Evaluation of Wound Healing Using a Combination Therapy of NSAID and an Antioxidant on Mice Model

A project submitted

by

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This work is dedicated to my parents and my respected project supervisor Fabliha Ahmed Chowdhury (Lecturer, Department of Pharmacy, BRAC University) from whom I received the most support.

Certification statement

This is to certify that, the project titled ‘Evaluation of Wound Healing Using a Combination Therapy of NSAID and an Antioxidant on Mice Model’ submitted for the completion of the precondition for the degree of Bachelor of Pharmacy to the Department of Pharmacy, BRAC University, contains my personal work under the supervision of Fabliha Ahmed Chowdhury, Lecturer, Department of Pharmacy, BRAC University. Proper acknowledgement goes to those from whom I got the ideas.

Signed,

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Counter signed by the supervisor

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Acknowledgement

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Abstract

Skin is the first line of defence mechanism in our body. When the protective barrier of skin breaks we can define it as a wound. Wounds need to be healed very fast in order to restore body's normal defence mechanism. For healing purpose different cell types and multiple steps are involved. Wound healing mechanism contains three main phases which are inflammation phase, proliferation phase and regeneration phase. In inflammation phase macrophage and neutrophils works, in proliferation phase, several growth factors like TGF (Transforming Growth Factor)-beta1, EGF (Epithelial Growth Factor), VEGF (Vascular Endothelial Growth Factor) etc and finally in regeneration, reconstitution of skin occurs. There are several drugs used for wound healing but all those need a longer time period for wound recovery. Along with that they have several side effects too. To minimize these side effects and to make the wound healing faster we came up with a combination therapy. We have used mice models for this experiment. Wound was created on the back of the mice and medicines were administered intraperitoneally (IP). There were four groups of mice. One group is treated only with saline, another one with Diclofenac-Na, next one with the antioxidant Vitamin C and last one with a combination therapy of Diclofenac-Na and Vitamin C. For determining the effect on inflammation phase did WBC counting, for proliferation we did quantification of VEGF and for regeneration we measured wound closure and weight of skin. The result of the study shows that for WBC counting the combination therapy has shown the most effective value. In quantitative analysis of VEGF also the combination therapy has shown better result than the monotherapies. But in this test, Vitamin C has shown better result than Diclofenac-Na and the concentration of VEGF was higher in earlier stage on day 2, 4 and 7. Finally, for regeneration phase we have measured the wound closure. In this case also combination therapy showed most effective result. Healing rate was faster on day 7 and 9. For regeneration, we did weight variation test. In that case also combination has shown better result amongst all. From all these results it has come to a conclusion that combination therapy has shown better result in wound healing. So, we can consider this combination therapy as an alternative medication for wound healing although this needs further investigation.

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NSAID: Non-steroidal Anti-inflammatory Drug

COX: Cyclooxygenase

WBC: White Blood Cell

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

PAMP: Pathogen Associated Molecular Pattern

DAMP: Damage Associated Molecular Pattern

RNA: Ribo-nucleic Acid

DNA: Dioxy Ribo-nucleic Acid

TLR: Toll like Receptors

NK: Natural Killer

MAPK: Mitogen activated Protein Kinase

ICAM1: Intracellular Adhesion Molecule 1

VCAM1: Vascular Cell Adhesion Molecule 1

IL: Inter-leukin

TNF: Tumor Necrosis Factor

TGF: Transforming growth factor

EGF: Epithelial growth factor

VEGF: Vascular endothelial growth factor

IP: Intra-peritoneal

SMA: Smooth Muscle Actin

PDGF: Platelet derived growth factor

BFGF: Basic fibroblast growth factor

ROS: Reactive oxygen species.

Chapter One: Introduction

Initially, skin protects our internal organs from hostile environment. Just after an injury it is important that wound heals quickly along with that damaged skins also regenerate rapidly to restore the defence mechanism. The prerequisite of effective repair entails connection and inter-relationship between different cell types and this process is controlled by multiple steps (Reinke and Sorg, 2012). The wound healing process embodies four successive but co-occurring phases: homeostasis (0-several hours after injury), inflammation (1-3 days), proliferation (4-21 days), and regeneration phase (21-1year) (Sen et al, 2012). Disturbance in any of these phases impairs wound healing (Kloeters et al, 2006). Contemporary remedy for impaired wound healing mainly focuses on optimization of controllable healing factors. For instance, mechanical protection, clearance of infection and nutritional supplements. Only a handful of approaches have been developed that includes topical application of growth factors. However, this has a limited clinical efficacy (Sun et al, 2014). Now it's very much necessary to identify new therapeutic target as well as developing more efficacious treatment as the conventional treatment shows delayed healing and more side effects.

What is wound?

A wound can be defined as breakdown of protective function of the skin by the degeneration of epithelial tissues along with or without damage in underlying tissues (e.g. muscle, nerves or bones) (Leaper and Harding 1998). This damage can occur by following matters like, by surgery, a cut, heat/cold, chemicals, result of disease, friction/ shear force, pressure (Hutchinson 1992).

There are mainly two types of wound.

1. Acute wound

2. Chronic wound

1.1 Types of wound

Acute Wound: Acute wound can be described as disruption in the skin integrity and tissues underneath which heals with an uneven time period. An acute wound is one type of injury that occurs unexpectedly not with a longer period. The rate of healing process is predictable and expected. Acute wounds can occur anywhere in the body. It can happen either just a superficial scratch or a deep wound that can damage muscle, blood vessels, nerves or many other parts of body. (Lazarus et al, 1994)

Chronic Wound: When an acute wound fails to repair within the predictable and expected time this turns into a chronic wound. Chronic wounds can be categorized into pressure ulcers, diabetic ulcers, and vascular ulcers (arterial and venous ulcer). (Demidova-Rice et al, 2012). Despite having a lot of technological advancement doctors till now being challenged with the healing of chronic wounds. Various drugs and non-surgical approaches were made in the management of wounds.

1.2 Mechanism of wound healing

Wound healing often describes as a continuous process that contains different cell types. Wound healing comprises of three main phases.

1. Inflammation phase
2. Proliferation phase and
3. Regeneration phase.

1.2.1 Inflammation Phase

Inflammation phase follows several pathways COX pathway, WBC pathway, oxidative pathway.

COX Pathway: Prostaglandins are one among many of the contributing agents in the inflammatory process; mediating the process by increasing blood flow, chemotaxis and decreasing activity of tissues. The synthesis of prostaglandins is triggered by a noxious response. Following injury, phospholipids, a substance of the cell membrane gets converted into arachidonic acid by an enzyme called phospholipase A2. Arachidonic acid acts as a substrate for two enzymes, cyclooxygenase or COX and 5-lipoxygenase. COX occurs in the body in two isoforms- COX 1 and COX 2 and are responsible for producing prostaglandins and thromboxane. The presence of COX 1 can be found throughout the body however, COX 2 only occurs at the site of injury. COX 2 derived prostaglandins mediate inflammation, pain and fever (Williams et al, 1999).

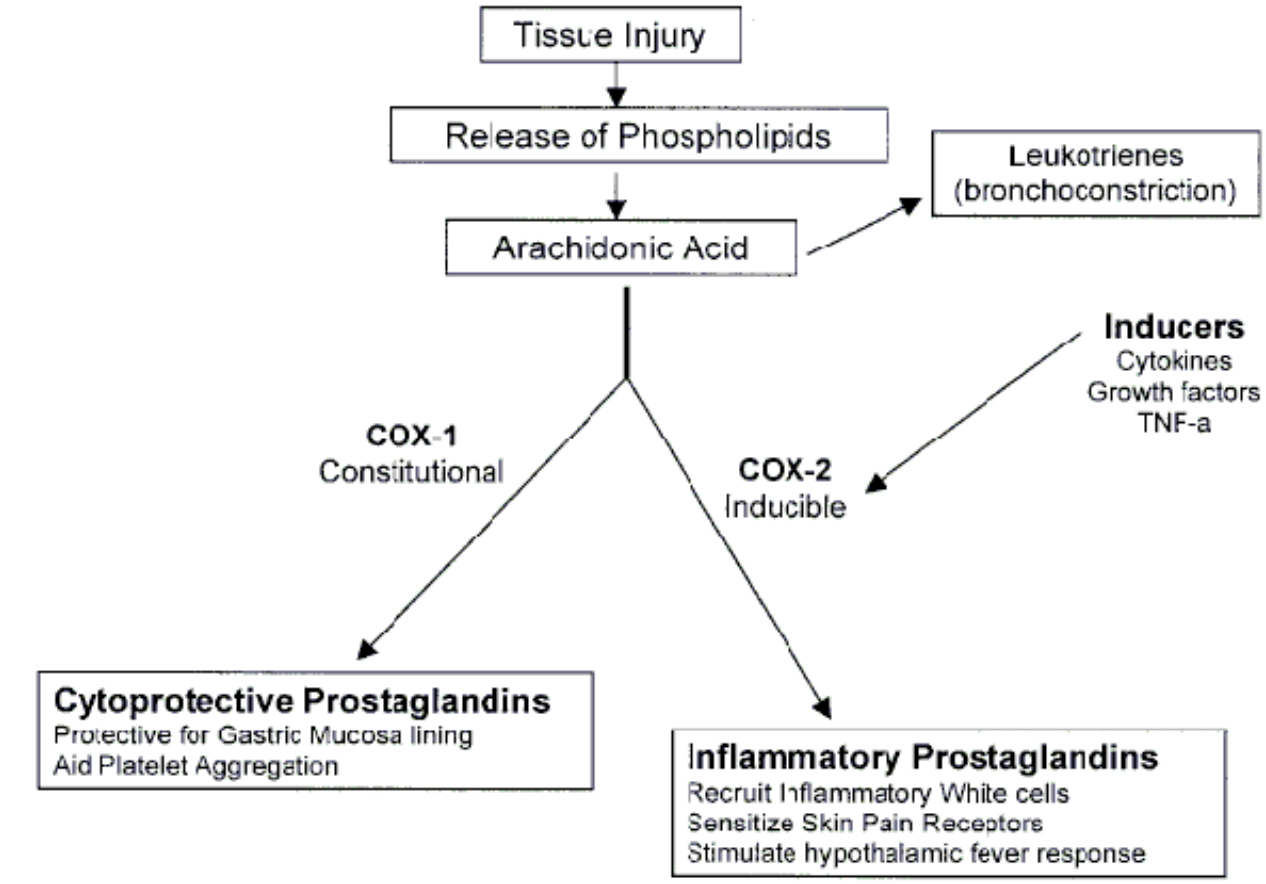


Figure 1.1: The Cox Pathway to Induce Inflammation (Wang & Dubois, 2009)

WBC Pathway: Among the blood cells, white blood cells are the protagonists when it comes to protecting the body against infection and foreign substances through their association with the immune system. All WBC originate from multiple cells in the bone marrow, known as the hematopoietic stem cells. Leukocytes penetrate the connective tissue and migrate to the site of injury through a process called chemotaxis, an ability of leukocytes to sense chemical signals and respond to it. The agents for chemotactic signaling include- neutrophils (the products of complement system), monocytes (the bacterial exudates, neutrophil cationic substances). Lymphokines discharged from the sensitized lymphocytes, other complement substances (C567 complex, C3a and C5a) (Zeka et al, 2006).

Macrophages play an important role in the regulatory cell for repair and delineates its effect in the subsequent stages of inflammation. Apart from phagocytic cell they contribute as dominant manufacturers of growth factors accountable for proliferation: Formation of the extracellular

matrix by fibroblast of smooth muscles of the endothelial cells resulting in angiogenesis. They also promote the release of proteolytic enzymes (e.g. collagenase) and facilitate debriding of wound. Dwelling period for normal skin wound healing ranges between 2-5 days and halts upon discarding deleterious stimuli's. Progressive metamorphosis of specific leukocyte involvement and affairs are discerned throughout the whole course of wound healing (Strbo et al, 2014). The disparate arm of immunity, alias the Adaptive Immunity comprises of postponed but concrete response generated by B and T cells. Apart from emission of antibodies B cells executes several other factors influencing immune response like: breeding diversified cytokines and growth factors, antigen presentation, govern T-cell activation and differentiation and constraining lymphoid organization (Lipsky 2001). B cells are said to be contemporary in injury site (Cowin et al, 1998) and depicts a critical role in wound healing (Iwata et al, 2009).T-lymphocytes performs as growth factor-producing cell in congruence with immunological effector cell during cell reconstruction (Gillitzer et al, 2001).

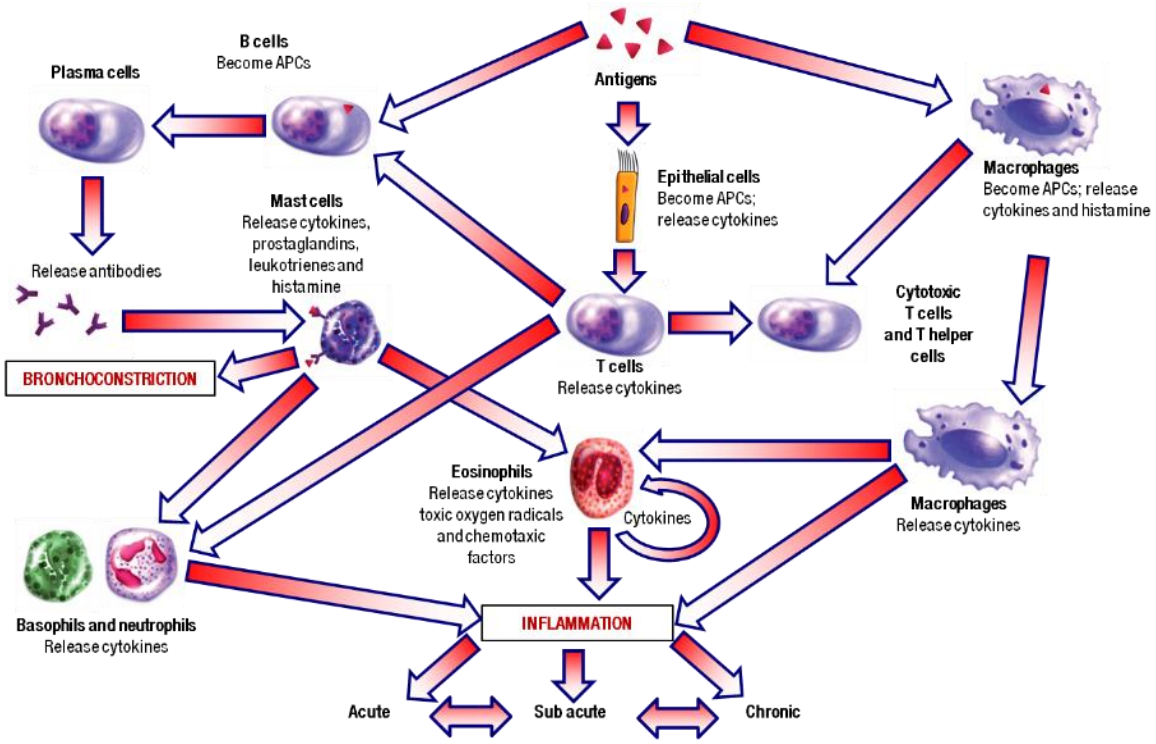


Figure 1.2: The WBC Pathway of Inflammation (Zeka et al, 2006)

Oxidative Pathway: An alternative way to induce inflammation is through producing free radicals. Tailing inflammation, neutrophils migrate to the vicinity and kill the microbes either

through an oxygen dependent mechanism or an oxygen independent mechanism. Oxygen is a must in the oxygen dependent mechanism and it starts with an oxidative burst, converting oxygen to superoxide and then to hypochlorous acid (HOCl) through an enzyme called NADPH (Nicotinamide adenine dinucleotide phosphate) oxidase. (Rosenson & Stafforini, 2012).

The free radicals bring about inflammation by disintegrating cell membrane. They receive an electron from the lipid of the cell membrane as it has an unpaired electron. The lipid loses electrons and triggers a chain reaction resulting in lysis of cell membrane and causing inflammation (Rosenson & Stafforini, 2012).

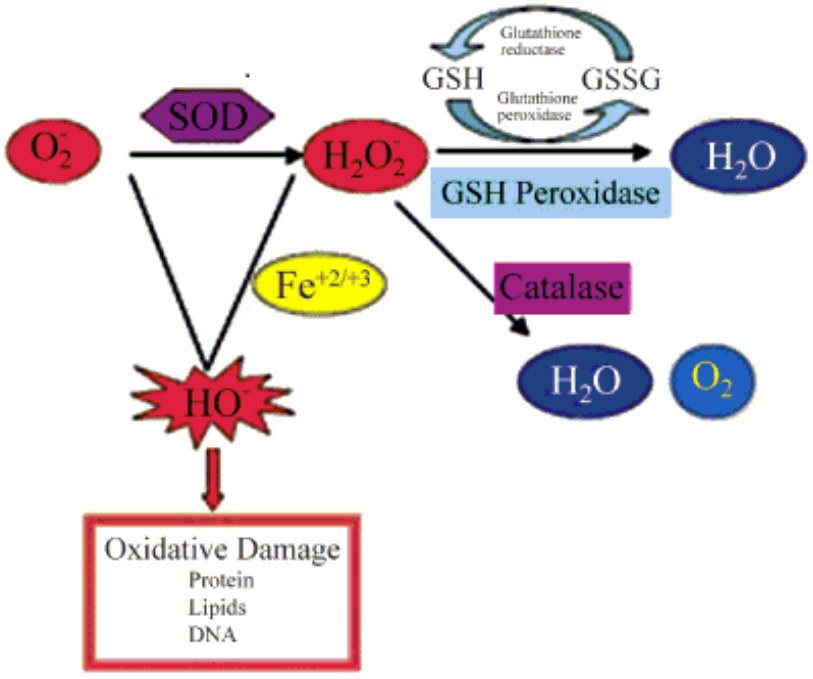


Figure 1.3: Formation of Superoxide and Subsequent Cell Destruction (Lee & Wolfgang, 2015)

The inflammation stage commences with the migration of the neutrophils and macrophages into the wound site which actuates the innate immune system. This stage is delineated as the beforehand stage of wound healing which is contemporary with homeostasis (Eming et al, 2014). As an aftermath to abrasion, resident skin cells e.g. keratinocytes, macrophages, dendritic cells and mast cells are susceptible to danger signals which in general is divided into two classifications: (a) Damage associated molecular pattern (DAMPs) i.e. molecules released by stress cells undergoing

necrosis, such as the intercellular protein, DNA and RNA. (b) Pathogen associated molecular pattern (PAMPs) found in the host cells are pathogen specific molecules e.g. bacterial essential polysaccharides and polynucleotides. (Strubo et al, 2014). Upon skin abrasion the pattern recognition receptor apprehends these danger signals amid which Toll-like receptors (TLRs) are noteworthy. The perpetual expression of TLRs on the host cell and its stimulation begets the actuation of the intracellular signaling pathways, encompassing the nuclear factor kappa-light-chain enhancer of activated B cell (NF-kB) and mitogen-activated protein kinase (MAPK) pathways, engendering the expression of large scales of genes, entailing cytokines, chemokines and antimicrobial peptides instituting and perpetuating the inflammatory response. Fledging of chemokines such as: fragment of extracellular matrix, transforming growth factor-β, complement Components (e.g. C3a, c5a), formyl-methionyl peptide product from bacteria, compliment and by-product of bacterial degradation, neutrophils to the injury site occurs as feedback during the premature stages of inflammation in a multistage process.

Adhesion of the molecules e.g. intercellular adhesion molecules 1 (ICAMI), vascular cell adhesion molecule 1 (VCAMI) and e-selection (SELE), on endothelial cells catalyzing the attachment of neutrophil on the wall of blood vessels upon articulation induced by Chemokines (15). Revamping of the cytoskeleton of the neutrophil occurs upon clinging to epithelial cells and manifestation of chemokines consequences neutrophil extravasation. Upon efflux from blood vessels, manifestation of neutrophils to chemokine gradients in-nard the skin and voyage towards the centralization occurs, making the origin of the chemokine release i.e. the wound site. It should be put under consideration that all leucocytes refer to this methodology for localization (Vestweber et al, 2015). The residence period of neutrophils usually dwells between 2-5 days if the abrasion is uninfected and accomplish phagocytosis with a view to banish pathogens and cellular debris. Annihilation of the microbe's digestion transpires by toxic cationic enzymes and oxygen metabolites when microbes are encapsulated and engulfed in the phagosome of the neutrophil (Wilgus et al, 2013). To sophisticate the inflammatory response cytokines e.g. Tumor necrosis factor (TNF)-α, Interleukin (IL)-1β and IL-6 are catalyzed by Neutrophils (Eming et al, 2014) and facilitates debridement by a wide scale antimicrobial agent. Monocytes are fledged nearly about three days from abrasion and alchemize to macrophages and proliferate rehabilitation. Phenotype alteration occurs in the blood monocytes and amends to form tissue macrophages. Certain specified chemoattractant entice monocytes the abrasion site such as: complement, clotting

components, fragments of immunoglobulin G, breakdown products of collagen and elastin, cytokines (e.g. Leukotrienes B4, platelet factor IV, platelet-derived growth factor, transforming growth factor-β).

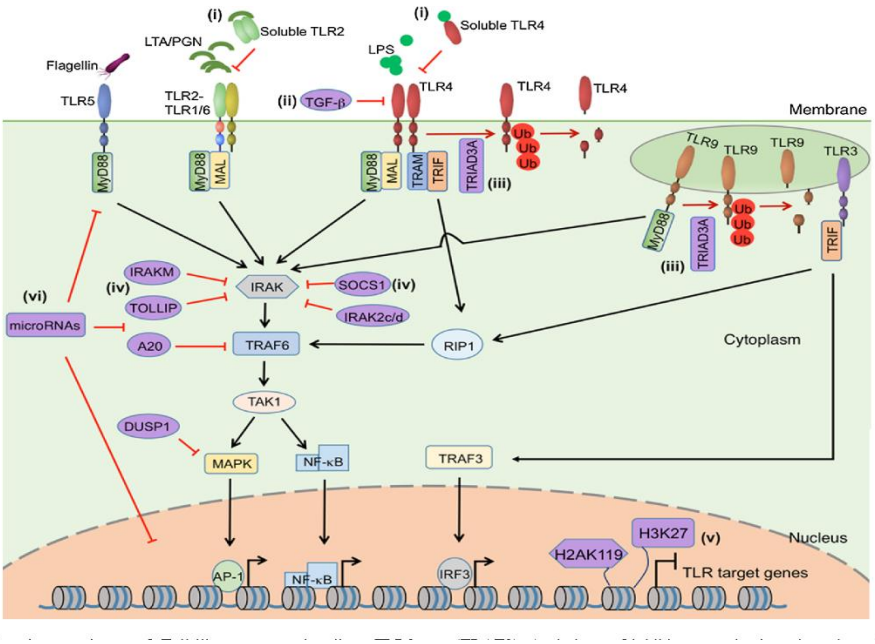


Fig 1.4: Inflammatory Phase of Wound Healing (Ning et al, 2016)

1.2.2 Proliferation Phase

The aftermath of Inflammation paves the way proliferation plays a considerable role in covering the surface of the wound (re-epithelialization), regeneration of vascular network and formation of granulation tissue.

Re-epithelialization:

Kerationyctes voyage and proliferation are the prerequisites for Re-epithelialization. Subsequently after abrasion, migration of the existing wound-edge keratinocyte and proliferation of cells occur to mantle the injury where keratinocytes at the basal layer of the wound edge and eptithelia stem cells from nearby hair follicle or sweat glands play a pivotal role (Lau et al, 2009). Provocation of migration commences with the diminution of contact inhibition and physical at cell adhesion structures i.e. desmosomes and hemidesmosomes, sparking membrane-consociated kinase, culminating membrane permeability for calcium which flags the methodization of cytoskeleton

propelling migration. Emancipation of migrating cells from the origin transpires by the collagenase and elastase (Jacinto et al, 2001).

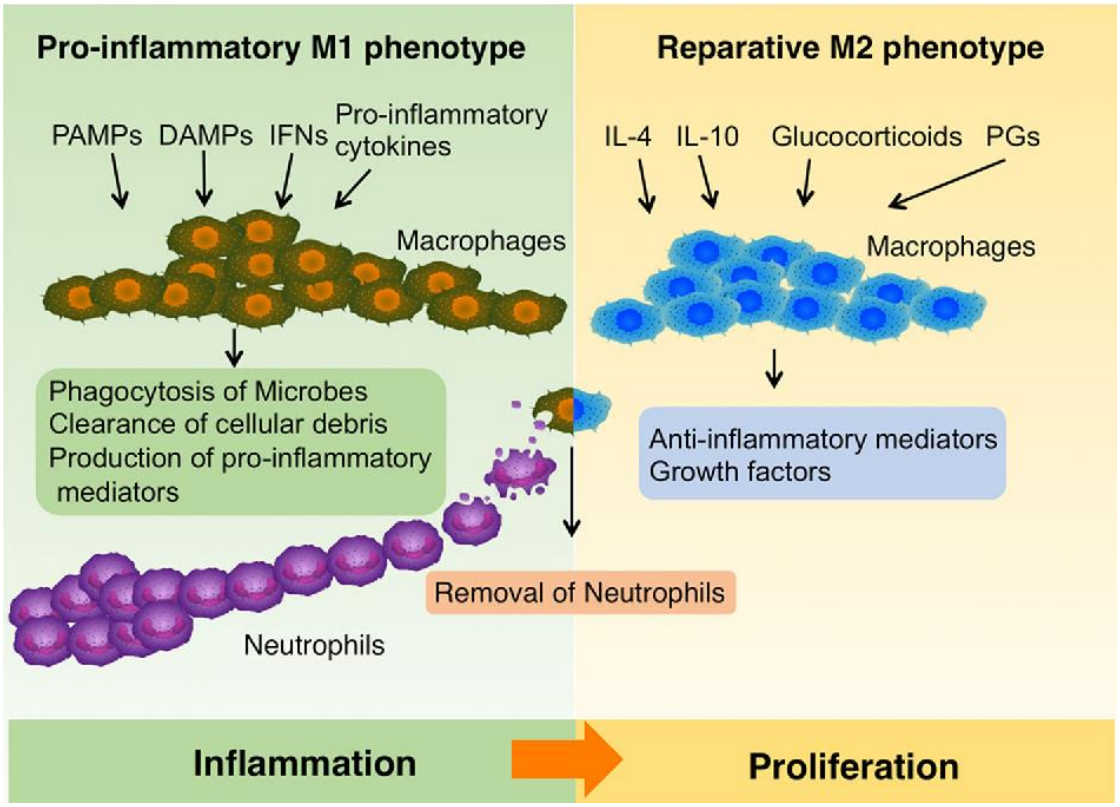


Figure 1.5 Transition from inflammation to proliferation (Ning et al, 2016)

Reconstruction of the basement membrane upon secretion of Keratinocyte and cessation of migration occurs when cells come in contact and new adhesion structure materializes (Jacinto et al, 2001). A hefty assortment of wound related signals such as: Nitric acid primarily synthesized by macrophages (Witte et al, 2002), cytokines, encompassing epidermal growth factor (EGF), KGF, IGF-1 and several other growth factors incoherence with nerve growth factor, emanated from multiple cell type in wound (Barrientos et al, 2008) are triggered by Re-epithelialization.

Fibroblast Migration:

Affixing of fibroblast to the wound occurs by a handful number of factors comprising the platelet derived growth factor and transforming growth factor-beta ensuing an injury. Upon entry to the

wound fibroblast accumulation culminates in the formation of the matrix protein fibronectin, hyaluronan and later collagen and proteoglycans aiding the concocting the new extracellular matrix propping additional ingrowth of cells and is indispensable for the repair process. Substantial interaction between the fibroblast and extracellular matrix is pivotal in ordinance for additional production of extracellular matrix and sequent remodeling.

Formation of Extracellular Matrix: Inward growth, movement and differentiation of cell is decisively maintained and endowment of substratum for cell adhesion is done by the extracellular matrix apart from rendering tugor to soft tissues and rigidity to bones. Along with the fibrous structural proteins (collagens, elastin) extracellular matrix comprises of the interstitial matrix orchestrated with adhesive glycoprotein integrated in a proteoglycan ad glycosaminoglycan gel. Assembling of the arbitrary arrays of interstitial matrix in the connective tissue encompassing the epithelial cell, endothelial cell and smooth muscle cell concocting a specialized basement membrane (involved in cell polarity channelization and subsequent sequential renewal of the epithelial tissue).

Collagens: Ensuing puissance and integrity to all the tissue, Collagens the most ample family of protein and synthesized by fibroblast has marked effect on wound repairing and reconstruction and its instigation commences during the proliferation and remodeling stage by the platelet derived growth factor, basic fibroblast growth factor, transforming growth factor-beta, interleukin-1 and tumor necrosis factor (TNF). Collagens comprehends a three protein alpha chains entwining a rope-like triple helix and due to the presence of glycine molecule at each every third molecule each alpha polypeptide facilitate individual chain firm intertwining. Around 18 different collagen type (some of which may be unique to specific cells and tissues) are generated by 18 disparate group of alpha-chains. A distinct number of collagens (e.g. i, iii, v) are associated with fibril formation in relation to lateral crosslinking of the triple helices and constitute majority of the connective tissues in wound healing. On the contrary other collagens are non-fibrillary (e.g. type iv) and are integrant of the basement membrane.

Adhesive Glycoprotein: Comprised of fibronectin, laminin and thrombospodin adhesive glycoproteins are morphologically varied proteins that associates the constituents of the extracellular matrix to each other and to the cell.

In association with the cell surface, basement membrane and the pericellular matrix, Firbronectin are classes of disulphide linked heterodimer incorporating unambiguous domains in unison with broad spectrum of constituents of extracellular matrix (e.g. collagen, fibrin, preoteoglycan) alongside with the cell integrin. Incongruence with aggrandizing sensitive to discrete cells (e.g. endothelial cells) for the proliferation of growth factors initiating intracellular pathways Firbornectin also conciliates adjunction, spreading and migration of cells.

Playing a significant role in the consolidation of function linking the extracellular matrix and the cytoskeleton, are a group of alpha/beta heterodimeric glycoproteins called Integrin's mediating cell-cell and cell-matrix adhesion.

Proteoglycans: Attached to the protein backbone they are glycoprotein (e.g. dermatan sulphate, heparan sulphate) are associated in modulation of the structure and permeability of the extracellular matrix and involved with tempering of the growth and distinction of cell. (e.g. syndecan binds to extracellular matrix collagen, fibronectin, thromobospodin and basic fibroblast growth factor and associates with intracellular actin cytoskeleton to prolong the normal morphology of epithelial sheets). Devoid of protein core (e.g. hyaluronan) glycosaminoglycan are consequential constituents of the extracellular matrix.

Restoring the vascular network:

Nutrients and oxygen play a considerable role in the regeneration of blood vessels also termed as Angiogenesis galvanized by the growth factors, e.g. Vascular endothelial growth factors (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and serine protein in the wound accompanying the activation of the endothelial cells of the existing vessels (Li et al, 2003). Secretion of proteolytic enzyme is followed by the dissolution of the basal lamina thereby paving the way for the absconding of the endothelial cells form the existing vessels enhancing proliferation and migration towards the angiogenic stimulus origin (Li et al, 2003). They pullulate from vessel lumen and segregate to arteries and venules and fully fledges by recruiting of pericytes and smooth muscle cells furthermore bone marrow derived endothelial progenitors results in formation of vessels de novo by a process vasculogensis (Ashahara et al, 1999).

With a view to retrieving the composition and function of the lacerated skin the provisional wound tissue formed amid the hemostasis superseded by the granulation tissue cohering numerous fibroblast, granulocytes, macrophages, blood vessels in compilation with collagen bundles (Schultz et al, 2009). Role centralization is done by fibroblast in granulation tissue conformation which migrate to the wound site as a feedback to cytokines and growth factor, e.g. PDGF, transforming growth factor (TGF)- beta and bFGF, catalyzed by the platelets and macrophages in the wound (Hinz et al, 2007) from adjacent dermis. Fibroblast a bone marrow-derived mesenchymal progenitor cell extrapolated from fibrocytes in case prolonged wound condition (Abe et al, 2001). Migration of circulating fibrocytes to the region of skin lesion and elevates healing and inflates the angiogenesis by both through as subset of fibroblast in wound and engendering cytokines, chemokines, growth factors and serving as antigen presenting cells (Blakaj et al, 2012). With a view to mortify the provisional matrix (Gill and Parks, 2008), fibroblast accumulate and produce proteinases, e.g. matrix metalloproteinases (MMPs) whilst sublimating collagen and other extracellular matrix (ECM) components e.g. proteoglycans, hyaluronic acid, glycosaminoglycan's and fibronectin in tissue formation (Xue and Jackson, 2015), with a view to filling up the wound void and furnish scaffolding for cell adhesion, migration, growth and differentiation amid wound repair (Barker, 2011) upon migration to provisional wound matrix.

VEGF (Vascular Endothelial Growth Factor):

The members of VEGF are VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PLGF). They show their biological function by three different transmembrane tyrosine kinase receptor which are VEGFR-1, VEGFR-2, VEGFR-3 (GALE et al, 1999). After various in-vivo and in-vitro experiment it has shown that VEGF-A has the greater activity in vasculogenesis and angiogenesis during wound healing (GALE et al, 1999).

Expression of VEGF-A and Its Receptors in Skin Wounds

Expression of VEGF-A gene was shown to be strongly induced with keratinocytes and macrophages after cutaneous injury (Brown et al, 1992). Along with that the receptors were seen in the blood vessel within granulation tissue (Lauer et al, 2000). This expression pattern reveals VEGF-A is stimulated in a paracrine manner during wound angiogenesis. In wound angiogenesis VEGF-A is very much important which has been supported by several studies and the reduced and

accelerated level of VEGF-A is found regarding wound healing defects. (Frank et al, 1995). Moreover, in the treatment of ischemic wounds with VEGF-A or VEGF-A overexpressing fibroblast enhances wound healing and adenovirus mediated VEGF-165 gene increases wound healing in diabetic mice (Ross et al, 1974)

A Role for VEGF-A in Wound Angiogenesis

In a recent study the importance of VEGF-A in wound healing was revealed by neutralizing VEGF-A antibodies causes lessening of wound angiogenesis, fluid accumulation and granulation tissue formation (Howdieshell et al, 2001). Along with that, when VEGF neutralizes in human body after injury the angiogenic activity present in body fluid is strongly inhibited (Nissen et al, 1998). Finally, retroviral delivery of VEGFR-2 which is a dominant negative gene causes a strong reduction in angiogenic activity in mice wound as well as granulation tissue formation (Tsou et al, 2002). On the contrary, wound closure is not affected due to increased wound contraction.

VEGF-A plays an important role in wound healing as it promotes early steps in angiogenesis specially in formation of endothelial cell migration and proliferation. VEGF-A transcription and secretion along with VEGFR increased in the acute wound. After injury platelet activates and release VEGF-A. Along with that macrophage also releases VEGF-A during wound healing. (186). Furthermore, keratinocytes, TNF-a, fibroblast, cytokines and other growth factors helps in releasing VEGF-A.

Expression of VEGF-C and Its Receptor in Healing Skin Wounds

Along with the formation of new blood vessel lymphangiogenesis also occurs during wound healing of skin. VEGFR-3 and its ligands VEGF-C and VEGF-D regulates the formation of lymphatic vessels (Karkkainen et al, 2002). In recent study inside the wound granulation tissue VEGFR-3 positive lymphatic vessels were found (Paavonen et al, 2000). These vessels were found in wound along with blood vessels but relapse earlier. The accountable ligand is VEGF-C which is found in normal and wounded mice (Paavonen et al, 2000). In a nutshell member of VEGF family are major regulators for angiogenesis in wound healing

1.2.3 Regeneration Phase

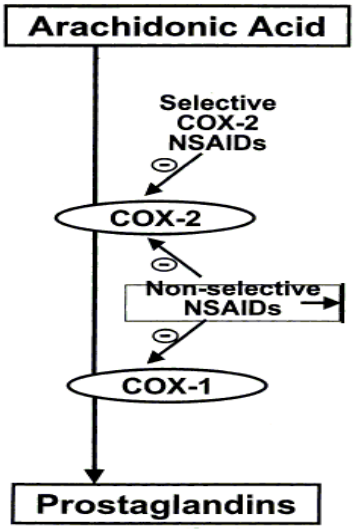
The remodeling phase commences later to the granulation tissue development. Differentiation into myofibril sequels in manifestation of alpha-smooth muscle actin (SMA) along with diminution of the abrasion prompted by the mechanical tension and cytokines TGF-beta impelled by fibroblast (Hinz, 2007) and myofibrils endures apoptosis amidst cessation of recovery (Xue and Jackson, 2015). Expeditious swapping of collagen I with that of collagen III in ECM entailing higher tensile robustness and entails prolonged time for sedimentation thus initiating dwindling of new blood vessels and blood circulation and leads to the emergence of a fully-fledged avascular and acellular ambience (Greenalgh, 1998). However, recuperation of skin constituents such as hair follicle and sweat glands after severe injury doesn't occur only 80% of the original tensile strength can be achieved(Schilling, 1976).

1.3 Drugs used in wound healing and their mechanism of action

1.3.1 NSAID

Indication: In acute and chronic diseases where pain and inflammation is involved, NSAIDs are the most common choice of drug to be used. The instances for their application include osteoarthritis, rheumatoid arthritis, low back pain, mild-to-excess pain due to inflammation and tissue trauma, inflammatory arthropathies, tennis elbow, migraine, headache, acute gout, dysmenorrhoea (menstrual pain), postoperative pain, metastatic bone pain, muscle stiffness and trauma because of Parkinson's disease, pyrexia (fever), renal Colic and ileus etc. Neonate infants suffer from ductus arteriosusis and macular edema etc. are also administered with NSAIDs. Aspirin is the only NSAID which can irreversibly inhibit both COX-1 and COX- 2 pathways (Silverstein et al, 2000).

Mechanism of action: Regular NSAIDs inhibit both COX-1 and COX- 2 non-selectively. NSAID's selectivity is based on their inhibition of the two isomeric forms of the cyclooxygenase enzyme. Tissue trauma stimulates the formation of arachidonic acid which goes on to produce prostaglandins responsible for inflammatory response via the COX pathway. NSAIDs bars the synthesis of prostaglandins by blocking the COX pathway.



Side effects: Side effects of NSAIDs include increased risk of gastrointestinal complications and renal diseases. About 10–20% of NSAID patients suffer from dyspepsia. High doses of NSAIDs were related with serious upper gastrointestinal side-effects which include bleeding. NSAIDs, like all drugs, may interact with other medications. Other side-effects of NSAIDs include nausea, vomiting, diarrhoea etc (Wang & Dubois, 2009). NSAID induced renal dysfunctions include decreased glomerular perfusion, decreased glomerular filtration and acute renal failure. The risk of ARF was increased nearly twofold for all NSAIDs (nonselective and COX-2 selective NSAIDs) within 30 days of initial use/prescribing in a nested case control study of older adults,.(Schneider, Lévesque, Zhang, Hutchinson, & Brophy, 2006)

1.3.2 Anti- oxidants

Indications: Anti- oxidants are a common element of general dietary intake. Vitamin C, Alpha lipoic acd, Glutathione are examples of anti- oxidants. Vitamin C is assumed to treat common cold and has been proved to treat scurvy. Studies have established the anti- cancer properties of ascorbic acid and its effectiveness in the treatment of cardiovascular disease and rheumatoid arthritis. (Nimse & Pal, 2015).

Mechanism of action: Oxidative stress, which is the imbalance of reactive oxygen species (ROS) and their neutralization by antioxidant system is another contributor in inflammation. There is an increased presence of ROS in inflamed regions which results in the occurrence of oxidative stress.

Anti- oxidants exert their anti- inflammatory action by decreasing ROS formation. (Nimse & Pal, 2015).

Side effects: Most notable side effects comprise of constipation, diarrhea and upset stomach. These are temporary side effects and disappear after a period of time. Stool color may turn black due to iron, but that is not harmful. Severe side effects are not observed in this case. (Nimse & Pal, 2015).

1.3.3 Pentoxifylline which is a methylxanthin is used in treating patients with ulcers resulting from peripheral vascular disease. It elevates peripheral vascular bed perfusion and lowers blood viscosity and aggregation of platelets to improve capillary microcirculation. It also exerts inhibitory action on tumor necrosis factor α. There are also other instances of its use such as sickle cell ulcers, livedoid vasculitis, and necrobiosis lipoidica.

Indication:

Pain, numbness and tingling in the legs. Also, for treating blood vessel problems.

Side effects:

Burping, bloating, dizziness, gas, headache. Allergic reactions, chest pain, abnormal heart beat, severe dizziness are rare side effects.

1.3.4 Iloprost, which is an analogue of prostacyclin, is a recognized drug for treating intermittent claudication severe limb ischaemia, and prevention of imminent gangrene, and to reduce the pain and clinical symptoms associated with Raynaud's disease. If administered intravenously, it is helpful in curing arterial ulcers and vasculitic ulcers resulting from connecting tissue abnormalities.

Indication:

Pulmonary arterial hypertension

Side effects:

Allergic reactions, peeling skin, trouble in breathing, chest pain, coughing up blood, abnormal heartbeat, back pain, flushing, muscle cramps, cough etc.

1.3.5 Glyceryl trinitrate, which is a nitric oxide donor is used in managing chronic anal fissures. It causes vasodilation and has a possible role of glyceryl trinitrate in treating chronic wounds of ischaemic aetiology.

Indication:

Angina, renal failure, CHF in the setting of MI, hypertensive crisis etc.

Side effects:

Headache, tachycardia, dyspnea, dizziness, flushing, blurred vision, hypotension etc. are common ones. Methemoglobinemia (rare), Syncope, Prolonged bleeding time, Unstable angina, Rebound hypertension, Thrombocytopenia are severe side effects.

Systemic corticosteroids are used in treating secondary ulcer as they can attenuate excessive inflammatory response.

1.3.6 Phenytoin, applied topically, assists in wound healing through inhibition of the enzyme collagenase. It is effective in some low-grade pressure ulcers and trophic ulcers due to leprosy. The possibility of systemic absorption and toxicity has limited its use. It is indicated for seizures.

1.4 Non-Surgical Treatments

Bandages and hosiery: Compression bandages are used in lymphoedema, lower limb ulcer to venous insufficiency. There are two types of bandages. They are single layer compression bandages and multiple layer compression bandages.

Caution in use of compression bandages

- Necessary clinical evaluation is needed before prescribing any compression bandage.
- Continuous use can lead to severe complications that includes limb gangrene.
- Cautious steps should be maintained in case of patient with peripheral neuropathy.

Ulcers	Treatment
Venous	Appropriate compression bandages
Venous ulcer with eczema	Medicated bandage such as zinc paste bandage containing calamine, coal tar, or ichthammol
Arterial	Pentoxifylline, iloprost
Vasculitic	Iloprost, glyceryl trinitrate, calcium antagonists, corticosteroids
Exudating cavity	Vacuum assisted closure
Ulcer secondary to lymphoedema	Compression bandages, intermittent pneumatic compression device
Ulcer with slough and non-viable or necrotic tissue	Any form of debridement (sharp, mechanical, autolytic (honey), enzymatic, or biosurgery (maggots))
Necrobiosis lipoidica	Psoralen and ultraviolet light therapy, pentoxifylline

Figure 1.6 Different types of ulcers and their treatment (Stuart et al, 2006)

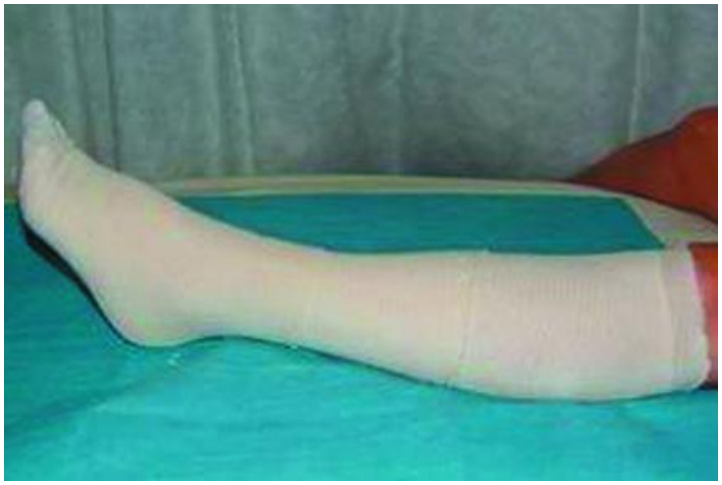


Fig 1.7: Single layer compression bandage

Vacuum assisted closure: It is a non-invasive negative pressure technique. It is widely used for chronic wound healing.

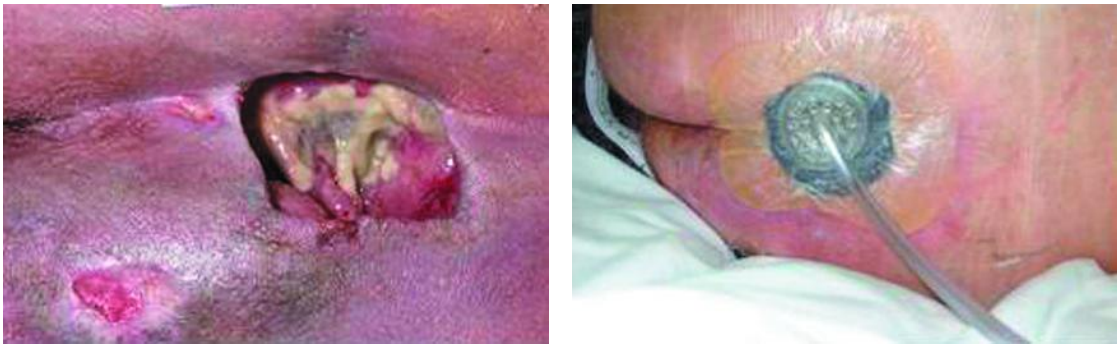


Figure 1.8

Left: Grade 4 sacral pressure ulcer suitable for vacuum assisted closure therapy. Right: Vacuum assisted closure in situ

Hyperbaric Oxygen: Hyperbaric oxygen therapy is used as adjunct therapy for the treatment of various non-healing wound because most of the non-healing wounds are hypoxic. In this treatment atmospheric pressure is increased and patient is given to breathe 100% oxygen. In this treatment some side effects have been observed like seizures and pneumothorax.

Biosurgery: Here sterile maggots (usually of the green fly *Lucilia sericata*) which digest the narcotic material from the wound without damaging the healthy tissues. They are useful in the treatment of pressure, arterial and venous ulcers.

Chapter Two: Rationale:

The aim of this study is to identify whether new drug therapy for wound healing can be establish or not. The rationale behind this study are given below:

- Previously no work has been done with the combination of Diclofenac-Na and Vitamin C. this would be totally a new work with this combination for the therapy of wound healing.
- Wound healing is a complex and much slower process. All the drugs that have been used for wound healing they take a lot of time to heal. This new combination might giver faster healing activity. As well as, the conventional drugs give side effects along with the therapeutic effect. This combination therapy might show lesser side effects. NSAIDs alone gives a lot of side effects like gastric irritation, heart burn, ulceration, dizziness, headache, kidney disfunction, hypertension, leg swelling etc (Wheeler, 2005). For this reason, we wanted to observe whether with the combination of Vitamin C can reduce the dose and duration of NSAID treatment can be changed or not. (Abbas et al, 2014).
- Previously in one experiment the combination of Diclofenac-Na and Vitamin C has shown the best effect as anti-inflammatory drug amongst other anti-inflammatory drugs. As inflammation is the first phase of wound healing so we wanted to find that whether this combination works in whole wound healing process.
- There are two types of wounds acute and chronic. In this experiment combination therapy is going to be used for acute wound. If this experiment become successful, then it can further lead to the experiment where it can be tested for chronic wound therapy. When acute wound does not heal in expected time and rate this wound converts into chronic wound. We wanted to check whether this acute wound converts into chronic wound or by using this combination therapy this wound heals within the time.

Therefore, for above mentioned reasons this study was conducted. If this new regimen can be established there will be a new treatment therapy for wound healing.

Chapter Three: Methodology and Materials

3.1 Animal

To perform this experiment *Swiss albino* mice were selected. The average age of the mice were 4-6 weeks which is considered as mature for the experiment. The average weight of the mice were 16-18gm per rate. They were collected from ICDDRB (International Centre for Diarrheal Disease Research, Bangladesh) situated in Mohakhali, Dhaka Bangladesh. They were kept in animal house under laboratory condition in the temperature of 22^0C- 25^0C and also humidity was maintained between 60 ± 10%. The mice were kept under Light and Dark condition in 12hr/day cycle. Therefore, there were no disturbance in their normal life. Their food were collected from ICDDRB

Figure 3.1 *Swiss albino* Mice

The food contained sufficient nutrient for mice with this they can stay in normal health condition. The water which was supplied for the rat was pure and served in plastic bottles for drinking purpose. Total 96 mice were taken for the experiment. Each group consisted of 24 mice and kept in separate cages. They were marked by using black marker pen. Before starting the experiment all the mice were weighed individually in weighing machine. All the experiments were done under the ethical committee of ICDDRB.

Groups of Mice

Day	Negative	Diclofenac-Na	Vitamin C	Combination
Day-2	6	6	6	6
Day-4	6	6	6	6
Day-7	6	6	6	6
Day-9	6	6	6	6
	Total= 24	Total= 24	Total= 24	Total= 24

3.2 Materials

Syringe: 1ml insulin syringe was used for drug administration to the mice. Total volume of the syringe was 1ml and the capacity was 100 IU. The syringe has very fine needle which was suitable for drug administration in mice model.

Cotton and Antiseptic agent: Cotton was used to anesthetized mice with ether. A piece of cotton was soaked in ether and kept in a closed container to anesthetized mice. Along with that cotton was also used to clean the place of wound and syringes with antiseptic. Antiseptic agents were used to disinfect the materials and mice skin. 5% ethanol was used as antiseptic agent.

Punching machine: 4.5mm diameter punching machine was used for creating the wound in mice.

Surgical knife: Surgical knife was used to collect the skin above the wound.

Electrical trimmer: Electrical trimmer was used to shave the dorsal skin of the mice.

Eppendorf: Medium and large Eppendorf was used to collect the samples from each mouse.

3.3 Reagents

Distilled water: For rinsing and cleaning purpose distilled water was used. It was used to avoid all kinds of contaminations.

Saline water: 0.9% sodium chloride solution (normal saline) was used as negative control. Along with that it was used for dissolving all the drug products. The saline was packed in a plastic bag. It was bought from Tamanna Pharmacy in Mirpur 10 Dhaka-1216.

ELISA Kit: Mouse VEGF ELISA kit was used for determining the concentration of total VEGF content in the sample. The kit was bought from Boster Bio.

Figure 3.2 Mouse VEGF ELISA Kit

RIPA Lysis Buffer: RIPA Lysis Buffer was used for tissue lysis. It was bought from Boster Bio.

Bio-ureate test kit: Bio-ureate test kit was used for the estimation of total protein in the sample.

3.4 Drugs and Dosage:

NSAID and anti-oxidant were used in this experiment. All the drugs were given in a definite dose.

All these drugs were in pure form and as active pharmaceutical ingredient. They all were collected from Incepta Pharmaceuticals Ltd, BD. There is a brief description about the dose of drugs used.

3.4.1 Anti-oxidant

Vitamin C (Ascorbic acid or L-ascorbic acid) was used as anti-oxidant. It was given in a concentration of about 0.25mg/kg. The Vitamin C was dissolved in 0.9% sodium chloride solution and made a solution. This solution was injected to mice at its intra-peritoneal site.

3.4.2 NSAID

Diclofenac-Na was used as NSAID. It was given in a concentration about 5mg/kg. It was dissolved in 0.9% sodium chloride solution and this solution was injected to mice.

3.4.3 Combination

In combination therapy both Diclofenac-Na and Vitamin C was used on their respective dose. Doses were Diclofenac-Na 5mg/kg and Vitamin C 0.25mg/kg. Together they were mixed and dissolved in 0.9% sodium chloride solution.

3.5 Methodology:

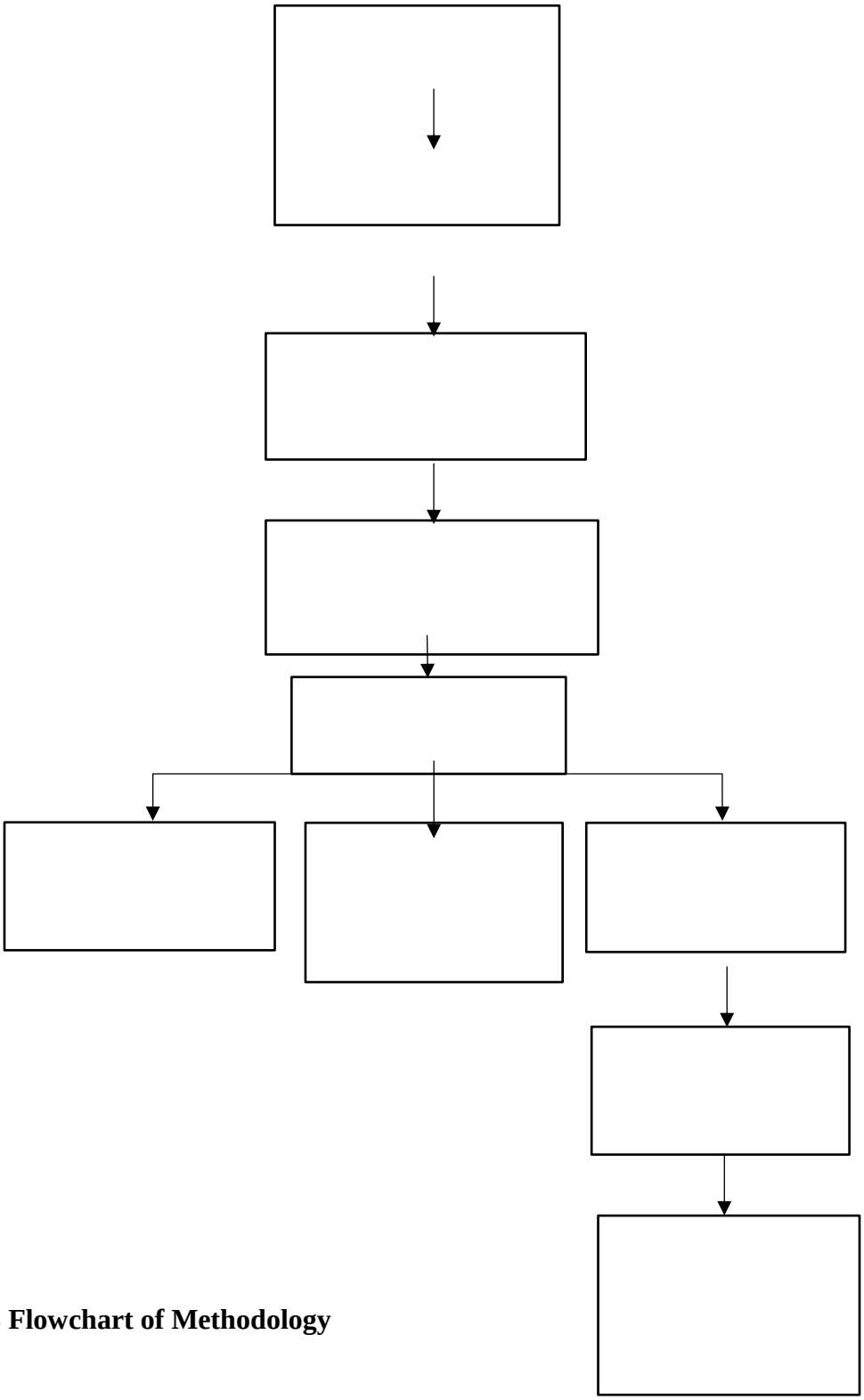


Figure 3.3 Flowchart of Methodology

Swiss Albino mouse having a mean weight of 16-18 gm of 4-6 weeks old were taken from ICCDDRB for analysis. Cropping of the dorsal hair was done accompanied by anesthesia of the mouse with 1ml Diethyl ether. Fumigation of the skin was done by 70% ethanol swabbing for mustering the skin for abrasion and a laceration of full diameter of 4.5 was done with succor of a diameter punch and indistinguishable congruous skin abrasions were made on the dorsal surface. Four groups comprised of twenty-four mice and referred to as: Negative Control being treated once solely with saline water, Diclofenac-Na group medicated with 5mg/kg Diclofenac-Na, Vitamin-C group dosed with 0.25mg/kg Vitamin-C and last but not the least was the Combination group treated with a amalgamation of both Diclofenac-Na and Vitamin-C.

Figure 3.4. Wound creation in mice

3.5.1 Wound size evaluation

For the appraisal of wound size, digital photographs of the two, four, seven and nine days were analyzed following the injection and was encapsulated by the vertical orienting the camera over the abrasion. To curtail the error, camera fixation was done at 60cm distance with the aid of a stand and for calibration comprehended a ruler in centimeter and was in proximity of the wound accompanied by the scrutinizing of the digitalized photographs by thee Image J program. Correction in accordance with the perspective scaling was done and calculation of pixels per area

of the photograph was done in commensuration with each wound. Comparative studies were conducted between the inceptive defect size and the fraction of wounds on day two, four, seven and nine.

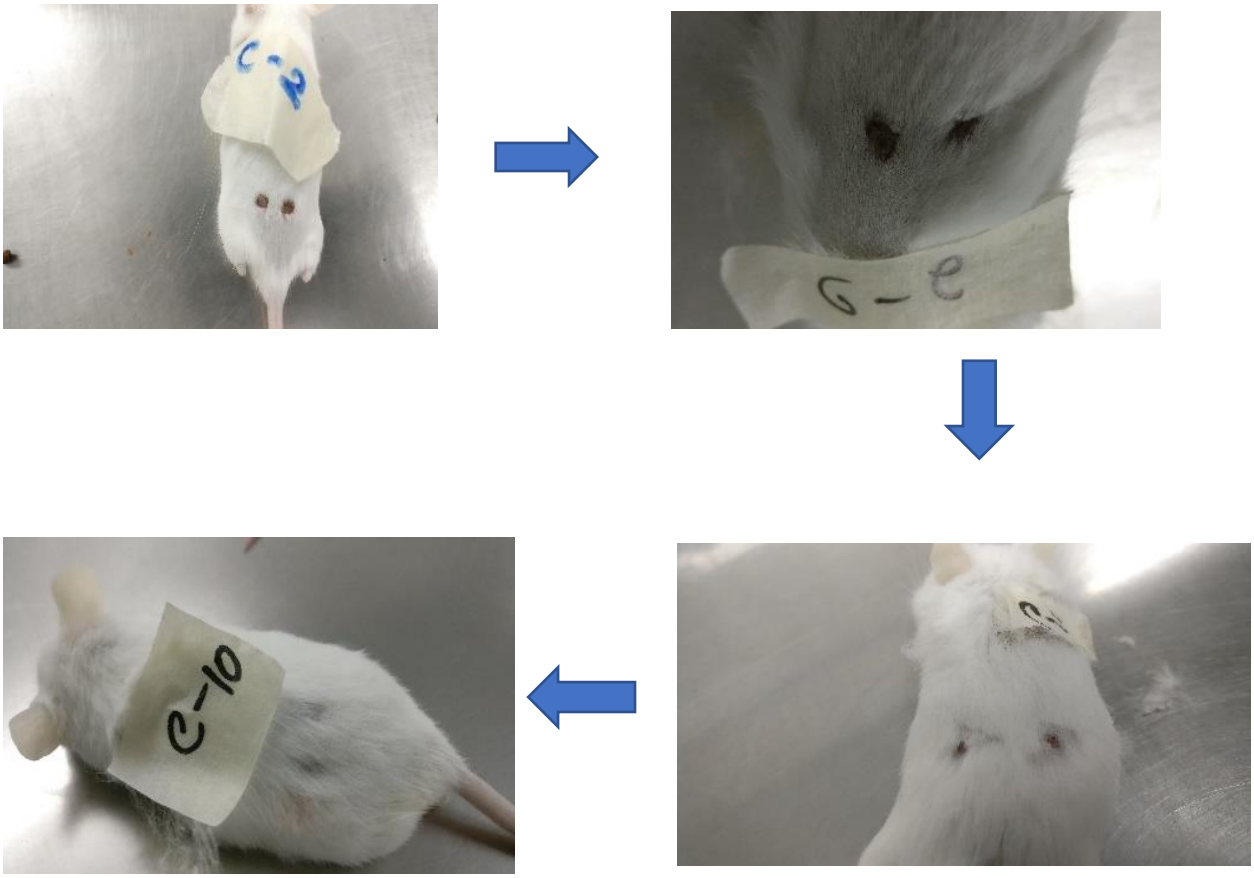


Figure 3.5 Wound Healing in Mice

3.5.2 Blood collection

Venous blood withdrawal was done from 6 mice of respective groups for WBC counting.

WBC Counting Process

Amalgamation for 5-7 minutes was conducted with 50µl of blood sample in a test tube with 950µl WBC reagent. 10µl was withdrew from the test-tube and was accompanied by placing it into the haemocytometer with a coverslip for the WBC counting.

3.5.3 Tissue Collection and Cell Lysis (RIPA Buffer)

Fumigation of the abrasion was done with alcohol and povidone Iodine. Anesthetizing by Diethyl ether on the respective two, four, seven and ninth days and six mice from the respective groups were hecatomb on each day and excision of the wound was done retaining 2mm unwounded skin around the abrasion. The fresh tissue was propped and sluiced several times in the PBS and followed by the mincing of the tissue. Correspondingly addition of the RIPA buffer was done in the ratio of 5:1 (Add 5 mL chilled lysis buffer per gram of tissue.) and if the prerequisite is a concentrated protein extract then use smaller volume of reagent. This is followed by homogenization until no tissue debris is seen to exist. Incubation on ice is done in auxiliary to centrifugation at 10000 x g for 10 minutes and latter transfer of the supernatant to a test tube is done for further scrutiny.

3.5.4 Total Protein Test:

Several test-tubes were taken for carrying out this test. One test-tube is for blank, another one is for standard and rest are for samples. 1ml of bio-ureate reagent was taken on each of the test tube. Then in standard labelled test tube 20µL of standard solution was given. On sample test tube 20µL of sample was given. Then all the test tubes were kept in dark for 20 minutes for colour development. Once the colour is developed after 20 minutes all the blank, standard and samples were taken for measuring the absorbance. The absorbance was measured in 540nm. From the absorbance value using equation concentration was calculated.

3.5.5 Quantitative analysis of VEGF with ELISA

Reagent Preparation:

Standard Solution: 10,000pg/mL: Add 1mL of sample diluent buffer into one tube of standard (10ng per tube) and mix thoroughly.

5000pg/mL: Mix 0.3mL of 10,000pg/mL with 0.3 mL of sample diluent buffer and mix thoroughly.

Perform similar dilution until the standard solution of following concentrations (pg/mL) are made.1250, 625, 312, 156 and 78. Add 100 µl of each of the diluted standard solutions to the appropriate empty wells and repeat in duplicate for accuracy.

Biotinylated Antibody: Calibration was done of the volume entailed for the assay by the multiplication of 0.1 mL/well and the number of wells required. 2-3 extra wells were added supplementary to the calculated wells in case of any pipetting spillage. Guesstimate the extent of the dilution antibody by performing a 1:100 dilution (For each 1 µL concentrated antibody, add 99 µL antibody dilution buffer) and blend thoroughly.

Avidin-Biotin-Peroxidase Complex (ABC): Calibration was done of the volume entailed for the assay by the multiplication of 0.1 mL/well and the number of wells required. 2-3 extra wells were added supplementary to the calculated wells in case of any pipetting spillage. Guesstimate the extent of the dilution of ABC solution by performing a 1:100 dilution (For each 1 µL concentrated ABC solution, add 99 µL ABC dilution buffer) and blend thoroughly.

Sample dilution: Sequentially dilute the sample with blocking buffer (5% w/v non-fat dry milk in PBS buffer) promptly before use.

Preparation of Phosphate Buffer:

Deliquescing 8gm of NaCl, 0.2g of KCl, 1.44 Na_2HPO_4

Assay Protocol:

Assay protocol As a precursor to the experiment it is advised that all the all reagents and materials be equipoised to 37°C/room temperature. All the reagent and preparation standard were arranged. Then all the surplus microplate strips were discarded form the plate. These surplus microplate strips were secured and put away in the authentic packing. 100 µl of the standard, samples, or control per well were prefixed and two clones of standard, sample, or control is recommended at minimum followed by sealing of the plate sealer along with incubation for 120 minutes at RT (or 90 min. at 37 °C). The cover was taken off and the waste of the well was disposed in a suitable waste container. Afterwards the plate on the benchtop was inverted on to

a paper towel to get rid of residual liquids. It is to be noted that the well should not be totally dry during the time of removing the plethora. 100 µl of the prepared 1x Biotinylated Anti-Mouse Vegfa antibody was added to each well accompanied by covering with plate sealer and incubation for 90 minutes at RT (or 60 minutes at 37°C). The plate was cleaned 3 times with the 1x wash buffer. Once more the liquid that has accumulated in the well is removed along with inversion of the plate so the residual liquid is discarded. 300 µl of the 1x wash buffer was affixed to each assay well. (For cleaner background incubate for 60 seconds between each wash). This whole cleaning of liquids along with addition of wash buffer is repeated two times. Then 100 µl of the prepared1x Avidin-Biotin-Peroxidase was added and complexed onto each well along with 40 minutes at RT(or 30 minutes at 37°C). Afterwards the plate is cleansed 5 times with the 1x wash buffer. Again the liquid in the well was properly disposed also the remaining liquid was removed through inversion of the plate onto a paper towel carefully. 300 µl of the 1x wash buffer was added to each assay well. (For cleaner background incubate for 60 seconds between each wash). It is to be noted that the well should not become completely dry while removing the liquids. This whole process of removing the liquid and adding 300 µl buffer was repeated 5 times. Now 90 µl of Color Developing Reagent was added to each well and incubated in the dark for 30 minutes at RT (or 25-30 minutes at 37°C). (The optimum incubation time must be empirically ascertained A guideline to look for is blue shading the top four standard wells, while the remaining standards remain clear.) 100 µl of Stop Solution was added to all the wells individually. The color instantly altered to yellow. At last within 30 minutes of halting the reaction, the O.D. absorbance should be read with a microplate reader at 450nm.

Chapter Four: Result

4.1 WBC Count

Table 4.1 WBC Count for Negative (N) control Group in Day 2, 4, 7 and 9

Sample Number	Day-2	Average
N-1	20250	20325
N-2	20450	
N-3	20150	
N-4	20050	
N-5	20650	
N-6	20400	
	Day-4	
N-7	14400	14425
N-8	14350	
N-9	14500	
N-10	14550	
N-11	14450	
N-12	14300	
	Day-7	
N-13	7000	7000
N-14	7100	
N-15	6900	
N-16	6750	
N-17	7050	
N-18	7200	

	Day-9	
N-19	5150	5250
N-20	5250	
N-21	5300	
N-22	5050	
N-23	5350	
N-24	5400	

Table 4.2 WBC Count for Diclofenac-Na (D) in Day 2, 4, 7 and 9

Sample Number	**Day-2**	**Average**
D-1	14150	14366.67
D-2	14550	
D-3	14500	
D-4	14250	
D-5	14450	
D-6	14300	
	Day-4	
D-7	11500	12150
D-8	15050	
D-9	10100	
D-10	10600	
D-11	10750	
D-12	14900	
	Day-7	

D-13	4000	4133.33
D-14	3600	
D-15	4950	
D-16	4250	
D-17	3900	
D-18	4100	
	Day-9	
D-19	1850	3125
D-20	2800	
D-21	5850	
D-22	2950	
D-23	3200	
D-24	2100	

Table 4.3 WBC Count for Vitamin C (V) group in Day 2, 4, 7 and 9

Sample Number	**Day-2**	**Average**
V-1	18450	18525
V-2	18150	
V-3	18400	
V-4	18450	
V-5	19250	
V-6	18450	
	Day-4	
V-7	10500	13050

V-8	16750	
V-9	12900	
V-10	12400	
V-11	15600	
V-12	10150	
	Day-7	
V-13	3000	
V-14	6350	
V-15	6450	5775
V-16	6700	
V-17	6400	
V-18	5750	
	Day-9	
V-19	4550	
V-20	4500	
V-21	4450	4566.667
V-22	4750	
V-23	4500	
V-24	4650	

Table 4.4. WBC Count for Combination (C) Group in Day 2, 4, 7 and 9

Sample Number	Day-2	Average
C-1	12450	12291.67
C-2	12400	
C-3	12150	
C-4	12050	
C-5	12550	
C-6	12150	
	Day-4	
C-7	13550	11316.67
C-8	12050	
C-9	11000	
C-10	10700	
C-11	10450	
C-12	10150	
	Day-7	
C-13	2300	3216.667
C-14	3600	
C-15	3550	
C-16	2900	
C-17	3400	
C-18	3550	
	Day-9	
C-19	4100	

C-20	1200	2500
C-21	4350	
C-22	1800	
C-23	1950	
C-24	1600	

4.2 Total Protein Test

Table 4.5 Total protein for Negative (N) control group in Day 2, 4, 7 and 9

Sample	Abs Day-2	Concentration (mg/dL)	Average	Standard Deviation
N-1	0.317	5.17	5.20	0.03
N-2	0.319	5.20		
N-3	0.321	5.23		
N-4	0.323	5.27		
N-5	0.318	5.18		
N-6	0.317	5.17		
	Day-4			
N-7	0.313	5.10	5.10	0.01
N-8	0.312	5.09		
N-9	0.313	5.10		
N-10	0.314	5.12		
N-11	0.312	5.09		
N-12	0.314	5.12		
	Day-7			

N-13	0.396	6.46	6.44	0.01
N-14	0.395	6.44		
N-15	0.394	6.42		
N-16	0.395	6.44		
N-17	0.394	6.42		
N-18	0.395	6.44		
	Day-9			
N-19	0.332	5.41	5.41	0.01
N-20	0.333	5.43		
N-21	0.332	5.41		
N-22	0.331	5.40		
N-23	0.332	5.41		
N-24	0.333	5.43		

Table 4.6 Total protein for Diclofenac-Na (D) Group in Day 2, 4, 7 and 9

Sample	Abs Day-2	Concentration (mg/dL)	Average	Standard Deviation
D-1	0.452	7.37	5.95	0.77
D-2	0.324	5.28		
D-3	0.32	5.22		
D-4	0.325	5.30		
D-5	0.382	6.23		
D-6	0.387	6.31		
	Day-4			

D-7	0.425	6.93	6.56	0.42
D-8	0.427	6.96		
D-9	0.434	7.08		
D-10	0.376	6.13		
D-11	0.377	6.15		
D-12	0.376	6.13		
	Day-7			
D-13	0.443	7.22	6.71	0.70
D-14	0.442	7.21		
D-15	0.441	7.19		
D-16	0.35	5.71		
D-17	0.442	7.21		
D-18	0.352	5.74		
	Day-9			
D-19	0.412	6.72	6.71	0.01
D-20	0.412	6.72		
D-21	0.41	6.68		
D-22	0.413	6.73		
D-23	0.411	6.70		
D-24	0.412	6.72		

Table 4.7. Total protein for Vitamin C (V) group in Day 2, 4, 7 and 9

Sample	Abs Day-2	Concentration (mg/dL)	Average	Standard Deviation
V-1	0.325	5.30	5.86	0.29
V-2	0.352	5.74		
V-3	0.366	5.97		
V-4	0.385	6.28		
V-5	0.365	5.95		
V-6	0.365	5.95		
	Day-4			
V-7	0.274	4.47	4.62	0.34
V-8	0.331	5.40		
V-9	0.273	4.45		
V-10	0.275	4.48		
V-11	0.275	4.48		
V-12	0.273	4.45		
	Day-7			
V-13	0.398	6.49	6.46	0.02
V-14	0.395	6.44		
V-15	0.397	6.47		
V-16	0.394	6.42		
V-17	0.398	6.49		
V-18	0.397	6.47		
	Day-9			

V-19	0.475	7.75	7.40	0.34
V-20	0.431	7.03		
V-21	0.433	7.06		
V-22	0.435	7.09		
V-23	0.474	7.73		
V-24	0.475	7.75		

Table 4.8. Total Protein for Combination (C) group in Day 2, 4, 7 and 9

Sample	Abs Day-2	Concentration (mg/dL)	Average	Standard Deviation
C-1	0.376	6.13	6.11	0.01
C-2	0.375	6.11		
C-3	0.374	6.10		
C-4	0.375	6.11		
C-5	0.375	6.11		
C-6	0.374	6.10		
	Day-4			
C-7	0.435	7.09	7.10	0.04
C-8	0.437	7.13		
C-9	0.439	7.16		
C-10	0.437	7.13		
C-11	0.432	7.04		
C-12	0.431	7.03		
	Day-7			

C-13	0.452	7.37	7.40	0.06
C-14	0.453	7.39		
C-15	0.452	7.37		
C-16	0.452	7.37		
C-17	0.451	7.35		
C-18	0.462	7.53		
	Day-9			
C-19	0.575	9.38	8.94	0.95
C-20	0.418	6.82		
C-21	0.576	9.39		
C-22	0.572	9.33		
C-23	0.575	9.38		
C-24	0.574	9.36		

4.3 ELISA Analysis for VEGF

Table 4.9 ELISA Analysis of VEGF for negative (N) control group in Day 2, 4, 7 and 9

Sample	Blank Abs	Sample Abs Day -2	Actual Abs	Average	Concentration	Average	Standard Deviation
N1A	0.009	0.015	0.006	0.006	12.37	11.64	0.56
N1B	0.009	0.015	0.006				
N2A	0.009	0.015	0.006	0.006	12.37		
N2B	0.009	0.015	0.006				
N3A	0.009	0.014	0.005	0.0055	11.75		

N3B	0.009	0.015	0.006				
N4A	0.009	0.014	0.005	0.005	11.12		
N4B	0.009	0.014	0.005				
N5A	0.009	0.015	0.006	0.005	11.12		
N5B	0.009	0.013	0.004				
N6A	0.009	0.014	0.005	0.005	11.12		
N6B	0.009	0.014	0.005				
		Day-4					
N7A	0.009	0.023	0.014	0.0135	21.75		
N7B	0.009	0.022	0.013				
N8A	0.009	0.023	0.014	0.0135	21.75		
N8B	0.009	0.022	0.013				
N9A	0.009	0.023	0.014	0.014	22.37		
N9B	0.009	0.023	0.014				
N10A	0.009	0.022	0.013	0.013	21.12		
N10B	0.009	0.022	0.013				
N11A	0.009	0.023	0.014	0.0135	21.75		
N11B	0.009	0.022	0.013				
N12A	0.009	0.022	0.013	0.0135	21.75		
N12B	0.009	0.023	0.014			21.75	0.36
		Day-7					
N13A	0.009	0.014	0.005	0.0045	10.5		
N13B	0.009	0.013	0.004				
N14A	0.009	0.015	0.006	0.0045	10.5	10.18	0.69

N14B	0.009	0.012	0.003				
N15A	0.009	0.014	0.005	0.0045	10.5		
N15B	0.009	0.013	0.004				
N16A	0.009	0.014	0.005	0.005	11.12		
N16B	0.009	0.014	0.005				
N17A	0.009	0.013	0.004	0.0035	9.25		
N17B	0.009	0.012	0.003				
N18A	0.009	0.013	0.004	0.0035	9.25		
N18B	0.009	0.012	0.003				
		Day -9					
N19A	0.009	0.012	0.003	0.003	8.62		
N19B	0.009	0.012	0.003				
N20A	0.009	0.012	0.003	0.0025	8		
N20B	0.009	0.011	0.002				
N21A	0.009	0.012	0.003	0.003	8.62		
N21B	0.009	0.012	0.003				
N22A	0.009	0.011	0.002	0.002	7.37		
N22B	0.009	0.011	0.002				
N23A	0.009	0.011	0.002	0.003	8.62		
N23B	0.009	0.013	0.004				
N24A	0.009	0.012	0.003	0.003	8.62		
N24B	0.009	0.012	0.003			8.31	0.47

Table 4.10 ELISA Analysis for VEGF for Diclofenac-Na (D) group in Day 2, 4, 7 and 9

Sample	Blank Abs	Sample Abs Day -2	Actual Abs	Average	Concentration	Average	Standard Deviation
D1A	0.009	0.026	0.017	0.0165	25.5		
D1B	0.009	0.025	0.016				
D2A	0.009	0.019	0.01	0.011	18.62		
D2B	0.009	0.021	0.012				
D3A	0.009	0.022	0.013	0.0135	21.75		
D3B	0.009	0.023	0.014				
D4A	0.009	0.027	0.018	0.0185	28		
D4B	0.009	0.028	0.019				
D5A	0.009	0.031	0.022	0.0215	31.75		
D5B	0.009	0.03	0.021				
D6A	0.009	0.028	0.019	0.0185	28		
D6B	0.009	0.027	0.018			25.60	4.33
		Day-4					
D7A	0.009	0.023	0.014	0.0145	23		
D7B	0.009	0.024	0.015				
D8A	0.009	0.023	0.014	0.015	23.62		
D8B	0.009	0.025	0.016				
D9A	0.009	0.022	0.013	0.0135	21.75		
D9B	0.009	0.023	0.014			23.41	0.85

D10A	0.009	0.025	0.016	0.0155	24.25		
D10B	0.009	0.024	0.015				
D11A	0.009	0.023	0.014	0.015	23.62		
D11B	0.009	0.025	0.016				
D12A	0.009	0.024	0.015	0.0155	24.25		
D12B	0.009	0.025	0.016				
		Day-7					
D13A	0.009	0.014	0.005	0.0055	11.75		
D13B	0.009	0.015	0.006				
D14A	0.009	0.014	0.005	0.0055	11.75		
D14B	0.009	0.015	0.006				
D15A	0.009	0.012	0.003	0.0045	10.5		
D15B	0.009	0.015	0.006				
D16A	0.009	0.013	0.004	0.0035	9.25		
D16B	0.009	0.012	0.003				
D17A	0.009	0.013	0.004	0.004	9.87		
D17B	0.009	0.013	0.004				
D18A	0.009	0.014	0.005	0.0055	11.75		
D18B	0.009	0.015	0.006			10.81	1.00
		Day -9					
D19A	0.009	0.013	0.004	0.0035	9.25		
D19B	0.009	0.012	0.003				
D20A	0.009	0.011	0.002	0.0035	9.25		
D20B	0.009	0.014	0.005			8.72	0.98

D21A	0.009	0.014	0.005	0.004	9.87		
D21B	0.009	0.012	0.003				
D22A	0.009	0.011	0.002	0.0015	6.75		
D22B	0.009	0.01	0.001				
D23A	0.009	0.013	0.004	0.003	8.62		
D23B	0.009	0.011	0.002				
D24A	0.009	0.012	0.003	0.003	8.62		
D24B	0.009	0.012	0.003				

Table 4.11. ELISA Analysis for VEGF for Vitamin C (V) group in Day 2, 4, 7 and 9

Sample	Blank Abs	Sample Abs Day -2	Actual Abs	Average	Concentration	Average	Standard Deviation
V1A	0.009	0.015	0.006	0.0065	13	12.51	0.98
V1B	0.009	0.016	0.007				
V2A	0.009	0.015	0.006	0.007	13.62		
V2B	0.009	0.017	0.008				
V3A	0.009	0.016	0.007	0.0065	13		
V3B	0.009	0.015	0.006				
V4A	0.009	0.014	0.005	0.0055	11.75		
V4B	0.009	0.015	0.006				
V5A	0.009	0.016	0.007	0.0065	13		
V5B	0.009	0.015	0.006				
V6A	0.009	0.015	0.006	0.00467	10.70		

V6B	0.009	0.017	0.008				
		Day-4					
V7A	0.009	0.01	0.001	0.002	7.37		
V7B	0.009	0.012	0.003				
V8A	0.009	0.013	0.004	0.0035	9.25		
V8B	0.009	0.012	0.003				
V9A	0.009	0.011	0.002	0.0045	10.5		
V9B	0.009	0.016	0.007			9.04	0.93
V10A	0.009	0.012	0.003	0.003	8.62		
V10B	0.009	0.012	0.003				
V11A	0.009	0.012	0.003	0.0035	9.25		
V11B	0.009	0.013	0.004				
V12A	0.009	0.013	0.004	0.0035	9.25		
V12B	0.009	0.012	0.003				
		Day-7					
V13A	0.009	0.011	0.002	0.002	7.37		
V13B	0.009	0.011	0.002				
V14A	0.009	0.011	0.002	0.002	7.37		
V14B	0.009	0.011	0.002				
V15A	0.009	0.013	0.004	0.0035	9.25	8.52	0.83
V15B	0.009	0.012	0.003				
V16A	0.009	0.012	0.003	0.0035	9.25		
V16B	0.009	0.013	0.004				
V17A	0.009	0.013	0.004	0.0035	9.25		

V17B	0.009	0.012	0.003				
V18A	0.009	0.011	0.002	0.003	8.62		
V18B	0.009	0.013	0.004				
		Day -9					
V19A	0.009	0.011	0.002	0.002	8		
V19B	0.009	0.012	0.003				
V20A	0.009	0.012	0.003	0.003	8.62		
V20B	0.009	0.012	0.003				
V21A	0.009	0.011	0.002	0.002	7.375		
V21B	0.009	0.011	0.002			8.31	0.59
V22A	0.009	0.012	0.003	0.002	8		
V22B	0.009	0.011	0.002				
V23A	0.009	0.012	0.003	0.003	9.25		
V23B	0.009	0.013	0.004				
V24A	0.009	0.012	0.003	0.003	8.625		
V24B	0.009	0.012	0.003				

Table 4.12. ELISA Analysis of VEGF for Combination (C) group in Day 2, 4, 7 and 9

Sample	**Blank Abs**	**Sample Abs** **Day -2**	**Actual Abs**	**Average**	**Concentration**	**Average**	**Standard Deviation**
C1A	0.009	0.021	0.012	0.013	21.12		
C1B	0.009	0.023	0.014				

C2A	0.009	0.028	0.019	0.0185	28	26.22	2.68
C2B	0.009	0.027	0.018				
C3A	0.009	0.028	0.019	0.0185	28		
C3B	0.009	0.027	0.018				
C4A	0.009	0.026	0.017	0.0155	24.25		
C4B	0.009	0.023	0.014				
C5A	0.009	0.026	0.017	0.018	27.37		
C5B	0.009	0.028	0.019				
C6A	0.009	0.028	0.019	0.019	28.62		
C6B	0.009	0.028	0.019				
		Day-4					
C7A	0.009	0.021	0.012	0.011	18.62	23.52	9.29
C7B	0.009	0.019	0.01				
C8A	0.009	0.019	0.01	0.011	18.62		
C8B	0.009	0.021	0.012				
C9A	0.009	0.019	0.01	0.0315	44.25		
C9B	0.009	0.062	0.053				
C10A	0.009	0.019	0.01	0.0115	19.25		
C10B	0.009	0.022	0.013				
C11A	0.009	0.023	0.014	0.012	19.87		
C11B	0.009	0.019	0.01				
C12A	0.009	0.022	0.013	0.0125	20.5		
C12B	0.009	0.021	0.012				
		Day-7					

C13A	0.009	0.013	0.004	0.0045	10.5	10.5	0.95
C13B	0.009	0.014	0.005				
C14A	0.009	0.015	0.006	0.006	12.37		
C14B	0.009	0.015	0.006				
C15A	0.009	0.014	0.005	0.0045	10.5		
C15B	0.009	0.013	0.004				
C16A	0.009	0.012	0.003	0.0035	9.25		
C16B	0.009	0.013	0.004				
C17A	0.009	0.014	0.005	0.0045	10.5		
C17B	0.009	0.013	0.004				
C18A	0.009	0.013	0.004	0.004	9.87		
C18B	0.009	0.013	0.004				
		Day -9					
C19A	0.009	0.016	0.007	0.0065	13	12.47	1.92
C19B	0.009	0.015	0.006				
C20A	0.009	0.017	0.008	0.0085	15.5		
C20B	0.009	0.018	0.009				
C21A	0.009	0.012	0.003	0.0035	9.25		
C21B	0.009	0.013	0.004				
C22A	0.009	0.017	0.008	0.0055	11.75		
C22B	0.009	0.012	0.003				
C23A	0.009	0.016	0.007	0.0055	11.75		
C23B	0.009	0.013	0.004				
C24A	0.009	0.015	0.006	0.007	13.62		

C24B	0.009	0.017	0.008				

4.4 Weight of skin of mouse

Table 4.13 Weight of skin for negative (N) control group in Day 2, 4, 7 and 9

Sample	Weight (mg) Day-2	Average	Standard Deviation
N-1	6	6	0.81
N-2	7		
N-3	6		
N-4	5		
N-5	7		
N-6	5		
	Day-4		
N-7	3	4	0.81
N-8	5		
N-9	4		
N-10	4		
N-11	3		
N-12	5		
	Day-7		
N-13	20	20.83	0.68
N-14	22		
N-15	21		

N-16	20		
N-17	21		
N-18	21		
	Day-9		
N-19	38	36.5	0.95
N-20	37		
N-21	35		
N-22	36		
N-23	37		
N-24	36		

Table 4.14 Weight of skin for Diclofenac-Na (D) group in Day 2, 4, 7 and 9

Sample	Weight (mg) Day-2	Average	Standard Deviation
D-1	13	11.83	0.89
D-2	11		
D-3	11		
D-4	13		
D-5	12		
D-6	11		
	Day-4		
D-7	7	6.16	0.68
D-8	7		
D-9	6		
D-10	5		

D-11	6		
D-12	6		
	Day-7		
D-13	43	42.66	1.37
D-14	40		
D-15	44		
D-16	42		
D-17	44		
D-18	43		
	Day-9		
D-19	58	56.66	3.03
D-20	56		
D-21	52		
D-22	54		
D-23	61		
D-24	59		

Table 4.15. Weight of skin for Vitamin C (V) group in Day 2, 4, 7 and 9

Sample	Weight (mg) Day-2	Average	Standard Deviation
V-1	6	6.16	0.68
V-2	5		
V-3	7		
V-4	7		
V-5	6		

V-6	6		
	Day-4		
V-7	4	4.16	0.37
V-8	4		
V-9	5		
V-10	4		
V-11	4		
V-12	4		
	Day-7		
V-13	40	41	1.63
V-14	41		
V-15	44		
V-16	42		
V-17	40		
V-18	39		
	Day-9		
V-19	54	52.16	3.23
V-20	56		
V-21	47		
V-22	55		
V-23	49		
V-24	52		

Table 4.16. Weight of skin for Combination (C) group in Day 2, 4, 7 and 9

Sample	Weight (mg) Day-2	Average	Standard Deviation
C-1	12	12.33	0.74
C-2	13		
C-3	13		
C-4	12		
C-5	11		
C-6	13		
	Day-4		
C-7	7	6.5	0.5
C-8	6		
C-9	7		
C-10	7		
C-11	6		
C-12	6		
	Day-7		
C-13	45	44.83	0.68
C-14	44		
C-15	46		
C-16	45		
C-17	45		
C-18	44		
	Day-9		
C-19	61		

C-20	59	57.5	2.56
C-21	53		
C-22	57		
C-23	56		
C-24	59		

4.5 Wound Closure

Table 4.17 Wound closure for Negative (N) Control in day 2, 4, 7 and 9

Sample	Wound Closure (mm) Day-2	Average	Standard Deviation
N-1	4.32	4.32	0.05
N-2	4.33		
N-3	4.326		
N-4	4.23		
N-5	4.41		
N-6	4.31		
	Day-4		
N-7	3.646	3.54	0.12
N-8	3.621		
N-9	3.526		
N-10	3.623		
N-11	3.265		
N-12	3.561		
	Day-7		

N-13	3.646	3.42	0.21
N-14	3.621		
N-15	3.656		
N-16	3.213		
N-17	3.214		
N-18	3.215		
	Day-9		
N-19	0	0	0
N-20	0		
N-21	0		
N-22	0		
N-23	0		
N-24	0		

Table 4.18 Wound closure for Diclofenac-Na (D) in day 2, 4, 7 and 9

Sample	Wound Closure (mm) Day-2	Average	Standard Deviation
D-1	4.233	4.13	0.04
D-2	4.11		
D-3	4.121		
D-4	4.12		
D-5	4.13		
D-6	4.111		
	Day-4		

D-7	3.194	3.19	0.31
D-8	3.865		
D-9	2.882		
D-10	3.124		
D-11	3.012		
D-12	3.111		
	Day-7		
D-13	2.761	2.46	0.31
D-14	2.999		
D-15	2.452		
D-16	2.215		
D-17	2.256		
D-18	2.123		
	Day-9		
D-19	0	0	0
D-20	0		
D-21	0		
D-22	0		
D-23	0		
D-24	0		

Table 4.19. Wound closure for Vitamin C (V) in day 2, 4, 7 and 9

Sample	Wound Closure (mm) Day-2	Average	Standard Deviation
V-1	4.235	4.28	0.05
V-2	4.237		
V-3	4.356		
V-4	4.367		
V-5	4.256		
V-6	4.287		
	Day-4		
V-7	3.289	3.33	0.09
V-8	3.456		
V-9	3.267		
V-10	3.4566		
V-11	3.245		
V-12	3.267		
	Day-7		
V-13	3.278	3.28	0.009
V-14	3.29		
V-15	3.268		
V-16	3.278		
V-17	3.289		
V-18	3.298		
	Day-9		

V-19	0	0	0
V-20	0		
V-21	0		
V-22	0		
V-23	0		
V-24	0		

Table 4.20. Wound closure for Combination (C) in day 2, 4, 7 and 9

	Wound Closure (mm) Day-2	**Average**	**Standard Deviation**
C-1	4.023	4.03	0.007
C-2	4.035		
C-3	4.047		
C-4	4.038		
C-5	4.038		
C-6	4.037		
	Day-4		
C-7	3.102	3.05	0.04
C-8	3.023		
C-9	3.0234		
C-10	3.021		
C-11	3.12		
C-12	3.021		
	Day-7		

C-13	2.382	2.26	0.10
C-14	2.378		
C-15	2.352		
C-16	2.123		
C-17	2.135		
C-18	2.245		
	Day-9		
C-19	0	0	0
C-20	0		
C-21	0		
C-22	0		
C-23	0		
C-24	0		

4.6 Summary

Table 4.21 WBC Count (Average Values)

Day	Mice	WBC count (mm3)
Day 2	Negative	20325
	Diclofenac	14366.66
	Vitamin	18525
	Combination	12291.66
Day 4	Negative	14425
	Diclofenac	12150
	Vitamin	13050
	Combination	11316.66
Day 7	Negative	7000
	Diclofenac	4133.33
	Vitamin	5775
	Combination	3216.66
Day 9	Negative	5250
	Diclofenac	3125
	Vitamin	4566.66
	Combination	2500

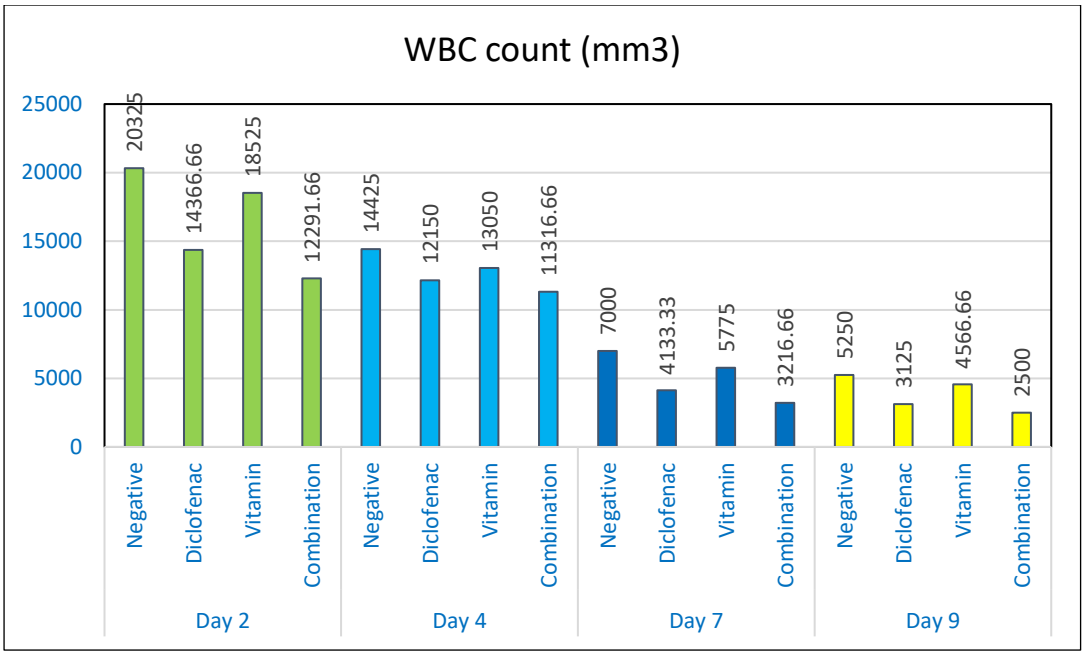


Figure 4.1 WBC Count of Average Value

Table 4.22 Total Protein (Average Values)

Day	Mice	Concentration (mg/dl)
Day 2	Negative	5.20
	Diclofenac	5.95
	Vitamin	5.86
	Combination	6.11
Day 4	Negative	5.10
	Diclofenac	6.56
	Vitamin	4.62
	Combination	7.10
Day 7	Negative	6.44
	Diclofenac	6.71
	Vitamin	6.46
	Combination	7.40
Day 9	Negative	5.41
	Diclofenac	6.71
	Vitamin	7.4
	Combination	8.94

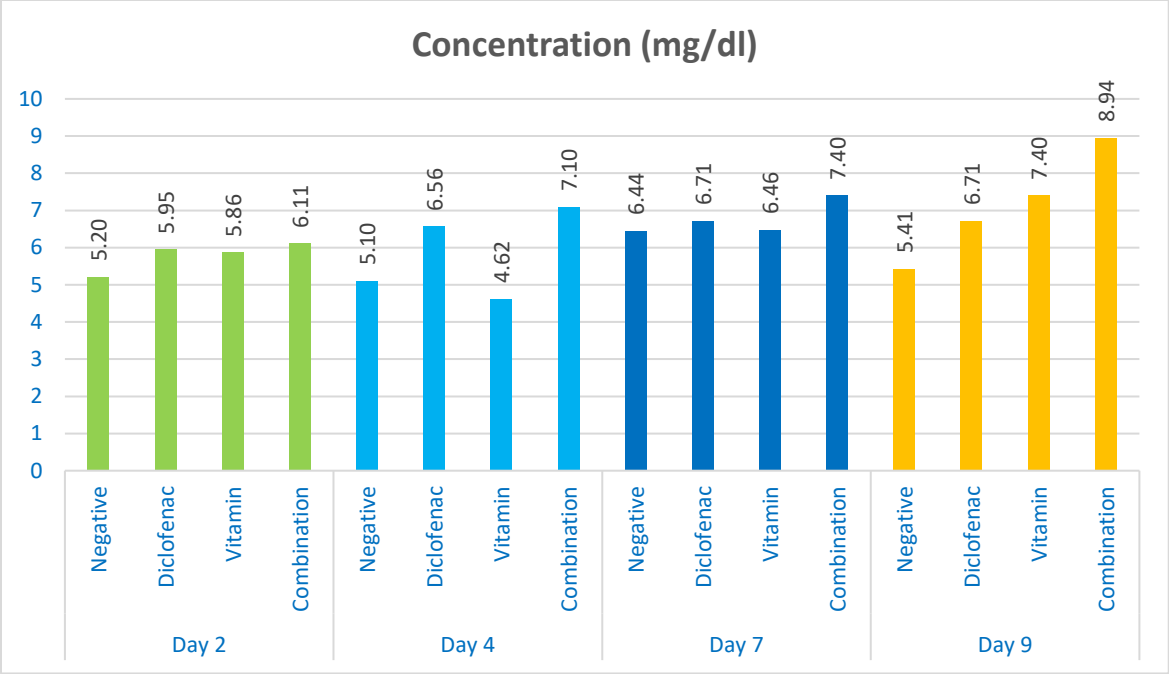


Figure 4.2 Total Protein Count of Average Value

Table 4.23 Total VEGF Concentration (Average Values)

Day	Mice	Concentration (mg/dl)
Day 2	Negative	11.64
	Diclofenac	25.60
	Vitamin	12.51
	Combination	26.22
Day 4	Negative	21.75
	Diclofenac	23.41
	Vitamin	9.04
	Combination	23.52
Day 7	Negative	10.18
	Diclofenac	10.81
	Vitamin	8.52
	Combination	10.5
Day 9	Negative	8.31
	Diclofenac	8.72
	Vitamin	8.31
	Combination	12.47

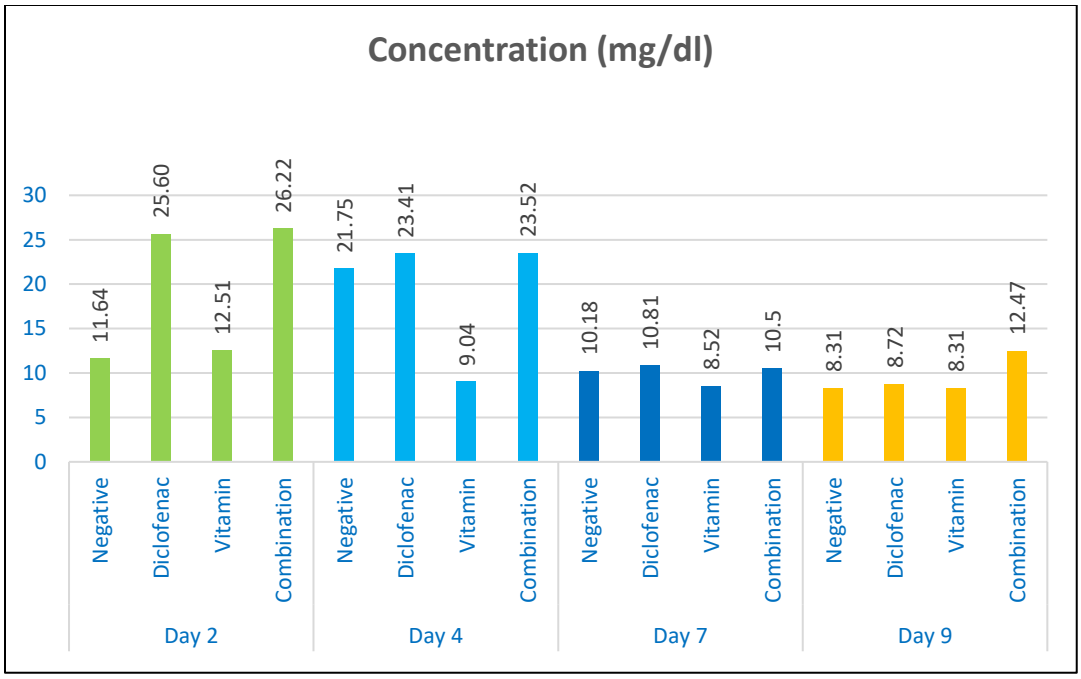


Figure 4.3 Total VEGF Concentration of Average Value

Highest reading was obtained from the first day of sampling with a gradual decrease. Diclofenac and combination therapy gave almost same results with combination therapy giving slightly higher results. Lowest concentration was found from the vitamin group.

Table 4.24 Weight variation (Average Values)

Day	Mice	Weight of skin (mg)
Day 2	Negative	6
	Diclofenac	11.83
	Vitamin	6.16
	Combination	12.33
Day 4	Negative	4
	Diclofenac	6.16
	Vitamin	4.16
	Combination	6.5
Day 7	Negative	20.83
	Diclofenac	42.66
	Vitamin	41
	Combination	44.83
Day 9	Negative	36.5
	Diclofenac	56.66
	Vitamin	52.16
	Combination	57.5

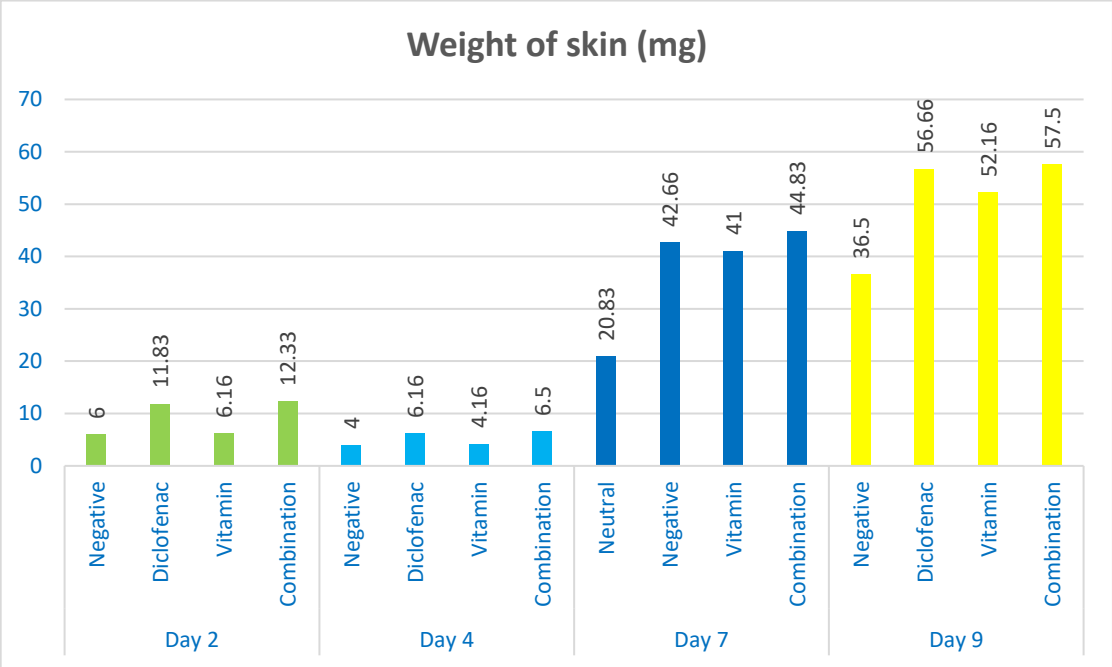


Figure 4.4 Weight of Skin of Average Value

Table 4.25 Wound Closure (Average Values)

Day	Mice	Wound closure (mm)
Day 2	Negative	4.32
	Diclofenac	4.13
	Vitamin	4.28
	Combination	4.03
Day 4	Negative	3.54
	Diclofenac	3.19
	Vitamin	3.33
	Combination	3.05
Day 7	Negative	3.42
	Diclofenac	2.46
	Vitamin	3.28
	Combination	2.26
Day 9	Negative	0
	Diclofenac	0
	Vitamin	0
	Combination	0

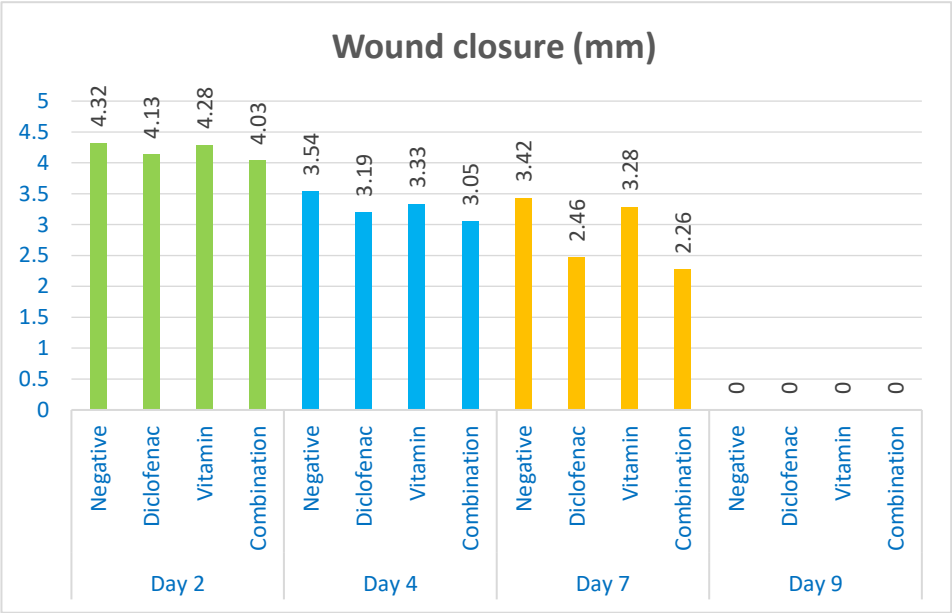


Figure 4.5 Wound closure of Average Value

Chapter Five: Discussion

The main objective of this study was to find out the effectiveness of the combination therapy of Diclofenac-Na and Vitamin C in wound healing on mice. To evaluate the effectiveness four groups were taken for the experiment. First group was negative control group where all the mice were treated only with normal saline. Followed by that another group was treated with Diclofenac-Na. Then next group was treated only with Vitamin C and finally, the last group was treated with the combination therapy of Diclofenac-Na and Vitamin C.

Wound healing mechanism comprises of three main stages. Initial stage in inflammation followed by that proliferation and finally regeneration. Inflammation phase starts with the activation of innate immune system by the migration of macrophage and neutrophils. This stage starts along with homeostasis. When the skin is injured several cells like keratinocytes, mast cell, macrophages comes to the wound site. After that in response to chemokines several complements and by product of bacterial degradation and neutrophils comes from circulation to wound area. On the activation of chemokines adhesion of neutrophil occurs on the wall of blood vessel. Macrophages plays an important role in inflammation. They contribute in the production of growth factors for proliferation stage. After inflammation next stage is proliferation. This stage mainly focuses on covering the wound surface and forming granulation tissue along with preparing vascular network again. For re-epithelization keratinocytes has to be migrated and proliferated. All the migrating cells released from collagenase and elastase. When all the cells adhere with the wound site migration stops and structures from. Followed by re-epithelization fibroblast migration occur. For this to be done platelet derived growth factor and transforming growth factor beta comes. After fibroblast migration formation of extracellular matrix starts. For forming the extra-cellular matrix collagens, adhesive glycoprotein and proteoglycan starts to work. Followed by that vascular network restores which is also known as angiogenesis. It is done by several growth factor platelet-derived growth factor (PDGF), vascular endothelial growth factors (VEGF), basic fibroblast growth factor (bFGF) and serine protein. After angiogenesis comes remodeling phase. This occurs after the cessation of granulation tissue development. In this phase the skin starts to become like original skin like prior wounding.

At first WBC counting has been done. In this case on day 2 highest number of WBC was found in negative control group. Followed by that Vitamin C then Diclofenac-Na and lowest number of

WBC was found in combination therapy. In wound healing initial stage was inflammation. Here number of WBC should have been highest but due to different therapy the number of WBC varies. The highest number of WBC was found in negative control group because no medication was given to that group. That's why that group of mice followed normal wound healing mechanism and released large number of WBC in accordance to initiate the inflammation stage. In Diclofenac-Na treated group the number of WBC was much less than negative control group because Diclofenac-Na is an anti-inflammatory drug for this reason migration of inflammatory cells are less in this case. Diclofenac-Na shows anti-inflammatory action by inhibiting COX pathways. Finally, in combination therapy WBC count is the lowest which was 12291.67. On day 4 number of WBC reduced from second day. Followed by that in day 7 and 9 number of WBC decreased rapidly. This has occurred because in the mechanism of wound healing inflammatory stage ceases within 1-3 days. After counting the WBC, it can be said that combination therapy gives better anti-inflammatory action as in all days of experiment WBC number is less in combination therapy. It has given the highest anti-inflammatory action even more than Diclofenac-Na. However, there isn't any significant action of Vitamin C in inflammation stage of wound healing. Despite that Vitamin C gives a synergistic effect along with Diclofenac-Na in combination therapy.

For proliferation stage total protein count was done. On day 2 protein count is highest in combination group and then in Diclofenac-Na. The lowest value was in negative control group. This shows that in combination group migration of growth factor was much higher than other groups and in negative control group the migration of growth factors and other proteins are significantly less. As day passes total protein concentration increases significantly because of initiation of proliferation stage. In combination the values on day 2, 4, 7, 9 were 6.11, 7.10, 7.40, 8.94 respectively. This is the highest value amongst all which proves the increased amount of protein around wound. Along with that the lowest value of protein was found in negative control group. This proves the lesser amount of protein was present there and ultimately healing was slower. This protein test was done to confirm the presence of growth factors. ELISA test was done for the confirmation of presence of VEGF during proliferation stage. During that test increased amount of VEGF was found on day 2 on each group. Among the four groups highest value was in combination therapy which represents the highest content of VEGF in combination group. On day 4, 7, 9 the values decrease in every group. But in every day of experiment the concentration of VEGF is higher in combination therapy. VEGF is a growth factor that releases when new vessel

growth is started. VEGF has a contribution in angiogenesis in both ways; directly and indirectly. Directly it effects on proliferation stage in the migration of endothelial cells and indirectly it effects on persisting vascular permeability on existing micro vessels. The main function of VEGF is to accelerate wound closure by mediating angiogenic activity during the proliferative phase of wound healing. In our experiment two 4.5mm diameter wound was created on the dorsal area of each mice with a punch. On day 2, 4, 7, 9 sizes of the wounds were measured. On day 2 decrease of the wound size were minimal on each group. Significant decrease on wound size was found in day 4 and day 7. Finally, on day 9 all the wound healed properly. Among all the groups combination therapy shows better healing regarding wound closure.

After concluding all the test parameters, it has concluded that among all the therapy combination therapy shows best action in every stage. Vitamin C basically works in collagenase production and Diclofenac-Na works in COX pathway. When both are given in combination they show synergistic effect. In a nutshell, the result of this experiment is that combination therapy is giving the best result for wound healing.

Table 5.1 Summary

Drug	**Day**	**WBC Count**	**Total Protein**	**Total VEGF Conc.**	**Weight of skin**	**Wound closure**
Diclofenac-Na	Day-2	14366.6667	5.955710956	25.60416667	11.83333333	4.1375
	Day-4	12150	6.567599068	23.41666667	6.166666667	3.198
	Day-7	4133.333333	6.717171717	10.8125	42.66666667	2.467666
	Day-9	3125	5.41996892	8.729166667	56.66666667	0
Negative	Day -2	20325	5.207847708	11.64583333	6	4.321
	Day-4	14425	5.107226107	21.75	4	3.540333
	Day-7	7000	6.442501943	10.1875	20.83333333	3.4275

	Day-9	5250	6.717171717	8.3125	36.5	0
Vitamin C	Day-2	18525	5.868686869	12.51388889	6.166666667	4.289666
	Day-4	13050	4.625874126	9.041666667	4.166666667	3.3301
	Day-7	5775	6.46969697	8.520833333	41	3.2835
	Day-9	4566.666667	7.405205905	8.3125	52.16666667	0
Combination	Day-2	12291.66667	6.116161616	26.22916667	12.33333333	4.036333
	Day-4	11316.66667	7.100621601	23.52083333	6.5	3.051733
	Day-7	3216.666667	7.402486402	10.5	44.83333333	2.269166
	Day-9	2500	8.947163947	12.47916667	57.5	0

Chapter Six: Conclusion

To conclude, the study shows alternative potential drug therapies for potentiating and mediating wound healing. The conventional agents for wound healing have many side-effects and there is harmful effect on long term use of these types of drugs. Also, these drugs slow down the wound healing process to an extent. So, to get rid of those problems establishing a new drug regimen has become necessary. In this study, antioxidant Vitamin C has been used to increase the therapeutic effects of conventional drug like Diclofenac. Antioxidants have actually potentiated the anti-inflammatory effects of the conventional anti-inflammatory drugs. They also work in the proliferation stage by initiating collagenase formation. There were some challenges to perform this study. We could not perform differential counting of WBC which could have helped us to know the presence of neutrophils and macrophage which would have helped us to understand specifically about the inflammation stage. We only measured VEGF in the proliferation stage that is one of the several growth factors, but we could have also measured EGF, TGF-β1 IGF-1 to properly understand the whole proliferation process. In the regeneration stage, we only measured the wound size however; we could have also performed histopathology so that we can understand the chemicals that have migrated to the wound site to form the new skin. However, we have tried to make the best use of our available facilities. We also have plans to carry on this work using different dose of this combination for seeing the wound healing effect of these combinations. We hope that the result from our study, even though in small scale, will contribute in bringing changes to the management of wound healing treatment.

Chapter Seven: Reference

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