats itself from the new vessels forming a plexus, supplying oxygen and nutrients to the wounded.
tissue. Angiogenesis is regulated by a complex interplay between the hypoxic environment, and growth factors such as basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) (74).

1.3.2.3 Fibroplasia - matrix deposition and collagen synthesis

Clot formation, provides the initial fibrin matrix to support cell migration. A variety of fibroblast-derived enzymes and serum derived plasmin, including plasminogen activator, interstitial collagenases [Matrix metalloproteinase-1 (MMP-1)], gelatinises [MMP-2] and stromelysin [MMP-3] aid the migrating fibroblasts. The endoplasmic reticulum and golgi apparatus retract to a perinuclear position within the fibroblast. This is followed by the formation of a loose extracellular matrix composed of fibronectin.

Under the influence of TGF-β the fibroblasts increase collagen production (75). Once abundant collagen matrix is deposited into the wound, fibroblasts cease collagen production despite the continuing expression of TGF-β. Grinnell in 1994 has demonstrated that the collagen matrix itself can suppress both fibroblast proliferation and collagen production (76). It has been found that many fibroblasts in a 10-day healing wound develop pyknotic nuclei, thus resulting in apoptosis (programmed cell death), but the triggers for wound fibroblast apoptosis has not been elucidated (77). This converts a fibroblast-rich granulation tissue to a relatively a cellular scar.

1.3.2.4 Epithelialisation

One or two days after injury, epithelial cells at the wound margin begins to proliferate. The epithelial cells undergo marked phenotypic changes, which include retraction of intracellular tonofilaments, dissolution of most inter-cellular desmosomes and the formation of peripheral cytoplasmic actin filaments which aid their mobility (78). Desmosomes are structures that interlink epithelial cells, thus providing strength to the epithelium. The binding between the epidermis and the dermis is also lost. This allows wound epidermal cells to extend pseudopodia from their free basolateral edge and move into the wound. Migrating wound epidermal cells do not contain the keratin proteins that are found in mature stratified epidermis or the matrix protein flaggrin, in which the keratin are embedded. Cells in all layers of the migrating tip contain the type of keratin that is normally found in the basal layer of the stratified epidermis (79). The stimulus for these changes are unknown, however, it has been postulated that the lack of neighboring cell contact may be the stimulus for both epithelial migration and proliferation, similar to the way re-endothelialisation occurs in large blood vessels after internal damage (78). Epidermal growth factor family (EGF), transforming
growth factor a (TGFa), and fibroblast growth factor family (FGF) may also play a role in the migration of keratinocytes (80). Once keratinocytes have migrated over the provisional matrix, there is re-establishment of the elements of the basement membrane (81). These include collagen type IV, heparan sulfate and the attachment factor laminin. The components of epidermal intracellular junctions, desmosomes and hemi-desmosomes are assembled, and the anchoring fibrils are replaced. The epidermis then enters a regenerative phase in which a mature stratum corneum is produced and is anchored to the underlying neodermis through type VII collagen (82). If the wound is superficial, there is preservation of epidermal appendages such as eccrine sweat glands, sebaceous glands, and hair follicles, these become the major source of epidermal cells, a principle utilised in the healing of donor sites after harvesting a split thickness skin graft (83).

1.3.3 The Maturation Phase

1.3.3.1 Tissue Remodelling

The final phase of wound healing is an ill-defined overlapping phase that begins at the time of matrix deposition in the wound and persists for many years after wound closure by the epithelium. By observing the strength of dermal scars in animal models with time from injury, it can be seen that the initial deposition of collagen fibres are random but soon align perpendicular to the wound providing strength to the scar tissue (84). There is also a change in the composition of the collagen bundles as the scar tissue matures. Early collagen, known as type III collagen, makes up to 30% of the granulation tissue; and contributes little to the wound's tensile strength (85). As the collagen matures, type III collagen is replaced by type I collagen, thereby exists a simultaneous process of matrix breakdown and synthesis. Fibroblasts are responsible in the regulation of these dual processes, by producing the extracellular components and MMPs, which are responsible for the degradation of the matrix (86). During this process the scar gains tensile strength (87). Imbalance in matrix synthesis and breakdown results in abnormal wound healing. This is seen in hypertrophic scars, keloid formation, and non healing chronic wound.

1.4 Wound Types

1.4.1 Acute Wounds

Acute wounds are caused by injury to intact skin and include bites, minor cuts, surgical wounds, abrasions and also more traumatic wounds such as lacerations and gun-shot injures.
(88). Injury limited to the epithelial tissue is classified as a superficial wound and will heal rapidly by regeneration of epithelial cells. A partial thickness wound involves the deeper dermal layer and includes damage to the blood vessels. A full thickness wound affects the subcutaneous fat layer and beyond and its healing will take longer as it requires the synthesis of new connective tissue (47). Acute wounds heal within a reasonable time frame after injury (89).

1.4.2 Chronic Wounds

Acute wounds can transform into chronic wounds [Figure 1.15] if the wound healing process is disrupted and they do not heal over the expected period of time irrespective of the cause. Additionally, they may form as the result of systemic infection, immune, vascular or nerve insufficiency or metabolic disorders such as diabetes (90). They are difficult to heal, may never heal or take years to heal (47). In chronic wounds the balance between production and degradation of collagen is lost and this is frequently caused by endogenous mechanisms, which disturb the integrity of dermal and epidermal tissue (87). There are several factors that may have an impact on wound healing. Such factors include age due to a decrease in inflammatory response and physiological processes such as blood circulation, reduction in collagen formation and basement membrane degradation (91). Malnutrition can also prevent wound healing by decreasing collagen production and other proteins needed for wound repair. Bacteria in high numbers at the wound bed produce toxic end products and compete with cells in the granulation tissue for available nutrients (92). Stress has also been implicated in the impaired healing process with decreased wound healing associated with pain and noise (93).

Chronic wounds may affect only the epidermis and dermis or they may affect tissue to the fascia. The majority of chronic wounds can be classified into three broad categories:

- Venous leg ulcers (VLUs),
- Pressure ulcers and
- Diabetic foot ulcers (DFUs).
Figure 1.15 Pathophysiology of Acute and Chronic Wound.
Several differences have been highlighted between acute and chronic wound environments. In comparison to acute wounds, chronic wounds are characterised by elevated levels of inflammatory cells, cytokines and proteases with diminished growth factor activity. These wounds are considered to be “stuck” in the inflammatory phase (Mast and Schultz, 1996 [94], Clark et al., 2007 [95]).

Diabetic Foot Ulcers (DFUs)

Approximately 20% of diabetic patients develop DFUs due to peripheral neuropathy, muscle atrophy, foot deformity and neuropathic fractures (96). These ulcers eventually became colonised or infected with different bacteria. Diabetic foot ulcers (DFUs) precede 85% of all diabetes-related lower-leg amputations (97). The moment a person with diabetes suffers a breach in the skin of their foot, they are at danger of amputation. Currently, there are approximately 100,000 limb amputations performed in the United States every year.

Venous Leg Ulcers (VLU)

Venous leg ulcers (VLU) are localised in the lower limb or an area of damaged skin below the knee. They are thought to be due to venous hypertension caused by the improper function of the valves of the veins to prevent blood from flowing backward. In venous hypertension, the difference between arterial and venous pressure reduces and blood is not pumped effectively (47, 98). Venous hypertension may also stretch veins and allow blood proteins to leak into the extravascular space, which isolates extracellular matrix molecules and growth factors,
preventing them from healing the wound (99). Other factors that contribute to venous leg ulcers such as arterial disease, vasculitis and diabetes, immobility, trauma and obesity (100).

**Pressure ulcers**

Pressure ulcers occurs when pressure on the tissue is greater than the pressure in capillaries causing obstruction of blood flow into the area. Patients with paralysis (temporary or permanent) that inhibit movement of body parts (heels, shoulder blades, and sacrum) are more prone to pressure ulcers (101, 102).

### 1.4.2.1 Pathophysiology of Chronic Wounds

The wound healing is related to local factors associated with the wound itself and also with co-morbidities (e.g. diabetes). Chronic wounds are associated with low levels of growth factors (103, 104) along with high levels of pro-inflammatory cytokines and matrix metalloproteinases (MMPs) (103); all these lead to impede wound healing process in the inflammatory phase. There is also evidence to suggest that a subset of chronic wounds do not heal because they fail to complete epithelialisation especially in elderly patients. For example, keratinocytes have been shown to exhibit an age-related reduction in mitogenic response and in vivo studies have shown that the rate of reepithelialisation is reduced in both aged rat and mouse models (105) and in humans (106). Ashcroft et al., (2002) have suggested that this may be related to low level of growth factors such as epidermal growth factor (EGF). Major factors that can lead to chronic wounds are ischaemia, reperfusion injury, and bacterial colonisation (107).

#### 1.4.2.1.1 Ischaemia

Ischaemia causes tissue inflammation and affected cells to release factors attracting neutrophils such as interleukins (108, 109), chemokines, leukotrienes, and complement factors which lead to tissue inflammation. Neutrophils, while fighting against pathogens, release damaging enzymes and inflammatory cytokines (110) and reactive oxygen species (ROS) to kill bacteria, by use of an enzyme called myeloperoxidase (107). These enzymes destroy extracellular matrix, and cytokines, prevent cell proliferation and wound closure by damaging DNA, lipids, proteins the that enhance healing (100). Neutrophils remain in chronic wounds for longer time than acute wounds, and this contribute to the fact that chronic wounds have higher levels of inflammatory cytokines and ROS (111, 112). Since chronic wound fluid
has an excess of proteases and free radicals, the fluid itself can inhibit healing by inhibiting cell growth and breaking down growth factors and proteins in the extracellular matrix (113).

### 1.4.2.1.2 Bacterial Colonization

Bacterial colonisation is another major factor that affect wound healing. It is known that healing and bacterial load is a complex equation involving the different type(s) and number of bacteria (114). Patients with decreased tissue oxygenation such as those who suffered hypothermia during surgery or diabetic patients have a higher risk for infection. Low levels of oxygen in the wound environment prevents white blood cells from producing reactive oxygen species essential for killing bacteria (108). The host’s immune response to the presence of bacteria delays healing by prolonging inflammation and causing damage to the tissue. Infection can cause not only the wound to become chronic but can cause also gangrene, loss of the infected limb, and death of the patient (115). Microbial colonisation and infection can further damage tissue by attracting a greater number of neutrophils to enter the wound site (110).

### 1.4.2.1.3 Growth Factors and Proteolytic Enzymes

The levels of proteolytic enzymes such as matrix metalloproteinases (MMPs) and elastase (116) in chronic wounds are higher than in acute wounds, while the concentration of growth factors such as KGF and PDGF are lower (112). Therefore, an important factor in chronic wound formation may be inadequate growth factors levels (90). Epidermal growth factor has been shown to be degraded in chronic wound fluid compared to acute fluid, which suggests again a direct link between high protease activity and poor tissue regeneration (104). For full healing, wounds require a certain level of elastase and proteases. However, too high a concentration of these enzymes is damaging (117).

Leukocyte in the wound area release elastase, which increases inflammation, destroys collagen, proteoglycans (118), growth factors, fibronectin (119). Leukocyte also release high levels of matrix metalloproteinases (MMPs), which may also cause wounds to become chronic (120). Matrix metalloproteinases destroy growth factors, ECM (120) and protease inhibitors. They increase degradation and reduce construction processes, which leads to balance disturbance (121). There is strong evidence that activity of MMP decreases as the wound heals (103). TiMP1 (Tissue inhibitor of metallopeptidase 1) is a glycoprotein that is expressed in tissue and involved in the degradation of the extracellular matrix. It is able to promote cell proliferation in a wide variety of cells. Ladwig et al (2002) have shown that the ratio of MMP to TiMP1 may be an important factor, which allows a prediction of a wound’s
ability to heal. Many common wound bacterial species produce a wide array of MMPs that additionally have a negative impact on wound healing (122).

1.5 Treatment of Ulcers and Types of Dressings (123)

The aim in the treatment of any type of wound is to achieve normal and timely healing. The large number of factors that may affect wound healing, such as tissue oxygen tension, wound hydration, temperature, and mechanical stress, together with the high degree of variation in wound characteristics and patient status may make it difficult to evaluate the effects of treatment. In some cases, wound healing techniques are not expected to result in complete wound closure. Rather, the treatment may be intended to advance the wound to a stage where healing is possible.

Hundreds of dressings are available in the market with more appearing each year. Wounds are dressed with materials to offer protection from outside contaminants, prevent wound desiccation, and provide an environment to facilitate wound closure. It is important to choose the right dressing for a wound, taking various factors into consideration like:

- The amount of moisture in the wound,
- The amount of discharge or pus,
- The condition of the skin around the wound,
- The location of the wound.

For over three decades, since the work of Hinman (124) a moist wound environment has been recognized as optimal for wound healing. Dressings have since been modified to maintain this environment while also controlling the growth of microorganisms, allowing gaseous exchange, and thermally insulating the wound. An ideal dressing should improve symptoms, provide wound protection, and encourage healing.

1.5.1 Negative Pressure Wound Therapy (NPWT)

1.5.1.1 History of NPWT

NPWT is one of the oldest forms of medicinal therapy. Wound drainage has been implemented for even longer reported cases trace back to 600 BC in Babylon and Assyria. Vacuuming via heated copper bowls began in 400 BC by the Greeks. In 1907, Dr. E. Klapp used a suction pump on a wound for the first time.

It was first used successfully by Raffl in the early 1950s to manage exudate and accelerate wound healing after radical mastectomies (125). A number of NPWT systems were then tried
in the 1980s, with both USA and Russia in the forefront of the technology. Miller et al. (126) quote a series of articles describing the treatment of wounds using negative pressure, the “Kremlinpapers” (127), that was published in the Russian literature in the 1980s. Negative pressure (−75 to −80 mmHg) was used in combination with aggressive debridement to significantly reduce bacterial counts in purulent wounds (128). In 1989, Chariker et al. (129) published their experience utilising NPWT in patients with incisional or cutaneous fistulae complicating ventral abdominal wounds. Where he placed moist gauze over the wound surface and flat drain placed over the gauze and both covered with an occlusive dressing. The flat drain was connected to vacuum line (wall suction) with continuous pressure set at approximately -60 to −80 mmHg. This method later became known as the Chariker-Jeter technique.

In the early 1990s, the first investigative studies into NPWT using foam as a wound contact layer were carried out by Fleischmann et al. (130). Argenta and Morykwas also carried out work on the use of foam contact layers in both patients and animals, and found that the use of NPWT with a pressure setting of −125 mmHg was likely to reduce healing time, reduce local edema and manage wound exudate (131). Their work led to the development of the first commercialized system, the V.A.C.® Therapy System (KCI, San Antonio, TX). Until 2003, this was the only commercially available NPWT system.

NPWT involves the controlled application of subatmospheric pressure to the wound bed through a wound filler (foam or gauze) placed in the wound. The wound is then sealed with an adhesive drape that allows pressure to be applied and helps provide a moist environment that supports wound healing. NPWT has become an integral part of modern wound care, and is used routinely in hospitals throughout the world.

### 1.5.1.2 Mechanism of Action

Normal wound healing progresses through the following phases: hemostasis, inflammation, proliferation, and remodeling. Both systemic and local wound factors can contribute to delay healing. Systemic factors (eg, wound ischemia, poor nutrition). Local wound factors that delays normal healing include desiccation, tissue edema, excessive exudate, poor tissue apposition (eg, grafts and flaps), and wound infection. Stagnant fluid is associated with cytogenetic factors that impede wound healing (131).

**Indications** — The application of negative pressure to assist in wound healing was first described in the management of soft tissue injury in association with open fracture (132). The demonstration of beneficial effects in animal models spurred the development of the negative
pressure wound systems that are widely available (133). NPWT has been applied to a wide range of clinical situations, including the open abdomen, following surgical debridement of acute or chronic wounds (eg, orthopedic, necrotizing infection, Venous leg ulcers, pressure ulcer), diabetic foot ulcers, and reconstructive surgery (eg, burns, skin graft, muscle flap).

**Advantages** — Compared to traditional wound care modalities, NPWT offers several clinical advantages compared to usual care (134,135).

- NPWT dressings are changed once every two to three days and anticipated pain can be managed preemptively compared to conventional dressing.
- Compared to other forms of wound dressing, NPWT is easier to tailor and maintain in position. Almost every configuration of wound including circumferential extremity wounds (ie, degloving injuries), and wounds located in proximity to orthopedic fixation frames can be managed with relative ease. As a result, NPWT may allow less complex modes of reconstructive surgery. Complex wounds that required a pedicle flap may, after NPWT, be converted to a wound requiring a rotation flap or skin graft.
- Accelerate wound healing by significantly reducing the time to wound closure in diabetic patients, returning these patients to baseline more quickly and improving quality of life.
- Reduced complexity of subsequent reconstructive procedures.

**Disadvantages** — From the patient's perspective, the main disadvantage of NPWT is the need to carry the portable pump. Light weight battery operated portable pumps are likely to be released in the market soon (patent pending –Devon medical products).

- NPWT systems are more expensive than traditional wound dressings.

**Contraindications** — NPWT should not be used when any of the following are present:

- Exposed vital structures — NPWT, in the presence of exposed organs, blood vessels, or vascular grafts, increases the risk for tissue erosion, which can lead to enteric fistula or hemorrhage (136). NPWT is generally avoided until an intervening granulation layer or tissue flap or graft provides coverage. Although some clinicians report success using barrier dressings, caution is advised when implementing this practice.
- Ongoing infection — Active infection should be treated prior to using NPWT.
- Devitalized tissue — Inadequate debridement with the presence of devitalized soft tissue or bone increases the risk for infection (137)
Malignant tissue — As with normal tissues, growth of malignant tissue is promoted in the presence of subatmospheric pressure. Malignant tissue is also more friable and prone to bleeding.

Adhesive allergy — NPWT requires an adequate seal to maintain the applied suction. The adhesive cover typically overlaps the skin 4 to 5 cm with a significant amount of adhesive in contact with the patient's skin. Sensitive patients can develop shearing of the skin and bullae formation.

Ischemic wounds — Although not absolutely contraindicated, no benefit has been demonstrated with the use of NPWT in patients with ischemic wounds (134). The application of negative pressure to these wounds would be expected to worsen tissue ischemia.

1.5.1.3 Clinical Applications

Chronic wounds

NPWT improves the healing of some types of chronic wounds/ulceration provided that they are well vascularized (138). Patients with extremity wounds and inadequate peripheral pulses should undergo noninvasive vascular testing to confirm adequate perfusion prior to instituting NPWT, especially patients with diabetes or other risk factors for peripheral artery disease. Venous stasis ulcers are uncommonly confused with other types of chronic ulcers. While these ulcers are associated with significant wound edema and exudate, they are managed with local wound care and compression therapy. Three randomized trials have evaluated the use of NPWT as an adjunctive therapy for the management of pressure (decubitus) ulcers (139). No statistically significant differences were identified with respect to quantitative wound healing measures (e.g., wound surface area reduction). However, NPWT improved patient comfort and was less labor intensive (140).

Skin graft/flap fixation

NPWT used to provide skin graft fixation instead of traditional bolstering methods (141). NPWT evenly distributes negative pressure over the surface of the fresh graft, immobilizing it with less chance of shearing. From observational studies (142) and two randomized trials, it has been shown that NPWT has improved qualitative skin graft take and quantitative improvements in skin graft success (e.g., reduced number of repeat grafts) (143, 144). In one of the trials, 60 patients were randomly assigned to conventional bolster dressing or NPWT following split thickness skin graft (SSG) (143). NPWT was associated with significant reduction in the loss of graft area (0 vs 4.5 cm² in the control group) and the median duration
of hospitalization (13.5 vs 17 days). Summary of the few negative pressure therapy studies shown in Table 1.1.

1.5.2 Limited Access Dressing (LAD)

In a study by Pramod Kumar the LAD was found to be a safe and effective alternative to conventional dressing methods. LAD retained all the advantages of moist wound healing and negative pressure dressings and avoided all of their disadvantages by manipulating the wound environment by providing limited access through tubes.

S1.5.2.1 Basic Principle and Design of LAD

LAD has been designed to reduce the pain and discomfort of conventional dressings, to reduce the frequency of dressing, to reduce the chances of wound infection and to improve the results obtained by negative pressure dressing (151-153). LAD combines the principles of moist wound healing and NPWT along with a provision of two additional ports (12-16Fr tube) for instilling antimicrobial solution of choice and alters the wound environment without any need to change the dressing. LAD utilizes definite intermittent negative pressure regime (30 minutes of negative suction and 3½ hours of rest i.e. no suction period; minimum 30mmHg of negative pressure).

1.5.2.2 Method of Application of LAD

For applying LAD over extremity wounds (151-153) either ethylene oxide presterilized customized polythene bag are used or based on the shape, size and site of the wound customized polythene bag is sealed using semi automatic plastic sealing machine and sterilized by immersion in 2.45% w/v glutaraldehyde solution for 20 minutes. Two thick nasogastric tubes (extra holes are made if required) after placed in between the bag and wound are brought out through the polythene cover at the predetermined site. The junction between tubes and polythene is sealed using hydrocolloid material and electrical insulation tape. Now at the mouth of the bag, the edges of polythene are sealed over extremity skin using hydrocolloid material and polyurethane film. Now, the tubes are connected to the suction machine using a ‘Y’ connection. [Figure 1.16].
Table 1.1 Summary of randomized clinical trials on topical negative pressure

<table>
<thead>
<tr>
<th>No.</th>
<th>Author</th>
<th>NPWT Control</th>
<th>Vs</th>
<th>Number of Patients</th>
<th>Follow up</th>
<th>Wound type</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild, 2008,145</td>
<td>TNP vs Redon suction Bottles</td>
<td>n=10 (5 vs 5)</td>
<td>8.4 d</td>
<td>Pressure ulcer grade ¾</td>
<td>The study was terminated after a post hoc analysis because of the significantly better results when using TNP and the substantially larger care effort needed in the Redon group. An increase in surface granulation tissue of 54% was observed with TNP and a reduction in the Redon group (P=.001). The Redon group showed an increase in fibrin tissue at the wound base of 21.8%, whereas in the TNP group, a 27% reduction was observed (P=.035). Necrosis was reduced in the TNP group, but this difference did not reach significance.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mody, 2008,146</td>
<td>B-TNP (intermittent) vs saline soaked gauze</td>
<td>n= 48 (15vs33)</td>
<td>8-70 d</td>
<td>Chronic wounds of different Etiology</td>
<td>No statistically significant differences in time to closure between the 2 treatment groups were observed except in a subset analysis of pressure ulcers (mean 10=7.11 days for treatment and 27=10.6 days in control group (P=.05). Direct costs to close a pressure ulcer were also lower in the GB-TNP treated group than in the control group. Significant smaller area of skin graft loss, less need for regrafting, and faster discharge after grafting in the GB-TNP group.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Llanos, 2006, 147</td>
<td>GB-TNP (80 mm Hg) vs same sponge but no drainage</td>
<td>n = 60 (30vs30)</td>
<td>11–22 d</td>
<td>Acute traumatic and burns covered with split skin grafts</td>
<td>No significantly faster granulation, wound surface reduction, or better bacterial clearance in the TNP group, but patient comfort was an important advantage. Time involvement and costs of nursing staff were significantly lower in the TNP group, but the overall costs were similar in both the groups. No significant difference in mean time to reach 50% of the initial wound.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Braaken, 2006, 148</td>
<td>TNP vs hydrocollois, alginates, acetic acid, eusol and sodium hypochlorite</td>
<td>n =65 (32vs33)</td>
<td>80 d</td>
<td>Full thickness acute and chronic wounds</td>
<td>Significant reduction in wound volume of 78% in the TNP group compared with 30% in the gauze group within 6 wks (P=0.038), no significant difference in complications.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Wanner, 2003, 149</td>
<td>TNP vs saline soaked Gauze</td>
<td>n=22 (11vs11)</td>
<td>15–56 d</td>
<td>Pressure sores of the pelvic region</td>
<td>No significant difference in mean time to reach 50% of the initial wound.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Joseph, 2000, 150</td>
<td>TNP vs saline 0.9% gauze dressing</td>
<td>n = 36 (18vs18)</td>
<td>42 d</td>
<td>36 nonhealing pressure ulcers in 24 patients</td>
<td>Significant reduction in wound volume of 78% in the TNP group compared with 30% in the gauze group within 6 wks (P=0.038), no significant difference in complications.</td>
<td></td>
</tr>
</tbody>
</table>
1.5.2.3 Clinical Applications

Intermittent negative pressure of LAD controls SIRS and organ failure

In a preliminary study (151) by Pramod Kumar (2010) on burns patients to prove leech effect, two identical groups of 54 thermal burns (less than 48 hrs duration) patients were made: Control group and LAD treated group each comprising of 27 extremities burn matching in age, sex and TBSA burn. There was no statistically significant difference in occurrence of SIRS on Day 1 (p value 0.742), but on Day 5 the difference in occurrence of both SIRS (p value 0.029) and occurrence of organ Dysfunction (p value 0.017) was significant. To alleviate the greater risk, odd ratio was analysed. The difference between two groups in SIRS on Day 1 was not significant, but on Day 5 was significant (p<0.05). The difference in organ dysfunction on Day 5 between two groups was also significant (p<0.05).

Role of LAD in preventing and treating wound infection

Negative pressure helps to eradicate the established infection LAD is very effective in eradicating both gram positive (100%) and gram negative bacteria (98.74% -100%) (154).

Wound isolation

Wound isolation by LAD polythene reduces the chance of bacterial colonization. Access of the microorganisms of the environment to the wound is limited by two long tubes and vice versa.
LAD wash

LAD wound wash with saline and removal of the wash fluid through the tubes reduces bacterial count over the surface and helps to prevent invasion.

Negative pressure reduces the chances of wound invasion

Negative pressure by providing channels with stronger negative pressure towards suction bottle as compared to that in venules and lymphatics, reduces the chances of wound invasion by colonized microorganisms and spreading of infection along with fascial planes/ sloughing tendons.

Intermittent negative pressure of LAD controls infection effectively

Negative pressure applied every 4th hourly for ½ hour (½ hour suction and 3½ hour no suction period) reduces chances wound invasion/ SIRS/sepsis/severe sepsis/ organ dysfunction and failure.

LAD produces ultra conservative debridement for limb salvage

Following necrosis, the natural process of proliferation of living tissue and separation of necrotic tissue leaving viable proliferating cells in wound bed results in minimal or no loss of viable tissue as compared to surgical debridements. During the period of auto separation of dead tissue from living tissue, unfavorable wound environment may produce SIRS and infection. These complications (SIRS, infection, sepsis) are controlled by LAD satisfactorily. Hence, though time consuming, debridement under LAD is ultraconservative as in the process there occurs minimal or no loss of viable tissue. Intra LAD ultraconservative debridement occurs in two ways (152).

• By phagocytosis and body enzymes in a moist environment. Viable cells proliferate and dead cells shed.

• Mechanical debridement occur during suction and LAD wash Slow autoseparation of necrotic tissue (time consuming) may be useful method to protect underlying structures (e.g. tendons over dorsum of foot/hand). Ultra conservative debridement conserves viable tissue maximally and hence LAD helps in diabetic foot salvage.
1.5.2.4 Advantages of LAD

Limited access dressing retains all the advantages of moist wound healing and negative pressure dressings (156).

The edge of LAD over other moist healing dressing is:

• The pus like gel (usually a source of concern to the patients in hydrocolloid dressings) produced due to dissolved hydrocolloid material is not a problem in LAD.

• Foul smell of moist dressing is reduced considerably due to LAD wash.

• Weak and limited absorbent capacity of moist dressing material is replaced by removal of secretion in an outside container by negative pressure that can be repeatedly replaced.

• Anaerobic growth is not a problem.

All the advantages negative pressure dressing is retained. Like other negative pressure dressings, consistently better graft take under LAD appears to be due to control of infection and compressions of graft between polythene sheet and recipient area. High levels of Vascular Endothelial Growth Factor (VEGF) associated with negative pressure dressing may also be responsible for better graft take (157). The average number of days required to prepare the wound bed under LAD is much less as compared to that in conventional dressing. Overall graft take under LAD was 99.87% (151).

The edge of LAD over other negative pressure dressing:

Additional advantages over other negative pressure dressing methods are:

• Intermittent low negative pressure makes LAD more acceptable and economical.
• Throughout treatment wound remains visible through polythene.
• Epithelialization is better than other negative pressure dressings where sponge is used
• Wound environment manipulation is possible in a variety of ways (155) (LAD wash, instillation of desired drug at desired site, aerobic/ anaerobic environment/ phototherapy).
• Since LAD is retained over the wound for a longer period, problems due to movements associated with frequent change of dressing in cases of compound fractures is avoided and makes it more suitable for compound comminuted fracture cases where early frequent dressings are required.
• Through transparent plastic sheet wound visibility is better.
• While hand/foot is still in LAD, physiotherapy (early) may be started.
• Intermittent negative pressure with the LAD may reduce the need for DVT prophylaxis (151) in immobilized patients with lower extremity wounds.

1.5.2.5 Disadvantages of LAD

LAD has following disadvantages that can be easily avoided by routine careful monitoring the progress of wound by an experienced person (155).
• Undesirable effects of negative pressure (pain, bleeding, ulceration)
• Ineffective negative pressure may invite undesirable effect of negative pressure (bad odor, anaerobic infection)

1.6 Factors Regulating Wound Healing

Factors that regulate wound healing are many and interrelated. Cells either secrete growth factors, enzymes or extracellular matrix components which coordinate wound healing. The cells and their secreted products in turn affect the functioning of other cells through several signaling pathways. The factors that regulate wound healing are described as follows.

1.6.1 Growth Factors

Growth factors are polypeptides that are secreted by various cells in response to stimuli. These factors present in serum and platelet extracts have played important roles in wound healing. The growth factors released from the cells during wound healing and their primary targets are listed in Table 1.2

1.6.1.1 Coagulation and Inflammation

Platelet derived growth factor (PDGF)

Platelet derived growth factor (PDGF), first identified by Ross et al in year 1974 (158). In the early phase of wound healing platelets release PDGF, a dimeric glycoprotein with a molecular mass ∼30 kDa composed of A (16 kDa) and B (14 kDa) chains. It exists in both forms as a heterodimer and as a homodimer. The major platelet and macrophage isoforms (AB and BB) of PDGF were found to stimulate fibroblasts to contract collagen matrix, while the major fibroblast isoform (AA) had no activity (159). It is undetectable in normal human plasma and has a very short half-life in vivo (158). Platelets, macrophages, endothelial cells (ECs) and vascular smooth muscle cells (SMCs) have been shown to secrete PDGF. PDGF has
mitogenic and chemotactic activity, on the target cells. Many cells express receptors for PDGF, including some microvascular ECs, dermal fibroblasts, and vascular SMCs [Figure 1.17]. Perhaps tissue macrophages release PDGF-BB or AB approximately 1 week after cutaneous injury, a time when myofibroblasts have filled the wound and are linked to each other and to the extracellular matrix. This then may be the signal for wound contraction to begin (160).

Table 1.2  Growth factors regulating wound healing. The cellular origin and the target cell of action of the growth factors in wound healing are shown in this table.

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Source</th>
<th>Primary target cells and effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>Platelets</td>
<td>Keratinocyte motogen and mitogen</td>
</tr>
<tr>
<td>TGF</td>
<td>Macrophages; Keratinocytes</td>
<td>Keratinocyte motogen and mitogen</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Macrophages</td>
<td>Keratinocyte motogen and mitogen</td>
</tr>
<tr>
<td>FGFs 1,2 and 4</td>
<td>Macrophages and damaged endothelial cells</td>
<td>Angiogenic and fibroblasts cell mitogen</td>
</tr>
<tr>
<td>FGF7 [KGF]</td>
<td>Dermal fibroblasts</td>
<td>Keratinocyte motogen and mitogen</td>
</tr>
<tr>
<td>PDGF</td>
<td>Keratinocytes; Macrophages; Platelets</td>
<td>Chemotactic for fibroblasts, macrophages; macrophage activation, fibroblast mitogen, and matrix production</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Platelets; Plasma</td>
<td>Fibroblast and Endothelial cell mitogen</td>
</tr>
<tr>
<td>VEGF</td>
<td>Macrophages; Keratinocytes</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>TGF-b1 and -b2</td>
<td>Macrophages; Platelets</td>
<td>Keratinocyte migration; chemotactic for macrophages, fibroblasts; fibroblast matrix synthesis and remodeling</td>
</tr>
<tr>
<td>TGF-b3</td>
<td>Macrophages</td>
<td>Antiscarring</td>
</tr>
<tr>
<td>IL-1 alpha &amp; beta</td>
<td>Neutrophils</td>
<td>Early activators of growth factor expression in macrophages, keratinocytes, and fibroblasts</td>
</tr>
<tr>
<td>TNF- alpha</td>
<td>Neutrophils</td>
<td>Similar to the IL-1</td>
</tr>
</tbody>
</table>

**Basic fibroblast growth factor (bFGF)**

Macrophages, smooth muscle cells, vascular ECs, fibroblasts, and some malignant tumor cells have all been shown to contain bFGF (161). It is a cell-associated protein that has been localized to the cytoplasm and extracellular matrix. bFGF can be released from heparan
sulfate binding sites on the cell surface and in the matrix, or by proteinase degradation of the extracellular matrix (162). bFGF has been shown to be angiogenic in vitro. Although the mechanism of release remains unknown, a unifying hypothesis in all of these cases is that bFGF may be either an effect of, or a reaction to, injury and thus aid in the healing process. bFGF has therefore earned the nickname of a "wound hormone".

1.6.1.2 Cell Proliferation and Matrix Deposition

Epidermal growth factor (EGF), PDGF and FGF all together influence re-epithelialisation. Keratinocyte growth factor (KGF), insulin like growth factor (IGF) and transforming growth factor (TGF) all regulate epidermal growth. Majority of the growth factors comes under in the EGF family, especially TGF-α (163), heparin-binding epidermal growth factor (HB-EGF) (164) and the FGF (165). Growth factors may be derived from macrophages/dermal parenchymal cells and act on epidermal cells, TGF-α, and other growth factors, originate from keratinocytes act directly on the adjacent epidermal cells (166).

Vascular endothelial growth factor (VEGF)

VEGF is the key factor that has been studied in angiogenesis of the neomatrix. VEGF is a 34 – 42 kDa homodimeric glycoprotein. VEGF primary target is a vascular endothelium that possesses two high-affinity tyrosine kinase receptors called Flt-1 and Flk-1 (167). Early studies of the expression patterns of VEGF in tumors revealed elevated expression in cells bordering necrotic areas of tumors. These observations, in turn, lead to the suggestion that VEGF might be regulated by low local oxygen tensions, i.e. hypoxia (168). A number of subsequent studies have shown this to be true and have begun to investigate the molecular basis of the hypoxic regulation of VEGF. The ability of VEGF to be regulated by hypoxia and the knowledge that the wound is particularly low oxygen environment suggest a role for VEGF in wound healing. In addition to its angiogenic capacity, VEGF has been shown to alter local protease production including plasminogen activator and interstitial collagenase (169). In addition, VEGF has been shown to induce monocyte migration and activation events critical to the successful wound healing response (170).

1.6.1.3 Matrix Remodeling

Transforming growth factor (TGF)

The effects of TGF-β on extracellular matrix are more complex and more profound than those of any other growth factor and an increasing the maturation and strength of wounds, (171).
Wound fibroblast, at dermal site is first stimulated to migrate chemotactically in response to very low concentrations of TGF-β at the periphery of the wounded area. This is activated transcriptionally by higher concentrations of TGF-β within the wound site. It also regulates the transcription of a wide spectrum of matrix proteins including collagen, glycosaminoglycans and fibronectins. TGF-β increases the breaking strength in incisional wounds in rats (172). The effect of TGF β on collagen synthesis is transient and parallels the increase in tensile strength.

Figure 1.17: Growth factors regulating wound healing. TGF-β1, β2 and β3 are secreted from keratinocytes and macrophages. KGF and FGF are secreted by fibroblasts, VEGF by endothelial cells and PDGF by macrophages and fibroblasts. The sources of growth factors and their sites of action are clearly depicted in this diagram (Adam J. Singer, 1999 [173]).

1.6.2 Collagen

Collagens are a family of glycoproteins containing triple helices, and are main components of ECM. At present, there are 18 collagen types designated type I to XVIII according to their chronological order of discovery (174). Type I collagen is the major structural component of skin, tendon, bone, and many minor structures. Type III collagen is present in skin in association with type I. Type VI collagen forms distinctive 100-nm periodic microfibrils intercalated between the types I and III collagen of the dermis (175). Type VII collagen forms the anchoring fibrils of epidermal basement membranes (176). Most studies on the collagen content of healing wounds have examined types I and III collagens. The function of collagen in the different phases of wound healing and regulation of synthesis are described below.
1.6.2.1 Coagulation and Inflammation

The inflammatory phase of dermal wound repair is initiated immediately following tissue injury by either activation of tissue complement or by activation of the coagulation cascade. Activation of both of these pathways leads to the recruitment of inflammatory cells into the area via the generation of C5a or the release of platelet factor, fibrin-degradation fragments. These chemotactic agents in vitro for specific cell types and may play a critical role in the early events of wound healing. Fibrillar collagens (types I and III) play a pivotal role in the initial stages of wound healing since they are believed to be a key element involved in the promotion of platelet aggregation following vascular injury (177). The binding of platelets to fibrillar collagen in the surrounding connective tissue results in the release of several large glycoproteins such as fibronectin and thrombospondin. This collagen-induced aggregation of platelets, along with other events in the coagulation cascade, results in the formation of a physical plug that provides hemostasis following vascular injury. Platelet aggregation also results in the release of the factors, such as PDGF, fibronectin which are chemotactic to inflammatory cells, fibroblasts and smooth muscle cells (178). Thus, fibrillar collagen plays a crucial role in the early stages of wound healing by hemostasis and recruitment of connective tissue cells.

1.6.2.2 Cell Proliferation and Matrix Deposition

Matrix deposition occurs in an ordered sequence - fibronectin, type III collagen, and type I collagen type IV collagen, together with other components, including a unique heparan sulfate proteoglycan and the glycoprotein laminin, makes up both the epidermal and endothelial basement membranes (179). Type VII V collagen forms anchoring fibrils that attach the basement membrane to underlying connective tissue (180). The migration of fibroblasts into the wounded area and rapid vascularization signal the initiation of granulation phase in the wound-healing. This phase is generally considered to begin 3-5 days after wounding and persist for 10-12 days, at which rapid synthesis of type I and III collagen and increase in the tensile strength of the wound (181). The collagenous matrix of early granulation tissue synthesized by fibroblasts in the wound, in conjunction with non-collagenous proteins such as fibronectin and fibrin, provides support for epidermal cell migration and proliferation (182, 183). Exposure of a granulating wound to air results in the formation of a scab (eschar), which is composed primarily of dead cells, and dehydrated serum, which attaches itself to the underlying granulation tissue.
Epithelial cells burrow between the eschar and granulation tissue by expressing collagenase and other hydrolases dissolving the collagenous matrix overlying them as they move (184). Once wound closure is affected, the scab is sloughed off. During wound closure, the provisional matrix provided by the early granulation tissue and serum-derived components is quite different from the basement membrane. This results in quite different cellular behavior characterized by lateral cell movement onto the wound bed and cell proliferation rather than the vertical movement and terminal differentiation characteristic of epithelial cells, when they rest on an intact basement membrane. Once cell closure of the epidermis is complete, a basement membrane is rapidly synthesized. This signals the end of rapid fibroplasia and the beginning of remodeling and maturation of the wound. During the first 3-4 days of wound healing, increased amount of type III collagen deposition is observed. Subsequently, a rapid increase in the amount of type I collagen was noted (185). The relative amounts of other collagen types that participate in the wound healing process are low compared with type I and III collagen. The synthesis of type V collagen by epidermal cells during this migratory phase has also been reported (186). The production of type IV collagen and regeneration of the basement membrane, including associated glycoproteins such as laminin, is delayed until the wound is covered and the epidermal cells are no longer in a migratory phase.

1.6.2.3 Matrix Remodeling

Remodeling and cross-linking of the collagen follows fibroplasia, resulting in generation of fibrillar collagen bundles or fibers. These fibers become oriented according to lines of stress and provide a slow increase in the tensile strength of the healing wound (Heughan, 1975) (181). This remodeling phase in the human ultimately results in the formation of scar.

1.6.2.4 Regulation of Collagen Production

The factors regulating collagen production during wound healing can be divided into those that participate in the early, intermediate, and later stages of the process. In the early inflammatory stage of wound repair, platelet factors released during clotting may play a key role in regulating collagen synthesis. One such factor is PDGF (187). This factor could promote collagen synthesis by recruiting connective tissue cells into the wound via its chemotactic activity (188) and causing them to proliferate via its mitogenic effect (189). Large-scale proliferative response of fibroblasts and associated deposition of connective tissue in the wound bed does not begin until the acute inflammatory phase begins to subside at 3-5 days (190). The released growth factors promote collagen synthesis by increasing the number of connective tissue cells in the wound site. Several other lines of evidence implicate
macrophage in the control of collagen deposition in wounds. Thus, it now appears that macrophages play an important role in fibroplasia and associated collagen deposition during the intermediate stages of wound healing by secreting a host of factors that enhance fibroblast proliferation. They promote these cells to synthesize and secrete extracellular matrix proteins. Another regulatory event of collagen production in the wound healing, particularly at the later stages of the process, is the catabolism of newly synthesized collagen. The rate of collagen degradation changes as wound healing proceeds, starting out at a relatively slow rate during the early stages and increasing as wound maturation occurs (191).

1.6.3 Enzymes

Wound healing is a dynamic reparative process involving debridement, inflammation and matrix deposition. All these metabolic processes require efficient expression and activity of enzymes. These include matrix metalloproteinases (MMPs) and enzymes involved in the free radical metabolism. The following section provides a detailed description of these enzymes and their role in wound healing. MMP’s involved in wound repair are shown in Table 1.3.

1.6.3.1 Matrix-metalloproteinases (MMPs)

MMPs are a family of zinc-dependent endopeptidases collectively capable of degrading components of extracellular matrix. The members of this family are divided into four groups such as collagenases, gelatinases, stromelysins and membrane type MMPs (192) according to their substrate type specificity and primary structure. Controlled breakdown of ECM by MMPs plays an important role in detachment and migration of cells as well as in tissue remodeling. MMPs play an important pathogenic role in excessive breakdown of connective tissue components e.g., arthritis, ulcers, dermal photo ageing, periodontitis, tumor cell invasion etc. In skin, different cell types produce MMPs are; keratinocytes, endothelial cells, mast cells, eosinophils, fibroblasts, macrophages, and neutrophils. MMPs are primary mediators of collagen turnover and are secreted in inactive form (zymogens) that can be activated by other proteases such as plasmin (192). This activation and inhibition of its enzymatic activity by protease inhibitors such as α-macroglobulin, have been proposed to play critical roles in the regulation of expression of collagenolytic activity in vivo (193). The activities of these enzymes are controlled by various inhibitor counterparts called tissue inhibitor of metalloproteinases (TIMP), which play an important role during development (194) and wound repair. Cytokines such as TGF β, PDGF, and IL-1 and the extracellular matrix itself may play an important role in the modulation of collagenase and TIMP expression in vivo (195).
## Table 1.3 MMPs Involved in Wound Repair

<table>
<thead>
<tr>
<th>MMP number</th>
<th>Enzyme</th>
<th>Secreted by (cell type)</th>
<th>Substrates</th>
<th>Biological effects in wound healing</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Collagenous-1</td>
<td>Keratinocytes, dermal cells, inflammatory cells</td>
<td>Type-1 collagen, MMP-2; MMP-9 Fibronectin.</td>
<td>Plate aggregation, keratinocyte migration and re-epithelisation, dermal cell migration, reduced cell adhesion and spreading, bioavailability of IGF1 and cell proliferation</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase A</td>
<td>Dermal cells</td>
<td>Fibronectin, laminin.</td>
<td>Dermal cell migration, epithelial cell migration, reduced cell adhesion and spreading, increased bioavailability of TGFβ1</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin</td>
<td>Keratinocytes, dermal cells</td>
<td>Fibronectin, perlecan, collagens (III, IV), decorin, laminin, plasminogen, IL-1 β; MMP-2/TIMP-2, MMP-7, MMP-8, MMP-9, MMP-13.</td>
<td>Cell migration, bFGF release, reduced cell, increased bioavailability of TGFβ1, angio- statin generation</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin</td>
<td>Dermal cells</td>
<td>Decorin, collagen IV fibronectin, laminin, plasminogen, Beta 4-integrin, MMP-1 MMP-2, MMP-9, MMP9/ TIMP-1.</td>
<td>Increased bioavailability of TGF-β1, generation of angio- statin</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Collagense-2</td>
<td>Inflammatory cells</td>
<td>Type1 collagen, fibronectin.</td>
<td>Neutrophil infiltration</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Gelatinase-B</td>
<td>Keratinocyte, inflammatory cells</td>
<td>Collagen IV, fibronectin, plasminogen,IL-1β.</td>
<td>Generation of angio- statin, anti and pro-inflammatory</td>
</tr>
<tr>
<td>MMP-10</td>
<td>stromelysin-2</td>
<td>Keratinocytes</td>
<td>Collagen III,MMP-1, MMP-8</td>
<td>Reduced IL-2 response,</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Macrophage elastase</td>
<td>Inflammatory cells</td>
<td>Collagen IV, fibronectin, vitronectin, laminin, plasminogen, Beta 4- integrin.</td>
<td>Generation of angio- statin</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagense-3</td>
<td>Inflammatory cells</td>
<td>Collagen III, plasminogen, fibronectin, MMP-9.</td>
<td>Release- bFGF, antiinflammatory</td>
</tr>
<tr>
<td>MMP-14</td>
<td>MT1-MMP</td>
<td>Dermal cells</td>
<td>Collagen (I-III), plasminogen, fibronectin, laminin, vitronectin, MMP-2, MMP-13.</td>
<td>Cell migration, antiinflammatory</td>
</tr>
</tbody>
</table>
1.6.3.1 72 kDa Gelatinase (MMP-2)

Gelatinase A is unique in that it is constitutively expressed by many cells, has a ubiquitous tissue distribution and has a cell surface mode of activation that differs from the other MMPs. Structurally this enzyme is similar to the rest of the MMPs. In addition to the shared domains, both gelatinases (A and B) have an additional fibronectin-like gelatin binding domain that provides substrate specificity of these enzymes (196).

1.6.3.1.1 Regulation of Gelatinase A activity

The activity of Gelatinase A is controlled by many regulatory mechanisms. They are as described as follows

a) Transcriptional Regulation of Gelatinase A Expression

Gelatinase A is constitutively expressed in many cells. The gelatinase promoter is characterized by the absence of AP-1, PEA-3 which is present in the other protease promoter regions. This promoter site has a unique TATA box as well as an enhancer element at −223 to −422 nucleotides. The distinctive features of this enzyme promoter include SP-1 sequence at 120bp from the start site and adenovirus E1A repressor element. As Gelatinase A is constitutively expressed and not well regulated it has led to the understanding that it acts as a housekeeping enzyme.

b) Post-transcriptional Regulation of Gelatinase A Expression

TGF β-1 suppresses the overall MMP activity but enhances the levels of Gelatinase A secreted by human fibroblasts and rat osteoblasts (197). It is shown that TGF β increases the stability of the gelatinase mRNA.

c) Regulation of the Activity of Gelatinase A by TIMP’s

Progelatinase A is usually found complexed with TIMP-2. It binds to the C-terminal domain of progelatinase A and regulates its activity. Recent studies show that TIMP-2 may mediate the cell surface activation of progelatinase A by binding to a MT1-MMP containing complex on the cell surface. Thus TIMP-2 facilitates the cell- surface mediated activation of the progelatinase-A and also the inhibition of the active enzyme.
1.6.3.1.1 Functions of Gelatinase A in Cellular Processes

a) Regulation of Cell Proliferation and Differentiation

In a mesangial cell model Turck et al (1996) have demonstrated that the expression of Gelatinase A coincides with the phenotypic transformation of the cells into those of the inflammatory stage (198). The inhibition of transcription of the Progelatinase A protein results in the failure of the cells to change into those of the inflammatory phase. Gelatinase A also cleaves fibronectin which is one of the key extracellular matrix components along with collagen (199).

b) Modulation of Cell Adhesion and Migration

Recent studies in melanoma and breast cancer cells have shown that gelatinase A decreases the adhesion of the cells to their substrates and increases their migration. Studies’ involving the cleavage of the Ln-5γ2 subunit of Laminin-1 by gelatinase A exposes the putative cryptic pro-migratory site that triggers cell motility but not cell adhesion (200). During cell migration as in tumor cell invasion, the degraded protein products serve as stimulus for cell movement.

1.6.3.1.2 92-kDa Gelatinase (MMP-9)

This enzyme is homologous to the gelatinase A and degrades the type IV collagen and gelatins. The protein consists of several structural domains. Both the gelatinases have an additional fibronectin like domain. The protein is maintained in the inactive state by a cysteine switch mechanism. When the interaction between the zinc and cysteine is disrupted the enzyme is activated. The activation depends on the state of the enzyme as it is mostly linked with TIMP-1. Gelatinase activation is also brought about by cathepsin G, trypsin, αchymotrypsin and stromelysin. The activation of the progelatinase-B TIMP-1 complex by APMAor trypsin results in poorly active Gelatinase B whereas activation by other MMPs results in a higher activity (201). Gelatinase B is widely thought of as a type IV collagenase as it cleaves native type IV collagen molecules. Other non-ECM substrates of Gelatinase B are Myelin basic protein, galactoside-binding proteins, aggrecan, a cartilage proteoglycan etc. While it is established that proteolysis is required for tumor invasion, certain studies suggest that excessive proteolysis may inhibit cell-matrix interactions and matrix signals that are required for migration and invasion.
1.6.3.1.2.1 Regulation of Gelatinase B activity

a) Regulation by Growth Factors and Cytokines

Most of the growth factors have been found to increase the activity of Gelatinase B, (e.g., TGF-β, EGF, bFGF, IL, TNF-α and IFN-γ. A number of proteins such as the granulocyte chemotactic protein and leukocyte inhibitory protein also induce the expression of this enzyme (201).

b) Regulation by Cell-Cell and Cell-matrix interaction

Gelatinase expression is also controlled by cell adhesion molecules, ECM and agents that change the shape of cells. Laminin peptide SIKVIV induces gelatinase expression in human monocytes. Cell-cell contact also up-regulates gelatinase B activity (202). Certain co-culture experiments have proven that the cell-cell contact is important for the expression of gelatinase B.

1.6.3.1.2.2 Functions of Gelatinase B in Cellular Processes

a) Tissue Injury, Inflammation and Wound Healing

Most of the inflammatory cells such as the neutrophils, lymphocytes, eosinophils, mast cells and macrophages express gelatinase B (203). The degradation of the subendothelial basement membrane during the process of inflammation is brought about by gelatinase B. Increased MMP-9 is found in infarcted heart, acute respiratory distress syndrome and burns. MMP-9 appears during the early phases of burns, whereas MMP-2 is expressed at a later stage along with stromelysin (204). Gelatinase B is expressed in the early inflammatory phase of tissue repair whereas Gelatinase A appears at a later stage.

1.6.3.2 Gelatinases as Applied to Wound Healing

MMP 2 and 9 are thought to play a key role in degradation of fibrillar collagens after initial cleavage by collagenases. MMP 2 cleaves native type I collagen into two fragments; N-terminal ¾ and C-terminal ¼. In addition, MMP 9 has been shown to cleave type I, II and V collagens in the N-terminal non-helical telopeptide. Therefore it is possible that MMP 2 and MMP 9 play a more important role in the remodeling of collagenous ECM. In a murine model the MMPs have been localized to the basal cells of the hyperproliferative epithelium (205).

The Gelatinase B is absent in the non-wounded epidermis. Gelatinase A is released later during the repair i.e. in the granulation tissue formation. Maximum activity of 72 kDa Gelatinase is seen between day 5-7, post- wounding (206). Thereafter the activity decreases
and by day 13 it reaches that of the normal skin. MT1-MMP is co-localized with 72kDa Gelatinase. It is a physiological activator of the 72kDa Gelatinase. This suggests that there is a high level of gelatinolytic activity in the provisional matrix. TIMP-1 which binds to activated MMPs is expressed with similar kinetics as 92kDa Gelatinase, collagenase, and stromelysins. TIMP-2 that is secreted as a complex with 72kDa progelatinase shows a constant level during entire time period. The various MMP’s released and their actions during wound healing are briefly summarized in Figure 1.18.

Figure 1.18: Enzymes acting during re-epithelialization. Matrix Proteases such as matrix metalloproteinase (MMP), urokinase type plasminogen activator (u-PA) and PA are secreted into the fibrin clot. This promotes re-epithelialization and fibroblast migration into the matrix (Adam J. Singer, 1999, [173])

1.7 Free Radicals and Reactive Oxygen Species

Free radicals are reactive compounds that can exert positive effects (e.g. on the immune system) when they are produced in lower concentration in the human or negative effects (e.g. lipids, proteins or DNA oxidation) when they produced excessive (207). Free radicals are toxic molecules, may be derived from oxygen, which are persistently produced and incessantly attack and damage molecules within cells; most frequently, this damage is measured as peroxidised lipid products, protein carbonyl, and DNA breakage or fragmentation. Collectively, the process of free radical damage to molecules is referred to as oxidative stress (208). A complex protection by antioxidant system which consists of antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase, glutathione s-
transferase) and non-enzymatic antioxidants (e.g. glutathione, thiol) can limit these harmful effects.

### 1.7.1 Formation of Free Radicals

Free radicals are very unstable and react quickly with other compounds, trying to capture the required electron to gain stability. Attacked molecule when loses its electron, it becomes a free radical itself, beginning a cascade chain reaction which cause disruption of a living cell.

### 1.7.2 Definition of Free Radicals

The outermost shell of a ground-state atom has complementary electrons which spin in opposite directions. An atom is considered to be ‘ground’ when its electrons fill the lowest energy orbital completely, and are arranged in pairs with antiparallel spin states before they begin to occupy higher energy orbitals (209).

### 1.7.3 The Octet rule

According to Lewis (1916), each bonded atom shares two or ‘paired’ electrons to form a stable molecule. Lewis (1916) called this discovery the ‘rule of two’, and subsequently Langmuis suggested the ‘octet rule’ (210). When a valence shell is filled completely and with eight electrons, the electron configuration corresponds to that of a noble gas, and the atoms are thought to be as stable as those of noble gases.

### 1.7.4 Redox Regulation in the Wound Environment

ROS may be involved in all stages of the wound healing process such as migration, adhesion, proliferation, neovascularization, remodeling, and apoptosis are main processes in wound healing regulated, or at least modulated, by ROS. Due to the underlying signaling and damage pathways, oxidative stress could result in disturbed wound healing. Enhanced ROS concentrations in chronic wounds are thought to drive a deleterious sequence of events finally resulting in the nonhealing state. In chronic wounds, there are numerous sources of ROS. Prolonged inflammation with neutrophiles migration into the damaged tissue generating superoxide anion radicals, hypoxia, and ischemia reperfusion are important mechanisms resulting in oxidative stress. Disruption of the redox balance and alterations to the level or activity of reducing enzymes that help to maintain the normal redox state could also contribute to poor healing and may even lead to damage to DNA within cells involved in the
normal healing process. Consequently, increased oxidative stress, together with elevated levels of ROS, could result in damage and strand breakage of cellular DNA (211).

### 1.7.5 Reactive Oxygen Species in wound healing

A majority of ROS in the wound environment are likely to be released by activated neutrophils and macrophages recruited to the site of injury (212). Under normal physiological conditions, ROS production is tightly regulated, and ROS participate in both pathogen defense and cellular signaling. However, insufficient ROS detoxification or ROS overproduction generates oxidative stress, resulting in cellular damage. Inflammation is an essential response in the protection against injurious insults and thus important at the onset of wound healing. However, circumstances arise when the antioxidant defense fails to neutralise these free radicals, resulting in a shift in the redox equilibrium and the onset of oxidative stress. Therefore, it is vital that a physiological balance between oxidant and antioxidant levels be maintained in order to prevent severe biological changes. [Figure 1.19]

![Diagram](image)

**Figure 1.19 Reactive oxygen species.**

Different ROS that are produced in inflamed tissues are shown together with the enzymes that generate or detoxify ROS as well as oxidized macromolecules. GSH: glutathione. (Reproduced from Schäfer M & Werner S, 2008 [213]).
1.7.6 Oxidative Damage to Lipids (Lipid peroxidation)

The peroxidation of lipids is basically damaging because the formation of lipid peroxidation products leads to spread of free radicals reactions. Polyunsaturated fatty acids (PUFAs) serve as excellent substrates for lipid peroxidation. Lipid hydroperoxides are non-radical intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters and cholesterol itself. Their formation occurs in enzymatic or non-enzymatic reactions involving activated chemical species known as "reactive oxygen species" (ROS) which are responsible for toxic effects in the body via various tissue damages. Byproducts are often used to measure oxidative stress is MDA. It is produced during fatty acid auto-oxidation which is accepted as the general marker of lipid peroxidation (214). This substance is most commonly measured by its reaction with thiobarbituric acid, which generates thiobarbituric acid reactive substances (TBARS). MDA levels have shown increased in chronic wounds due to increased lipid peroxidation by free radicals damage (215).

1.8 Role of Antioxidants in Wound Healing

The term “antioxidant” refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells. Humans have evolved highly complex antioxidant systems (enzymic and nonenzymic), which work synergistically, and in combination with each other to protect the cells and organ systems of the body against free radical damage. The antioxidants can be endogenous or obtained exogenously eg, as a part of a diet or as dietary supplements.

Enzymatic antioxidants involve glutathione peroxidase, glutathione s-transferase, catalase and superoxide dismutase (216). Nonenzymatic antioxidants include Vitamin E and C, thiol antioxidants (glutathione, thioredoxin and lipoic acid), melatonin, carotenoids, natural flavonoids, and other compounds (217). There is growing evidence to support a link between increased levels of ROS and disturbed activities of enzymatic and nonenzymatic antioxidants in diseases associated with aging. Antioxidants have been shown to promote wound healing (218). The magnitude of free radical generation and their disposal mechanism are known to be altered in impaired wound healing (219). It has been shown that oxygen free radicals play an important role in delayed ischemic wound healing (220).
1.8.1 Non Enzymatic Antioxidants

1.8.1.1 Glutathione and Thiol

Glutathione is a low-molecular weight tripeptide (c-glu-cys-gly) with two biologically important structural features: a thiol group and C-glutamyl linkage, formed from the amino acids glutamate, cysteine, and glycine. It serves as electron acceptor for hydrogen peroxides and becomes toxic, oxidized thiol (GSSG). The major thiol is an intracellular antioxidant and is considered to be the major thiol-disulphide redox buffer of the cell (221). It is abundant in cytosol, nuclei, and mitochondria, and is the major soluble antioxidant in these cell compartments efficiently scavenges ROS and free radicals preventing an increase in the oxidative stress process. In these reactions, the reduced GSH is oxidized, via the enzyme glutathione peroxidase, to form glutathione disulfide (GSSG). Measuring the plasma level of GSH or its oxidized form (GSSG) is a widely accepted method of detecting oxidative stress and can be reported as redox potential, GSH or GSSG concentration, or GSH/GSSG ratio and play a major role in the regulation of cellular integrity, proliferation and protein function, and therefore disruption of GSH homeostasis may be a key event leading to delayed wound healing (222). Both GSH and protein bound thiols contributes maximum to the total thiols pool in the cell (223). Low levels of total thiols pool have been shown to be associated with various disorders with increased generation of free radicals (217).

1.8.2 Enzymatic Antioxidants

Enzymes in Free Radical Metabolism

Cells possess various mechanisms to protect themselves from the free radical-mediated damage defined as scavengers. And among these, some enzymes have the role of antioxidants.

Expression and Function of ROS-Detoxifying Enzymes in Wound Repair

Several ROS-detoxifying enzymes in addition to low molecular weight antioxidants are crucial for the regulation of the cellular redox balance as shown in Table 1.4.

1.8.2.1 Glutathione Peroxidase and Glutathione-S-Transferase

There are two forms of this enzyme, one which is selenium-dependent (GPx, EC1.11.1.19) and the other, which is selenium-independent (glutathione-S-transferase, GST, EC2.5.1.18) (207).
Table 1.4: Decomposition of hydro peroxides and hydrogen peroxides by enzymes. The enzymes that catalyse the peroxidation reactions.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione peroxidase</td>
<td>Reduction of hydrogen peroxide</td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG}$</td>
</tr>
<tr>
<td>Glutathione-S-Transferase</td>
<td>Reduction of fatty acid hydroperoxide</td>
</tr>
<tr>
<td>Catalase</td>
<td>Reduction of hydrogen peroxide</td>
</tr>
<tr>
<td></td>
<td>$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$</td>
</tr>
</tbody>
</table>

Selenium-dependent glutathione peroxidase acts in association with tripeptide glutathione (GSH), which is present in high concentrations in cells and catalyses the conversion of hydrogen peroxide or organic peroxide to water or alcohol while simultaneously oxidising GSH. GPx also competes with catalase for hydrogen peroxide as a substrate and is the major source of protection against low levels of oxidative stress (217). GPx reduces the lipiddic and non-lipiddic hydroperoxides as well as hydrogen peroxide ($\text{H}_2\text{O}_2$) while oxidising glutathione. It is considered to be an essential factor in wound healing to defense against oxidative tissue damage and the cellular function. It was reported that GPx reduces oxidative stress in vivo, protecting diabetes-related complications and promoted wound healing (224).

Glutathione-S-transferase (GST) are thought to be of extreme importance in the cytoprotection during cutaneous wound repair which catalyses the conjugation of glutathione with toxic metabolites and xenobiotics compounds, which subsequently results in detoxification of toxic metabolites (225).

1.8.2.2 Catalase

Catalase has one of the highest turnover rates for all enzymes: one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute (220).

The $\text{H}_2\text{O}_2$, which is generated by SODs from superoxide radical anions, can be further detoxified by catalase which was expressed at particularly high levels in the hyperproliferative wound epidermis, but lower levels were also seen in the granulation tissue (220). Catalase and glutathione peroxidase activity decreased due to ROS-mediated inactivation in skin injured and immunocompromised rats (221).
1.9 Nitric Oxide (NO) and Wound Healing

NO is a free radical gas that is involved in many important biological functions and become one of the most studied molecule in biomedical sciences during the past few years. As a testimony to the rapidly expanding knowledge about its multiple biological roles, it was named as molecule of the year in 1992 after its discovery in 1987 (226). L-Arginine, the substrate for NOS, was first noted to trigger wound healing in 1978 (227). Subsequently, dietary L-arginine intake has been shown to improve collagen deposition and wound strength in both animals and humans (228). Accumulating evidence indicates that NO plays a key role in normal wound repair (229). Production NO metabolites, nitrite (NO$_2$) and nitrate (NO$_3$), are elevated early in the fluid of subcutaneous wounds (230), and increase in excretion of urinary nitrate after excisional wounding. Furthermore, the presence of nitrite and nitrate is directly correlated with collagen deposition within the wound and in dermal fibroblasts, suggesting that NO synthesis is critical for wound collagen accumulation and acquisition of mechanical strength.

1.9.1 Mechanisms of Nitric Oxide on Wound Healing

NO acts by multiple ways and different mechanisms. A definitive role of NO in wound healing has still not been established. One study even showed that NO inhibits collagen synthesis in wounds (231). On the other hand several studies have implicated that NO might play a vital role in all the phases of wound healing (232).

1.9.1.1 Role of NO in the Inflammatory Process

NO acts as a vasodilator, antimicrobial, and has a vital role in the inflammatory process by preventing platelet aggregation, induces vascular permeability and responsible for both the up regulation and down regulation of the inflammatory phase of wound healing. Post wound inflammation initiated by chemoattractant cytokines such as interleukin (IL)-8 , TGF-β1 (233), monocytes and neutrophils were modulated by NO (234). Neutrophils and monocytes begin to produce TNF- α and IL-1 (235) and high levels of NO may also be anti-inflammatory during the later phase of inflammation (236).

1.9.1.2 NO and Angiogenesis

Angiogenesis, the process of formation of new micro-vessels, is a vital component of normal wound repair. NO plays important crucial role in wound healing by enhancing post-wounding angiogenesis and vascular endothelial growth factor (VEGF) a potent angiogenic factor (237).
1.9.1.3 NO and Matrix Deposition and Remodeling

The final phases of healing require increased collagen synthesis and also regulates gene expression (238) and cellular differentiation (239). Recent evidence suggests that NO can also reduce cell proliferation by inhibiting ornithine-decarboxylase activity (240). Though NO is critical for wound collagen deposition, clear-cut enhancement of collagen synthesis has not been found (241).

1.9.2 Negative Effect of Nitroxidative Stress and Oxidative Stress in Wound Healing

An increase in the allantoin uric acid percentage ratio (AUR), a biomarker for increased oxidative/ nitroxidative stress in chronic wounds (242), and levels of 8-isoprostane were found to be higher in chronic venous ulcers than in acute wound fluid have been reported (243). Prolonged inflammation leads to impaired migratory, proliferative and extracellular matrix (ECM) synthetic properties of dermal fibroblasts and keratinocytes (244) and inactivation of epidermal enzymatic and non enzymatic antioxidant levels in wound tissues, stimulate neutrophil and macrophage chemotaxis, migration and also induce the expression of adhesion molecules in the capillaries (245).

1.10 Wound Surface pH

pH value is a key determinant for the metabolism/local chemical environment and enzyme activity during wound healing and thus also an important parameter for therapeutic interventions in wound care. The pH value within the wound-milieu influences indirectly and directly all biochemical reactions taking place in this process of healing.

1.10.1 pH value of Normal Skin

Hesus et al. in 1892 (246) found that under normal circumstances an acidic milieu was found on the skin surface and the same was later on confirmed again by Schade and Marchionini in 1928 (247). This acidic milieu varies depending on the anatomical location and age of the person between a pH of 4–6 and has always been seen as an important aspect of the skin’s barrier function (248). The physiological pH value results from amino acids, fatty acids and others produced and secreted by the keratinocyte layer and the skin appendages, which provide a local shift in the natural lactate -bicarbonate buffer system of the body towards an acidic milieu.
1.10.2 pH milieu Influences the Proteolytic Activity in Chronic Wounds

Physiological balance between tissue degradation and tissue reassembly is lost and catabolic processes predominate in chronic wounds. This explains the overabundance of proteolytic enzymes found in the milieu of chronic wounds. As long as the wound remains in the inflammatory phase, the catabolic enzymes remain active in the tissue. When the healing eventually progresses and inflammation subsides, the physiological balance slowly returns and protease activity diminishes. In this context a study by Trengove et al. demonstrated that activity of MMPs decreases consistently in venous leg ulcer patients as wound progress from non-healing to healing state (249). In addition, they found that the physiological opponent of MMPs, the tissue inhibitor of MMP (e.g. TIMP-1) raises to about tenfold the normal level in healing wounds (250). Other study by Greener et al. investigated the pH dependency of certain proteases like cathepsin-G, elastase, plasmin and MMP-2 that are relevant for matrix degradation and re-assembly in chronic wounds. A total of 19 secretion samples from patients with chronic wounds were collected. The samples demonstrated a pH value of 7.5–8.9. Half of the measured samples were in the pH range of 8.1 to 8.3. The optimum pH for MMP-2, plasmin and elastase is 8.0. A shift of the wound pH value down to 6.0 would entail a 40–90% decrease of activity of these enzymes and imply a profound impact on the biochemistry of proteolytic activity of wound (251, 252), [Figure 1.20].

1.10.3 pH milieu Influences the take-rate of Skin-Grafts

A study by Sayegh et al. (253) first correlates the skin graft takeover in wounded rats with the pH range within the wound milieu. At a pH value of 6.6 a 0–30% take-rate, at a pH value 6.8 a 50–100% take and at a pH value 7.0 a 87–100% take was noted. To further the investigation he recruited 25 patients with 2nd and 3rd degree burns for this part of the study. At a pH of 6.4 the overall 20 % of skin-grafts were successful, at a pH value of 6.6–35%, at pH value 6.8–66% at pH value 7.0–77% and at pH value 7.2–90%. Thus, their finding in rats could be confirmed in humans with burn wounds (253). Another investigation by Ye et al., who also looked at the take-rate of skin-graft in patients with acute and chronic wounds of different etiology. In a total of 90 patients, no case of skin-graft was successful in wounds with a pH value below 7.0, but, 99% of skin-grafts were taken in wounds with a pH value of 7.4 and higher (254). Important aspects of the different studies in this field are summarised in Table-1.5.
Figure 1.20 - b. Course of pH milieu in Acute & chronic wounds (Adapted from Schneider LA 2007 [252])
Table- 1.5 Take-rates of skin transplants in different studies compared with wound pH value 
(Adapted from Sayegh 1988 [253])

<table>
<thead>
<tr>
<th>Author</th>
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<td>Burn</td>
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1.11 Scope of the Study

The evidence supporting the use of NPWT in the treatment of chronic nonhealing wounds exists primarily in the form of nonrandomized, controlled trials; prospective and retrospective large and small case series; single-center studies; and single case studies, with few randomized, controlled trials (RCTs). Beside almost all the published evidence related to the use of the VAC device.

In our study we will explore the efficacy of LAD that is a combination of two newer forms of dressings- moist wound healing and NPWT. LAD utilizes definite intermittent negative pressure regime (30 minutes of negative suction and 3½ hours of rest i.e. no suction period; minimum 30mmHg of negative pressure). Clinically it has been claimed to have more effective and economical acceptable than conventional dressings. The present study was planned to evaluate the comparative efficacy of the Limited access dressing on chronic wound (wounds of more than 4 weeks). The efficacy of both groups will be evaluated at fixed interval by biochemical and histological scoring methods. Plan was made to recruit patient and randomly allocate into LAD and Conventional dressing (5% providone iodine soaked squeezed gauze dressing) group. Biopsies will be taken from granulation tissue for objective analysis using biochemical and histological methods. Chronic wound cases of all etiologies of 12 to 65 years (Gender-both inclusive) will be included for the study. Cases with any local and systemic specific problems that causes or influence wound healing will be excluded in the study.