THE EFFECTIVENESS OF NEGATIVEPRESSURE WOUND THERAPY IN INDUCING HYPOXIA COMPARED TOSTANDARD OCCLUSIVE DRESSING WOUND THERAPY IN THE HEALINGOF TRAUMATIC WOUNDS

DR. MATWA CHRISTOPHER ONDIEKI
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DECLARATION

This thesis dissertation is my original work and has not been submitted for degree award in any other university.

Candidate: **Dr. Matwa Christopher, Ondieki [BSc-Physio (hons); MBChB, UoN.]**

Registration Number: **H58/88012/2016**

Signature

25 / 06 20 21

Date (DD/MM/YY)

APPROVAL

This thesis has been submitted for examination with our approval as the University supervisors

Dr. Nangole Ferdinand W., [MBChB; Dip STI (UoN); MMed Surg. (UoN); FPRS (S.A);] FEBPRS (Brussels, Marseille).

Consultant Plastic Surgeon and Senior Lecturer,

Department of Surgery, Plastic Surgery Thematic Unit,

University of Nairobi

25/06/2021

Signature Date (DD/MM/YY)

Prof. Khainga Stanley O., [MBChB; MMed Surg (UoN); FCS (Plast); FCS (COSECSA).]

Consultant Plastic Surgeon and Senior Lecturer,

Chairperson of Plastic Surgery Thematic Unit, Department of Surgery,

University of Nairobi

25/6/2021

Signature Date (DD/MM/YY)

3. **Dr. Daniel Zuriel**

MBChB, MMED (Pathology) UoN, Diploma in Forensic Pathology, SA Board certified DermPath, EAMS-Frankfurt, and Senior Lecturer Anatomic Pathology

Signature._____ Date: 03/06/2021

DEDICATION

This thesis is dedicated to my mother Roseline Bochaberi and to the memory of my late father Ibrahim Ondieki for the vision they had for and hard work they put into raising and molding me.

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LIST OF ABBREVIATIONS

ECM: - Extra-cellular Matrix

ELISA: - Enzyme Linked Immune-sorbent Assay

FAP: - Fibroblast Activation protein

HIF: - Hypoxia Inducible Factor

HRP: - Horseradish Peroxidase

KAVI: - Kenya AIDS Vaccine Iniatiative

MMPS: - Matrix Metalloproteinases

NPWT: - Negative Pressure Wound Therapy

PBS: - Phosphate buffered saline

SODWT: - Standard Occlusive Dressing Wound Therapy

SPSS: - Statistical Package for Social Sciences

TMB: - Tetramethylbenzidine

VEGF: - Vascular Endothelial Growth Factor

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ABSTRACT

Background: Negative Pressure Wound Therapy has gained popularity as an effective adjunct in management of wounds. It accelerates wound healing by enhancing granulation tissue formation. Its mechanism of action was always postulated to increased perfusion and oxygenation to the wound. However, recent postulates seem to suggest that it reduces perfusion to the wound thus creating a hypoxic environment which stimulates vascularization and angiogenesis.

Objective: To establish the effectiveness of Negative Pressure Wound Therapy in inducing hypoxia compared to Standard Occlusive Dressing Wound Therapy in the healing of traumatic wounds.

Study Design: This was a randomized C;;;ontrolled trial

Settings: The study was undertaken at the Kenyatta National Teaching and Referral Hospital from the months of October 2020 to April 2021

Materials and Methods: The study participants were patients with traumatic wounds admitted in the surgical wards at the Kenyatta National Teaching and Referral Hospital and were divided into two groups where one group was subjected to Negative Pressure Wound Therapy and the other group subjected to Standard Occlusive Dressing Wound Therapy. The samples for analysis were obtained though punch biopsies of wounds at two sites; the wound edges and the wound bases and they were carried out at baseline day 0, day 3 and day 7 in each group. They were homogenized and stored at -80^0C until enough samples accumulated for analysis in a 96-well ELISA plate. ELISA assays for quantitative levels of Hypoxia Inducible Factor 1 alpha and Fibroblast Activation Protein was carried out to obtain optical densities which were converted to actual concentrations of the two elements through Standard Absorbance Curves.

Results: The concentration of Hypoxia inducible factor 1 alpha, both at the wound edges and wound bases, increased by 176% and 194% respectively from baseline to day 7 of therapy in wounds managed by Negative Pressure Wound Therapy. A slight concentration increase of 23% and 21% (respectively) was noted in wounds subjected to Standard Occlusive Dressing Wound Therapy. Similarly, the concentration of Fibroblast Activation Protein increased both at the wound edges and wound bases by 124% and 146% respectively from baseline to day 7 of therapy in wounds treated by Negative Pressure Wound Therapy. A slight increase by 37% in wounds subjected to Standard Occlusive Dressing Wound Therapy was noted at both the wound edges and wound bases.

Discussion: Our findings are additive to initial studies that demonstrated reduced blood flow and reduced transcutaneous partial pressure of oxygen in wounds subjected to Negative Pressure Wound Therapy. Our study further demonstrates the molecular mechanisms that result from the hypoxia induced in wounds managed by Negative Pressure Wound Therapy and the molecules generated enhance granulation tissue formation.

Conclusion: Hypoxia induction should be recognized as a mechanism of action through which Negative Pressure Wound Therapy through increases wound healing by enhancing granulation tissue formation.

CHAPTER ONE:

INTRODUCTION

1.1.1. WOUNDS AND WOUND HEALING

Wounds are tissue injuries characterized by a break or breaks in the normal continuity of the skin. The etiology is by pathologic processes within the body or external to it and trauma is one of the major external wound etiology (1). Deeper wounds expose underlying structures like blood vessels, nerves, muscle and bone predisposing them to complications like hemorrhage, inflammation, infection, sepsis and necrosis. The management of wounds with these complications can be clinically challenging (2).

A complex series of molecular events in succession and overlapping phases take place as the body attempts to repair the wound once an injury occur (3). The first phase is the inflammation phase which commences immediately after an injury and lasts 24 to 48 hours. It is characterized by vasoconstriction, platelets recruitment to form a primary plug and activation of the coagulation cascade to form a secondary (fibrin) plug in order to achieve homeostasis. Consequently, the platelets recruit inflammatory and immunity cells into the zone of injury to proliferate and secrete chemicals that stop and prevent infections (4). The second phase is the proliferation phase which commences after 24 hours and lasts up to 2 weeks. It is characterized by resolution of inflammation and recruitment of synthetic cells. Fibroplasia increases lay down of extracellular matrix (ECM) through collagen synthesis and matrix metalloproteinases (MMPs) degrade the overlying fibrin clot to create room for migration and mitogenic stimulation of endothelial progenitor cells that result in increased angiogenesis thus increased granulation tissues formation (5). The third phase and final phase is the remodeling phase which commences 2 to 3 weeks after injury and can last several months. It is characterized by ECM remodeling whereby collagen type III gets replaced by collagen type I, increased tensile strength through myofibroblast activity and epithelialization through keratinocyte differentiation, migration and proliferation. These processes are also facilitated by the proteolytic activity of MMPs and apoptosis. The tensile strength increases exponentially and reaches approximately 80% by the end of 90 days (6).

1.1.2. GRANULATION TISSUE

Formation of granulation tissue in wounds is the sign of normal and progressive wound healing (7). This phenomenon is important especially in wounds healing by secondary intention. It also the hallmark of readiness of wound for closure with skin grafts (8-9).Granulation tissue is a rapidly forming stromal tissue over a healing wound that comprises of new blood vessels (capillaries), inflammatory cells and ECM embedded in loose connective tissue (10). The main feature is sprouting of the new capillaries that protrude to the surface of the wound to produce minute red granules hence the granular wound appearance (11).

This process begins as early as the second day after injury and normally lasts 2 - 3 weeks. It coincides with the proliferative phase of wound healing. It is formed through mechanisms like

endothelial cell proliferation in vasculogenesis, fibroblast proliferation and extra-cellular matrix formation and lay down (12).

1.1.3. MANAGEMENT OF WOUNDS

For wounds to heal properly, they have to successfully transition through all the three phases of wound healing. Therefore, meticulous clinical management of wounds is paramount more so when managing complex wounds. The DIME principle which represents Debridement of devitalized or necrotic tissue, control of Inflammation and/or Infection, balance of wound moisture and preparation of wound Edges to maximize healing has been universally adopted as the governing principle in wound management by clinicians (13).

Several methods of wound treatment or therapy are available and they are tailored to address aspect(s) of the DIME principle. Occlusive Dressing is one of the methods of wound therapy which protects and maintains a sterile wound environment. A bare minimum is use of Standard Occlusive Dressing Wound Therapy (SODWT) which is effective in controlling inflammation, infection and moisture balance. In simple wounds, SODWT is a very effective method of wound management and wound therapists achieve SODWT by completely covering the wound (dressing) using a paraffin impregnated gauze as the primary dressing and secondary plain gauze applied on top (14-15)

However, in complex and extensive wounds, SODWT may not be adequate for effective wound management. This has necessitated use of adjunctive therapies to hasten the process of wound healing. One of the adjunctive therapies applied is Negative Pressure Wound Therapy (NPWT) or vacuum assisted closure (VAC) which is a mechanical adjunct (16).

1.1.4. NEGATIVE PRESSURE WOUND THERAPY

NPWT is a mechanical and clinical wound therapy technique used to hasten wound healing. It is very effective in enhancing wound healing by controlling inflammation and infection, balancing wound moisture and decreasing wound size through wound edge contraction (17). These effects commence after application of NPWT which stimulate a series of bio-molecular reactions locally within the wound (18).

The basic components of NPWT include porous materials which is used to fill the wound. The commonly used materials include foam made of polyurethane (ether or ester) or polyvinyl alcohol and gauze which are moistened with or without an antimicrobial. After application of the porous material, the wound is sealed with an adhesive clear drape in which an opening is created to attach a drainage port at a central point. A tubing with connectors is used to connect the drainage port to a vacuum pump whose modes of action can vary between continuous, intermittent and variable suction modes at different suction pressures (19). Modifications have been applied to improve NPWT including continuous irrigation especially with an antimicrobial and oxygenation of the wound for instance RNPWT as described by Moris Topaz (20).

1.1.5. MECHANISM OF ACTION OF NPWT

Application of NPWT as a mechanical adjunct in wound management results in several local changes to the wound and its immediate surrounding tissues. Micro-deformation is one of the

changes that occur within the wound. This refers to changes at cellular level due to mechanical forces of NPWT on wound surface. These forces comprise of compression, shear, tension and hydrostatic forces and the cell membrane transmits the mechano-transduction of these forces to intracellularly to activate the cytoskeleton (21-22).

The activated cytoskeleton in turn causes a downstream intra-cellular signaling cascade and a number of target molecules get activated with resultant secondary effects. These secondary effects include promotion of angiogenesis, vasculogenesis, increased cell division and elaborate production of local growth factors (23).

Increased angiogenesis has been shown to involve vast synthesis of VEGF. Studies comparing levels of VEGF in wounds managed with NPWT to levels of VEGF in wounds managed with standard conventional occlusive wound dressings have shown that NPWT treated wounds have higher levels of VEGF which is in tandem with increased granulation tissue formation and wound healing (24-25).

Other local changes that are effected by NPWT include macro-deformation in which the suction force created by NPWT over the wound surface causes the foam pores to collapse in the course of transmission of the negative pressure from vacuum machine to wound surface. This collapse of pores results to deformational forces on wound edges inducing wound shrinkage thus the wound contracts reducing its primary size (26-27).

NPWT also increases drainage of excessive interstitial fluid which in effect reduces wound and surrounding tissue edema. The elimination of this occlusive tissue pressure causes increased lymph and blood flow in effect leading to increased delivery of oxygen, nutrients and antibiotics (28).

Lastly, NPWT causes stabilization of the wound environment through local thermoregulation, reduction of contamination and reduction of wound bio-burden, maintenance of sterility and regulation of wound moisture (29).

LITERATURE REVIEW

1.2.1. HISTORY OF NPWT

Archaic forms of NPWT were practiced before and after medieval ages. The use of cups, oral suction and syringes has been well documented. In ancient Greece, Hippocrates described application of cups to poisoned wounds while in ancient Rome, Celsus performed ligatures above wound and then applied cup over the wound. Another Greek known as Machaon was made to orally suck the wounds of king Menelaus. In ancient Rome, three individuals: Psyli, Marsi and Ophegenes are said to have been deployed to orally suck wounds of Roman soldiers as they were considered to possess hereditary powers to heal wounds (30).

Later on in the 18th century, Dr. Anel developed triangular based suction syringes and Dr. Francis Fox in 1821 developed a concept of glass leech. In 1890, Dr. Gustav Bier developed a method involving cupping together with tubing and a bulb to induce negative pressure of wounds. Dr. Barry around this period while experimenting on rabbits and dogs did a controlled study by injecting strychnine to wounds and applied cups (31).

These archaic and un-orthodox methods of NPWT were forgotten in history until Morykwas and Argenta modified and redesigned NPWT in 1997. They carried out animal experiments and clinical trials and were able to demonstrate the effectiveness of NPWT in enhancing wound healing. They went further ahead to study the mechanisms through which NPWT exerts these benefits. They found out that NPWT was effective in increasing blood flow to wounds, stimulating granulation tissue formation, clearing wound bacteria and enhanced survival of random flaps. It also markedly reduced edema around and in the wound (32-33).

Increased blood flow and oxygen to wounds due to NPWT was a concept held as true until 2009 when Kairinos carried out series of clinical researches to study the effect NPWT on perfusion (blood flow) to wounds (34). He established that there was a reduction in blood flow to wounds subjected to NPWT. The reduced perfusion was found to be worse in wounds with compromised vascularity and also in circumferential wounds. Thus he cautioned against the use NPWT in these wounds which could further complicate the wounds (35). Other studies done in South Korea on trans-cutaneous measurement of partial pressure of oxygen in 2014 on healthy feet and 2016 in diabetic feet demonstrated a reduction in tissue perfusion (36-37).

1.2.2. HYPOXIA AND WOUND HEALING

Once tissues are injured, they are generally thrown into a state of hypoxia because of initial hemorrhage and vasoconstriction and these two events compound the reduction of blood flow to wounds. Hypoxia in wounds has always been associated with poor wound healing and development of chronic ulcers (38). However, recently it has been postulated that acute wound hypoxia is beneficial and hastens wound healing because it has been found out that it increases release of peptides that stimulate angiogenesis, modulate inflammation and enhance lay down of ECM. Chronic wound hypoxia, on the other hand, has been postulated to lead to development of the non-healing chronic wounds because it creates a state of tissue starvation of vital elements for growth, proliferation and differentiation (39).

Formation of new blood vessels in healing wounds, collagen deposition, epithelialization, fibroplasia and resistance to infection are key processes required for normal wound healing and all of them have been shown to require oxygen according to earlier studies (40-41) However, recently with increased studies on the survival of malignant tumors and development of keloids, both have been shown to thrive by inducing hypoxic cellular environment (42-43). This hypoxic cellular condition leads to increased angiogenesis because the hypoxia stimulates synthesis, release and accumulation of hypoxia sensitive factors. These hypoxia sensitive factors or elements partly work by activating the transcription of angiogenesis related genes. Increase synthesis of the angiogenesis stimulating factors results in formation of new blood vessels, therefore increasing metabolite delivery to the keloids and the malignant cells (44-46). Thus, acute hypoxia has been demonstrated to be a key factor in cellular proliferation and survival as is required in wounds.

1.2.3. ASSESSMENT OF WOUND HYPOXIA

Hypoxia sensitive elements have been well studied and researched. These elements accumulate in hypoxic cellular environment. Once normoxic conditions are re-established, these factors get broken down and their synthesis inhibited effectively reducing their levels. One of the well-researched hypoxia sensitive elements is Hypoxia Inducible Factor 1-alpha (HIF-1α). The Nobel Prize of 2019 in Medicine and Physiology was awarded to three individuals: Ratcliffe, Kaelin and Gregg; for their exemplary discovery of how cells adapt to hypoxic conditions (47). The other factor sensitive to hypoxia is Fibroblast Activation Protein (FAP).

1.2.3.1. HYPOXIA INDUCIBLE FACTOR 1-ALPHA

One of the hypoxia sensitive factors that have been widely studied is the transcriptional factor HIF-1α. It increasingly gets expressed and accumulates in cells subjected to hypoxia as the cells attempt to adapt to the hypoxic environment (48). Accumulation of HIF-1α in cells has been shown to cause increased transcription of angiogenesis related genes: - Vascular Endothelial Growth Factor (VEGF) gene, erythropoietin (EPO) gene and glycolytic enzyme gene - PDK1 (49; 50; 51). Vital in the process of angiogenesis is the VEGF gene that leads to synthesis of VEGF which is a key molecule in the synthesis of new blood vessels. This is an important event in granulation tissue formation during wound healing (52-53).

HIF-1α is one of the subunit of HIF-1 which is a hetero-dimeric protein. The other subunit is HIF-1β. Expression of HIF-1α is oxygen dependent whereas the expression of HIF-1β is constitutive (54). During cellular hypoxic conditions, the levels of HIF-1α increase exponentially. When the cellular environment becomes normoxic, oxygen dependent ubiquitination and proteasomal degradation processes get activated and target HIF-1α which quickly get degraded and levels reduce markedly (55). In this normoxic cellular condition, oxygen molecules activate deoxygenases that in turn hydroxylate HIF-1α on two proline residues. After hydroxylation, HIF-1α binds to von Hippel-Lindau protein which marks it for ubiquitination and subsequent proteasomal degradation (56).

Therefore, HIF-1α can be used indirectly as an endogenous marker of hypoxia to measure levels of cellular and tissue hypoxia (57). During hypoxic cellular conditions, inhibition of degradation of HIF-1α occurs and causes this hypoxia sensitive transcription factor to stabilize. HIF-1α

moves to the nucleus, in its abundance, and binds to its β counterpart subunit to complete the hetero-dimeric structure. HIF-1 then binds to hypoxia response element to activate hypoxia sensitive genes especially VEGF gene (58).

1.2.3.2. FIBRO-BLAST ACTIVATION PROTEIN

During the process of wound healing, proteolytic degradation of components of the ECM by proteolytic enzymes is one of the key processes especially in granulation tissue formation. These proteolytic enzymes are released mostly by matrix metalloproteinases (MMPs) and they activate or stimulate the release growth factors from ECM to enhance angiogenesis. Some of them also act directly on ECM to facilitate angiogenesis (59).

One such proteolytic enzyme is FAP which has both dipeptidyl peptidase and collagenolytic properties capable of degrading gelatin and type I collagen. It is a type II integral membrane protein of the serine protease family. It is not expressed in most adult mesenchymal cells but has increased physiologic expression in healing wounds and pathologic expression in malignant tissues (60). Hypoxia has been shown to a potent stimulus for FAP release (61).

The levels of FAP have been shown to parallel the amount of granulation tissue formed (62-63). Therefore, FAP can be used as an indirect biomarker to assess amount of granulation tissue formed during wound healing (64).

We could not find any published study throughout our study research that had been undertaken to establish the effect of NPWT on expression of FAP in healing of wounds.

In summary, the quantitative assay of these two hypoxia sensitive elements can indirectly be a measure of the status or level of wound hypoxia.

CHAPTER TWO:

2.1.1. RATIONALE AND JUSTIFICATION FOR THE STUDY

There is availability of a variety of adjunctive therapies used in wound management and they include: hyperbaric oxygen therapy, hydrotherapy, ultrasound stimulation, NPWT, compression dressing therapy, growth factors and electro-stimulation. It has been demonstrated that NPWT is the adjunct commonly used and applied for wound therapy more than the other locally available adjuncts (65).

Limited human studies have been conducted to establish the mechanism of action through which NPWT enhances wound healing in humans (66; 67).

The response of human wounds to NPWT by physiological nature is different from animal response to NPWT (68).

There are inconsistent findings in studies done to effects seen in wounds in response to application of NPWT. This is especially with regards to blood flow to the wounds where Morykwas and Argenta demonstrated an increase whereas Kairinos and South Korea studies demonstrated a decrease in blood flow and tissue oxygen partial pressure.

Throughout my extensive search for published articles, none could be found that established the effect of NPWT on production of FAP in healing of wounds.

This study aims to establish whether NPWT induces wound hypoxia as a mechanism of action through which it enhances healing of traumatic wounds. Hypoxia levels in wounds will be estimated indirectly by measuring levels of HIF-1α and FAP in wounds treated with NPWT and then compared to wounds treated with SODWT.

2.2.1. Study Question

Is Negative Pressure Wound Therapy more effective in inducing wound hypoxia in comparison to Standard Occlusive Dressing Wound Therapy in the healing of traumatic wounds?

2.3.1. Null Hypothesis

Negative Pressure Wound Therapy is not more effective than Standard Occlusive Dressing Wound Therapy in inducing wound hypoxia during the healing of traumatic wounds.

2.4.1. Alternative Hypothesis

Negative Pressure Wound Therapy is more effective than Standard Occlusive Dressing Wound Therapy in inducing wound hypoxia during the healing of traumatic wounds.

2.5.1. Broad Objective

To establish the effectiveness of Negative Pressure Wound Therapy in inducing hypoxia compared to Standard Occlusive Dressing Wound Therapy in the healing of traumatic wounds.

2.5.2. Specific Objectives

1. To determine the quantitative change in levels of HIF-1α in traumatic wounds subjected to NPWT compared to wounds subjected to SODWT.
2. To determine the quantitative change in levels of FAP in traumatic wounds subjected to NPWT compared to wounds subjected to SODWT.
3. To establish the relationship between expression of HIF-1α at the wound edge and expression of HIF-1α at the wound base in traumatic wounds managed by NPWT
4. To establish the relationship between expression of FAP at the wound edge and expression of FAP at the wound base in traumatic wounds managed by NPWT
5. To establish the relationship between the effect of NPWT on expression of HIF-1α and the effect of NPWT on expression of FAP in traumatic wounds.

CHAPTER THREE:

MATERIALS AND METHODOLOGY

3.1.1. STUDY AREA

This study was conducted at the Kenyatta National Teaching and Referral Hospital (KNH) in the surgical wards. This hospital is a level VI tertiary institution that serves as a National Referral and Teaching facility. It is located within Nairobi, the capital city of Kenya and is approximately 5 kilometers from the city center.

3.2.1. STUDY DESIGN

This was a randomized control trial (RCT).

3.3.1. STUDY POPULATION

The study participants were patients admitted at KNH in various surgical wards with traumatic wounds. Both male and female gender, children and adults were included without bias in the study.

3.4.1. INCLUSION CRITERIA

Following careful reviews and considerations, the following the criteria were used to determine participants to be included into the study:

- Patients with traumatic wounds only. This was to eliminate any bias that comes with wounds of different etiologies. The second reason was majority of patients present with traumatic wounds thus sufficient numbers for this study.
- Patients under inpatient care. This was to ease patient tracking and also NPWT is ideal in inpatient setting for continued monitoring.
- The inclusion into either experimental intervention (NPWT) or control (SODWT) group was subject to individual consent upon agreeing to participate in the study.
- Both male and female patients with traumatic wounds were included in this study

3.5.1. EXCLUSION CRITERIA

After careful reviews and considerations, the following criteria were used to exclude patients from the study (69):

- Patients who declined to consent after selection or patients who declined to be selected to participate in the study. It is important to note that although these patients were excluded from the study, they continued to receive their routine wound care as per prescription by various ward doctors.
- Elderly patients above 65 years were excluded from the study because of confounding factors that come with aging and degeneration whereby they result in variable outcomes in wound management. These factors include vascular changes like sclerosis, skin atrophy and other systemic degenerative disorders.
- Patients with wounds properties contraindicated for NPWT were also excluded from this study. These factors complicate wounds further when subjected to NPWT. They include:

- Necrotic tissue in the wound until after debridement,
- Untreated osteomyelitis,
- Malignant wounds,
- Unexplored non-enteric fistula,
- Exposed delicate structures – nerves, vessels and anastomotic sites and
- Patients with increased risk of bleeding – coagulopathy and anticoagulant therapy because these wounds were bound to be complicated with NPWT.

3.6.1. VARIABLES

3.6.1.1. INDEPENDENT VARIABLES

a. Male or female gender
b. Age
c. Wound size
d. Wound site or location
e. Traumatic wound etiology

3.6.1.2. DEPENDENT VARIABLES

a. HIF-1α quantitative levels
b. FAP quantitative levels
c. Wound hypoxia

3.7.1. SAMPLE SIZE

The following factors were considered during sample size calculation:

Previous studies by Erba *et al* and Grimm *et al* established that NPWT is more effective than SODWT in enhancing wound healing through increased stimulation of granulations tissue formation. Therefore this study was taken as a statistically superior trial because NPWT was the intervention. The α for null hypothesis (H_0) was taken to be 0.05 and the β value for alternative hypothesis (H_a) was taken to be 0.20 at a power of 0.80 thus H_0: $T - S = \delta$, H_a: $T - S > \delta$ and $Z = (d - \delta)/sd$ whereby:

- T is NPWT
- S is SODWT
- δ is clinically admissible margin of superiority
- d is the effectiveness difference between T and S
- SD is the standard error of d
- Z is obtained from z-tables and obeys the standard normal distribution.

This study comprised of two groups (an intervention group and a control group) with each having an equal number of study subjects and involves measurement of level of HIF-1α and FAP in digression from baseline levels pre-intervention in both groups. This made the study be of

continuous variable and for statistically superior design; the following formula was used to calculate the sample size:

$$N = 2(\{Z_{1-\alpha/2} + Z_{1-\beta}\}/\delta) \times S^2$$

Whereby:

- N was the size per group
- S^2 was the polled standard deviation of both comparison groups

From previous studies (Erba *et al* and Grimm *et al*) δ is 4 and S is 6 thus:

$$N = 2 \times (\{1.96 + 0.845\}/4) \times 6^2 = 35.406$$

Therefore each group comprised of 36 subjects and total sample size for this RCT was made up of 72 participants (70-71).

3.8.1. SAMPLING METHOD

Patients with traumatic wounds were identified through hospital and/ or ward admission records. Through a voluntary and non-coerced approach, these patients with traumatic wounds were selected to participate in the study and using simple random selection criteria. Those who gave consent to participate in the study were recruited and they were assigned tracking numbers irrespective of gender or age. This was done taking into consideration the inclusion and exclusion criteria for identification purposes and randomly assigned into either experimental intervention (NPWT) or control (SODWT) group. These patient tracking numbers served to aid patient follow-up and protection of identity of the patients (confidentiality), as recorded in data collection sheet (APPENDIX A) and consent form (APPENDIX B).

3.9.1. RECRUITMENT AND CONSENT

After agreeing to participate in the study voluntarily, each of the participants signed consent forms individually and allocated a tracking number as detailed in APPENDIX B. The consent forms were availed both in English and Kiswahili languages as detailed in APPENDIX B. No real or official names of the participants were recorded in the consent forms.

3.10.1. ETHICAL CONSIDERATIONS

Approval for this study was requested from and granted by the KNH-UON ERC (APPENDIX C). Permission to engage surgical patients in the wards as study participants was also requested and granted by the KNH administration for commencement of the study (APPENDIX D).

After agreeing to consent to the study, the participants were given the liberty to withdraw from participating in the study as per their wish and any time in the course of the study. This was based on the fact that the patients' participation was voluntary and there was no expected monetary compensation or gain. This was especially applicable to the control or comparative group of patients who were subjected to SODWT as they were not directly having the benefits of NPWT.

3.11.1. CONFIDENTIALITY

The names or in-patient hospital number of participants were not to be recorded on the data collection tools. Instead they were assigned a research tracking number.

An inventory of participants tracking system was kept by the principal investigator.

Confidentiality of the clinical information of the participants was ensured at all stages of the study research.

Collected data were kept safely by the principal investigator.

3.12.1. PARTICIPANTS' AUTONOMY, BENEFICENCE AND NONMALEFICENCE

Written consent was sought from the participants who had attained 18 years and above.

Participants were allowed to freely withdraw from the study at any stage if they chose to.

No extra cost unrelated to the management of the participant was incurred purely for the purposes of the study

No coercion or persuasion was used to persuade participants to take part in the study and those who decline consent had their choice and views given due respect.

No material gain was extended to participants.

3.13.1. DATA COLLECTION METHODS

Data from patients were obtained through wound punch biopsies and the wound samples converted to homogenate solutions that were ELISA assayed to determine quantitative levels of HIF-1α and FAP.

3.13.1.1. WOUND DRESSING MATERIALS

NPWT equipment was obtained from Smith & Nephew Public Limited Company, United Kingdom through a local agent. They included a vacuum pump, connecting tubing, valve seal, foam dressings and adhesive sterile clear drapes.

The SODWT material used was Bactigras ® chlorhexidine gauze dressing from Smith and Nephew Limited Company, United Kingdome also through a local sales agent.

3.13.1.2. ELISA ASSAY MATERIALS

HIF-1α ELISA assay kits and FAP ELISA assay kits (Invitrogen^{TM} ELISA kits for HIF-1α and FAP assay by Thermo Fisher Scientific)were purchased and shipped from the United Kingdom through a local sales agent.

A hand held tissue homogenizer (Bio-Spec 985370-07 Tissue-Tearor Homogenizer) was also purchased online and shipped from the United States of America.

3.13.1.3. WOUND BIOPSY PROCEDURE

- **Baseline Day 0**: The selected participants were briefed on the biopsy procedure that entailed cleaning the wounds with 0.9% normal saline solution and then infiltration

of a local anesthetic solution (adrenaline-lignocaine solution made up of 1:200,000 adrenaline and 2% lignocaine in the ratio of 1:20mls respectively). After 5 minutes following the local anesthetic infiltration, punch biopsies using a 4mm punch biopsy devices were performed at two locations of the wound, namely the wound edge and wound base. Pressure was applied till homeostasis was achieved. The participants selected for NPWT had initiation of the VAC therapy with continuous mode of negative pressure set at -120mmHg. The other participants selected for SODWT had their wounds dressed with bactigras and plain gauze.

- **Day 3 after initial therapy**: The old dressings were removed and the wounds cleaned with 0.9% normal saline solution. The local anesthetic agent was infiltrated and allowed to settle for 5 minutes. Repeat punch biopsies were done adjacent to initial biopsy sites both at the wound edge and wound base. To achieve homeostasis, pressure was applied. NPWT was reapplied to participants in the experimental intervention group and bactigras with plain gauze re-applied to the control or comparative group.
- **Day 7 after initial therapy**: The old dressings were removed and wounds cleaned with 0.9% normal saline solution. The local anesthetic agent was infiltrated and allowed to settle for 5 minutes. Repeat punch biopsies were done adjacent to initial biopsy sites both at the wound edge and wound base. To achieve homeostasis, pressure was applied.

3.13.1.4. BIOPSY PRESERVATION AND TRANSPORTATION

Immediately after the wound biopsy samples were obtained, they were put into tissue sample vials which were put into a cool box with ice packs. The samples were immediately transported to KAVI laboratories for homogenate preparation within 2 hours of collection.

3.13.1.5. HOMOGENATE PREPARATION

The tissue biopsies were rinsed in ice-cold 1xPBS to remove excess blood. Each of the biopsies was weighed and using the ratio of tissue to homogenizing solvent (1xPBS) of 10mg per 1mL of ice-cold 1xPBS, the biopsies were crushed using a hand held tissue homogenizer (BioSpec 985370-07 Tissue-Tearor Homogenizer) to make a 10% homogenate solution.

The homogenate solutions were stored overnight at -200C and the following day two freeze-thaw cycles were performed to break cell membranes. The homogenates were then centrifuged at 5000xG for 5 minutes and stored at -800C until a time adequate number of biopsies had been collected and processed.

3.13.1.6. ELISA ASSAY PROCEDURES

After obtaining the Invitrogen™ ELISA kits for HIF-1α and FAP assay by Thermo Fisher Scientific, they were transported in cool boxes and stored at 2^0C and -20^0C respectively.

3.13.1.6.1. HIF-1α ASSAY

The standard operational procedure (SOP) detailed in the insert brochure with each kit (APPENDIX E) was followed in preparation of samples, reagents and standards. The assay procedure was done by preparation of 96-well plates each with 8 standards to derive standard

curve and 40 samples to obtain optical densities (OD) also detailed in the SOP insert brochure. The standards and samples were done in duplicate wells.

Immediately after stop solution was applied, the OD values were obtained through Tecan® infinite m-200 ELISA absorbance micro-plate reader where the absorbance was set at 450nm and 550nm. The raw values obtained were recorded on a Microsoft Excel Spreadsheet.

To obtain the concentrations of HIF-1α, the OD value of the 8th standard which was a blank and served as the zero standards (0 pg/mL) was subtracted from rest of all OD values to eliminate background absorbance. This was done to derive corrected OD values. The corrected OD values of standards were used to plot a scatter graph against their known HIF-1α concentrations and a formula generated to convert the rest of the OD values of samples to concentration of HIF-1α in pg/mL. The higher OD values were omitted in graph formulation to in order to increase precision to recorded sample OD values. This process was repeated for each ELISA kit utilized.

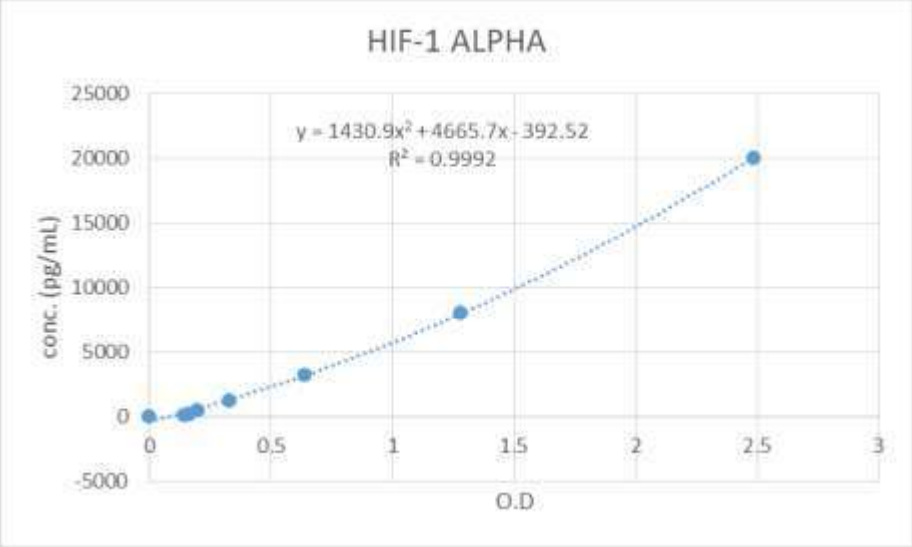

Figure 1 Scatter graph of HIF-1a ODs obtained from Standards to calculate conc. in pg/mL

3.13.1.6.2. FAP ASSAY

The standard operational procedure (SOP) detailed in the insert brochure with the kit (APPENDIX F) was followed in preparation of samples, reagents and standards. The assay procedure was done by preparation of 96-well plates each with 8 standards to derive standard curve and 40 samples to obtain optical densities (OD) also detailed in the SOP insert brochure. The standards and samples were done in duplicate wells.

Immediately after stop solution was applied, the OD values were obtained through BioTek® ELx-808 ELISA absorbance micro-plate reader where the absorbance was set at 450nm. The raw values obtained were recorded on a Microsoft Excel Spreadsheet.

To obtain the concentrations of FAP, the OD value of the 8th standard which was a blank and served as the zero standards (0pg/mL) was subtracted from rest of all OD values to eliminate background absorbance. This was done to derive corrected OD values. The corrected OD values of standards were used to plot a scatter graph against their known FAP concentrations and a formula generated to convert the rest of the OD values of samples to concentration of FAP in pg/mL. The higher OD value of the first standard well was un-recordable in all kits thus it was omitted in graph formulation. This process was repeated for each ELISA kit utilized.

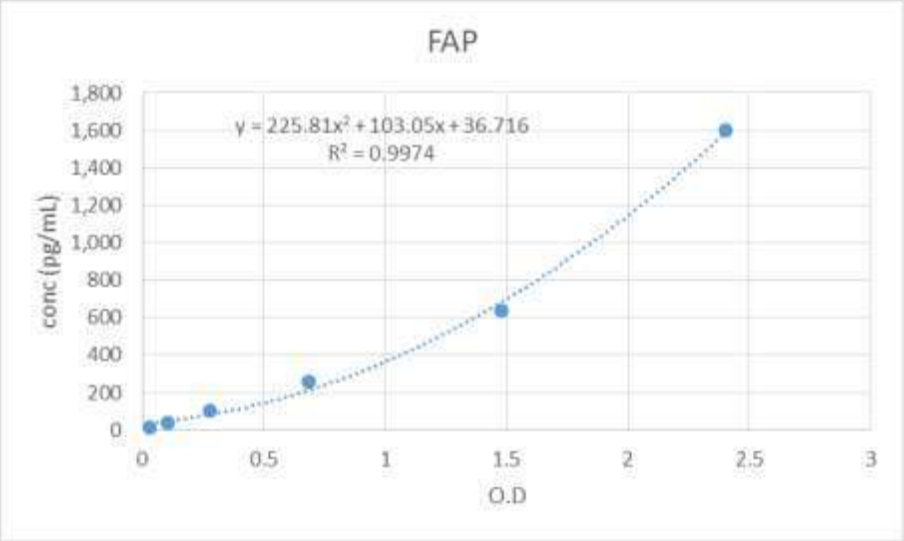

Figure 2 Scatter graph of FAP ODs obtained from Standards to calculate conc. in pg/Ml

3.14.1. DATA ANALAYSIS

After obtaining the quantitative results (concentrations in pg/mL) from ELISA assays, the results were transferred into Microsoft excel spreadsheet as raw data.

The raw data collected were put into SPSS version 24 where descriptive statistics, frequencies and cross tabulations were derived.

- Mean percentages and standard deviations of changes in FAP and HIF-1α were calculated for both NPWT and SODWT groups.
- Statistical significant differences between HIF-1α and FAP levels were analyzed using variance (pooled) T-test.
- A *p*-value of ≤ 0.05 was considered significant at 95% confidence interval at which point null hypothesis will be rejected.
- The findings are presented in tables and figures.

3.15.1. BENEFITS OF THE STUDY

Participants who underwent NPWT had accelerated wound healing without incurring costs of procuring materials and equipment for the VAC therapy.

Data and evidence obtained from this study will form a major contribution towards understanding the mechanism of action of NPWT.

This study will generate open ideas and avenues towards advanced molecular and genetic studies in relation to wound healing.

3.16.1. STUDY LIMITATIONS

This study needed expensive budgetary allocation towards purchase of materials and equipment.

There were noted few cases of sample mislabeling leading to error in identification.

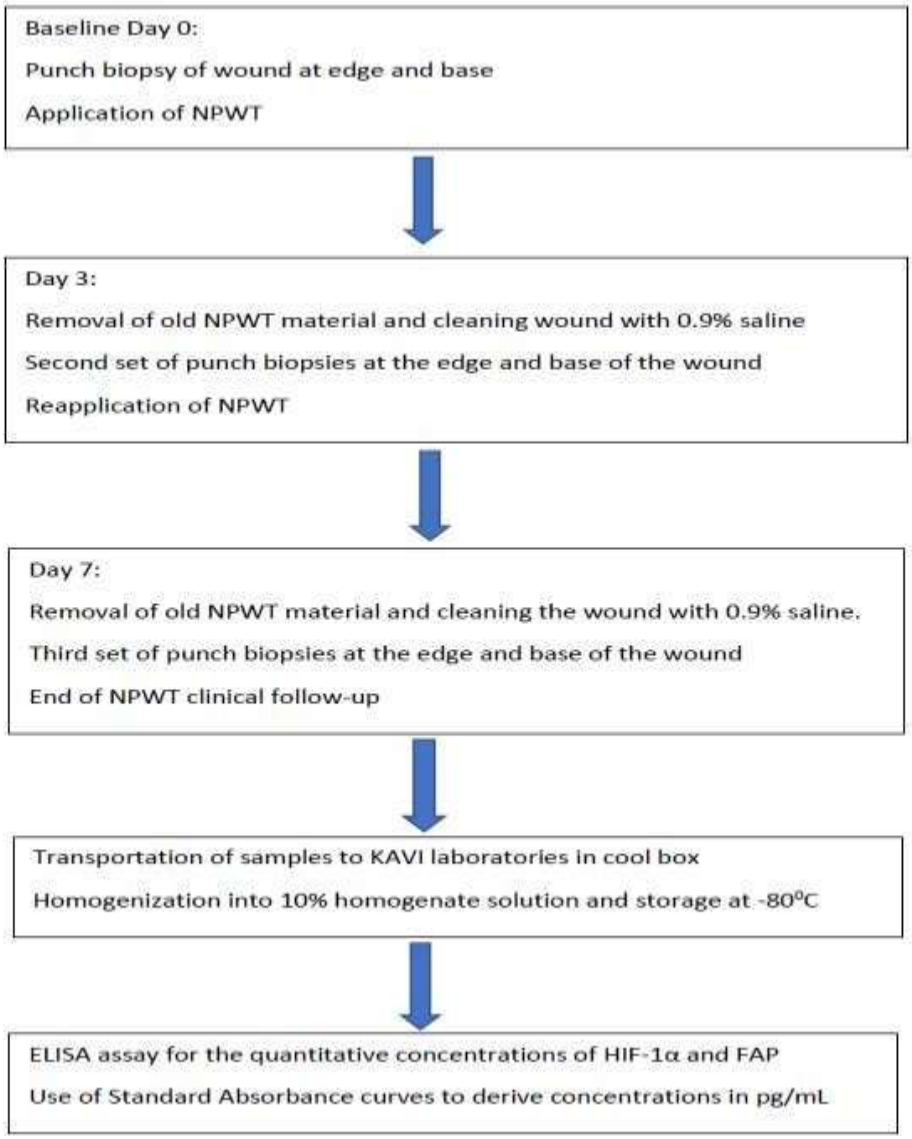

Figure 3: Flow chart of events in NPWT in traumatic wounds

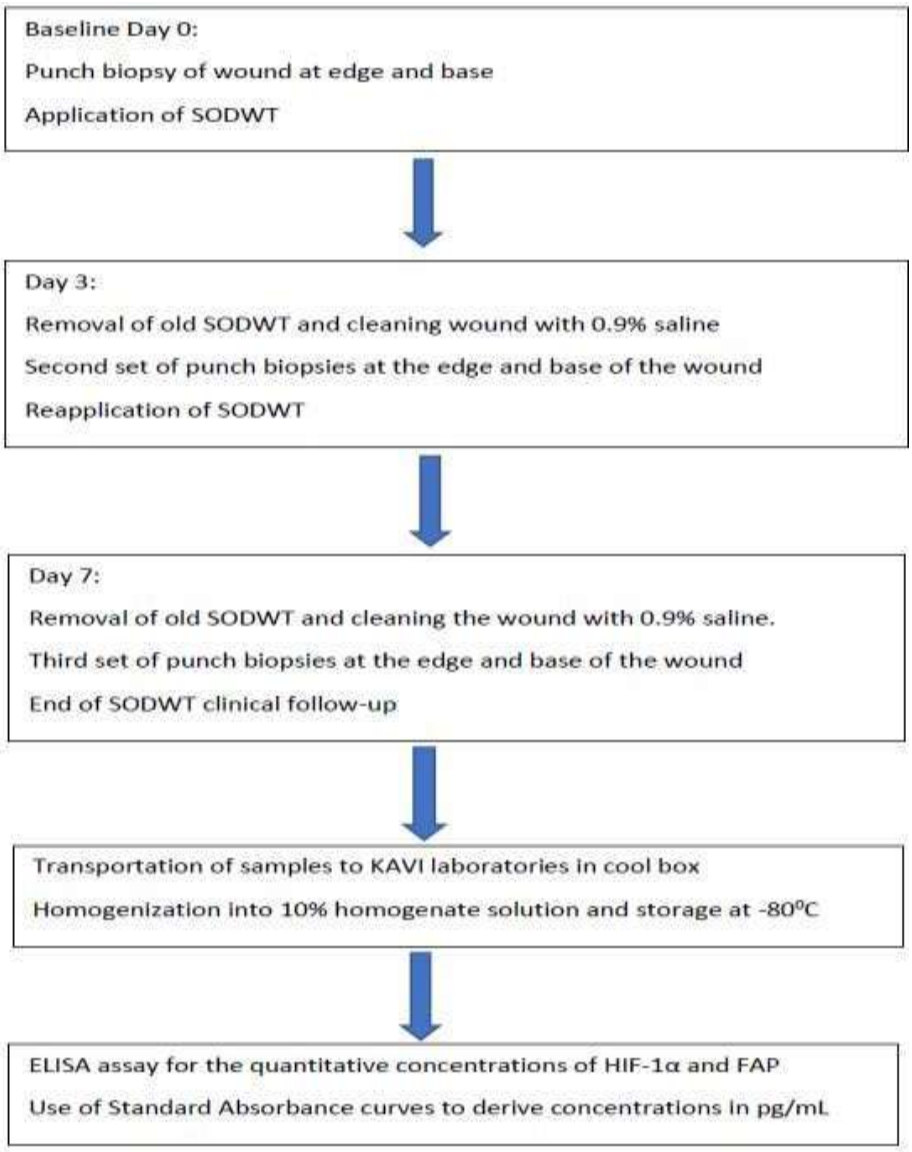

Figure 4: Flow chart of events in SODWT in traumatic wounds

CHAPTER FOUR:

RESULTS

4.1.1. INTRODUCTION

A total of 72 participants were involved in this study from October 2020 to April 2021. None of the participants withdrew from the study or developed any complications to warrant discontinuation from the study. All of them had traumatic wounds and voluntarily gave consent to be participants in the study.

4.1.1.1. AGE AND GENDER DISTRIBUTION

Majority of the participants were male (81.9%) and majority of the participants were in their third decade of life (42.4%male and 61.5%female).

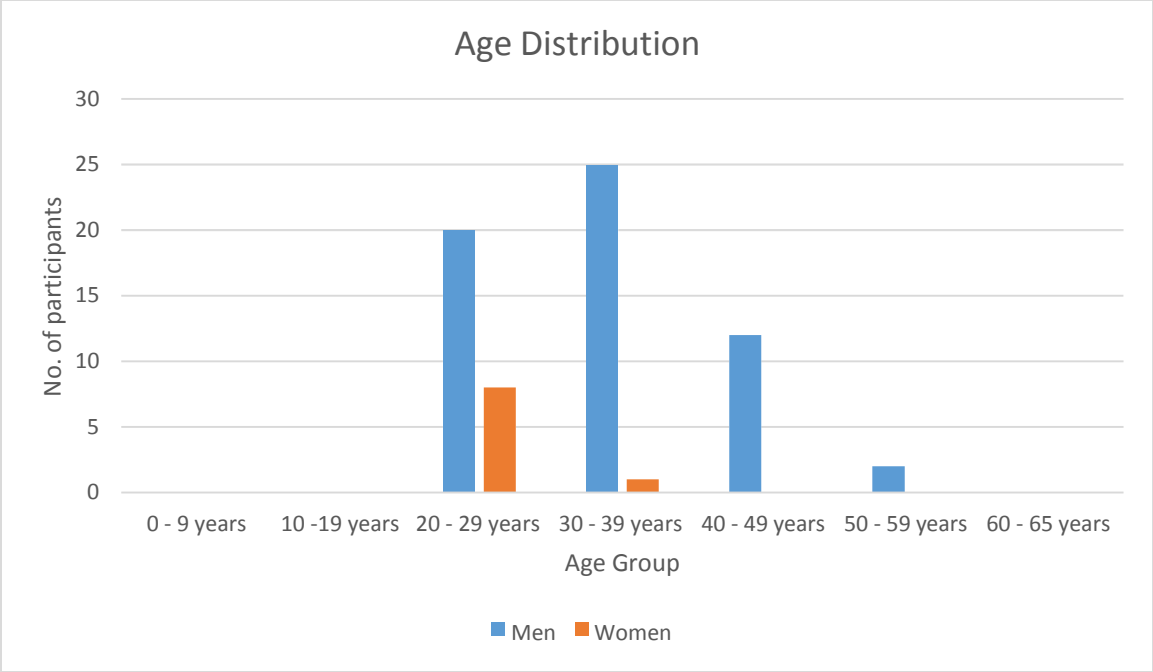

Figure 5: Bar Chart illustrating the age distribution

4.1.1.2. SITE OR LOCATION OF THE WOUNDS

Most of the traumatic wounds were located in the lower limbs (88%) while the rest were located in the upper limbs (12%).

None were located in the torso, head and neck regions.

4.1.1.3. WOUND SIZE

Majority of the wound had the largest diameter ranging from 1 to 10cm (97.2%). No wound had the largest diameter below 1cm and 2 participants had the largest diameter of their wounds above 10cm (2.8%).

4.1.1.4. TRAUMATIC WOUND ETIOLOGY

Majority of the participants got injured after getting involved in motor vehicle related traumatic events (87.5%). The rest acquired the wounds after assault and surgical procedures.

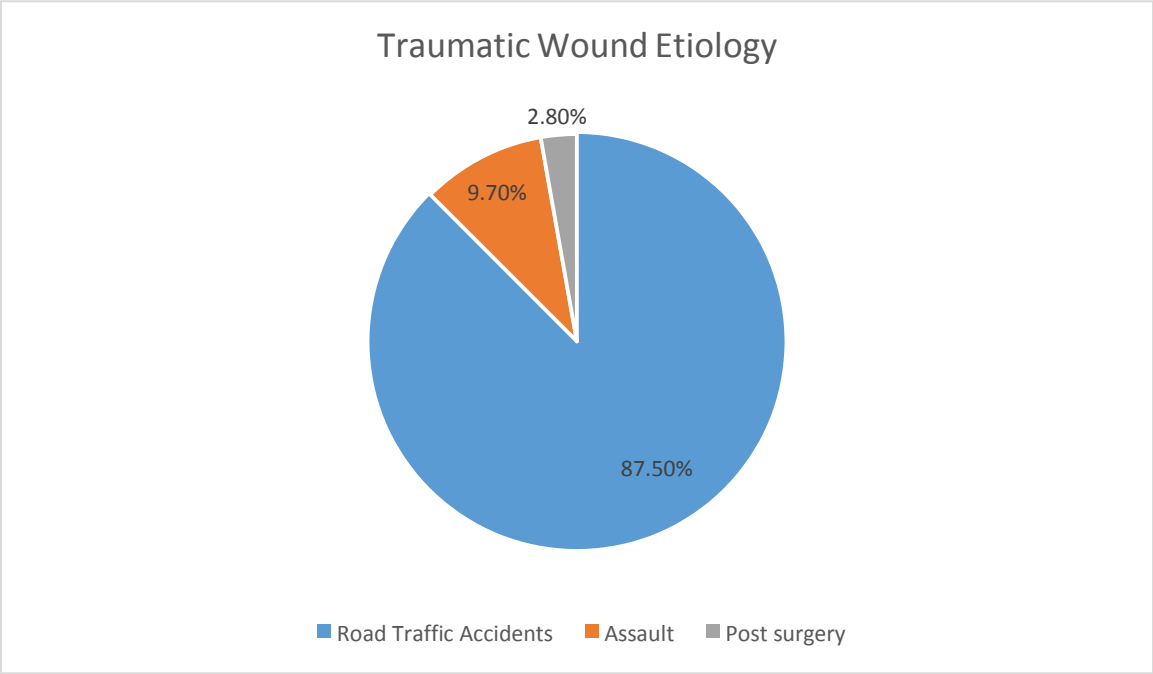

Figure 6: Pie Chart illustrating traumatic wound etiologies

4.1.1.5. TIME AT CLINICAL EVALUATION

Most of the patients were recruited into the study within one week after getting injured (51.4%). Four weeks was the longest time after injury in some patients who got recruited into the study (2.8%).

4.2.1. ELISA ANALYSIS RESULTS

With the use of optical densities (ODs) obtained from standard solution curves, the actual concentrations of HIF-1α and FAP were derived in pg/mL. This was achieved for both NPWT and SODWT groups, and the quantitative levels were demonstrated both at the wound edges and the wound bases.

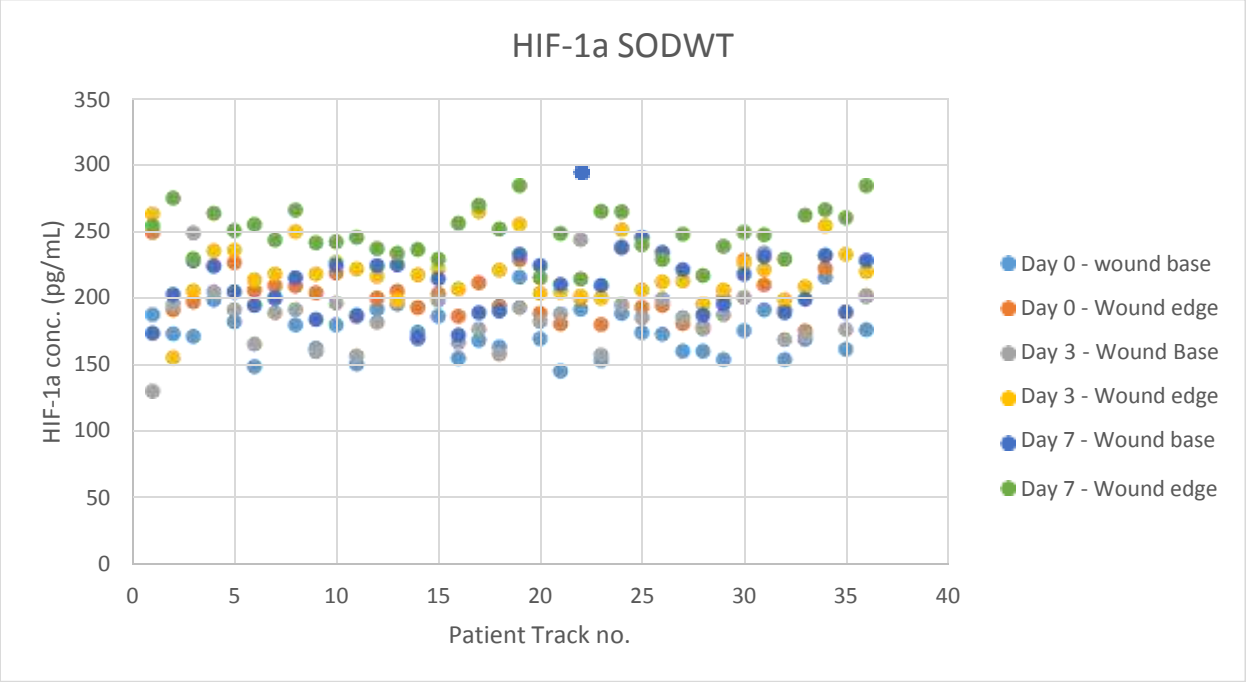

Figure 7: Scatter graph illustrating the distribution of HIF-1a in the group managed with SODWT

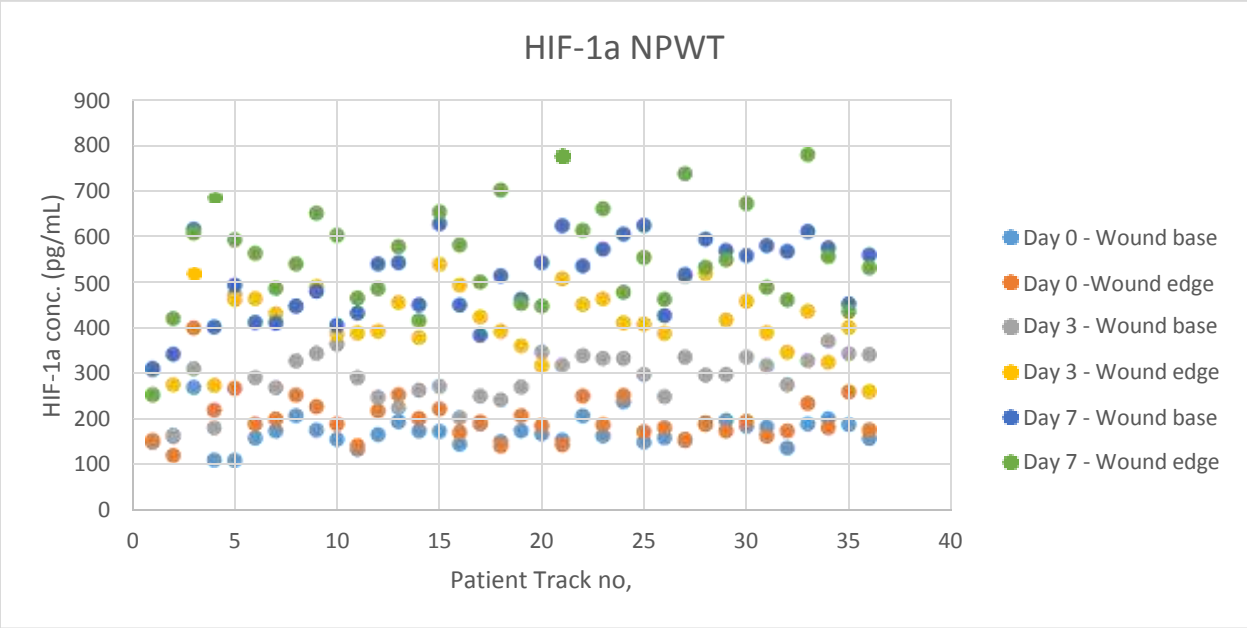

Figure 85: Scatter graph illustrating the distribution of HIF-1a in the group managed by NPWT

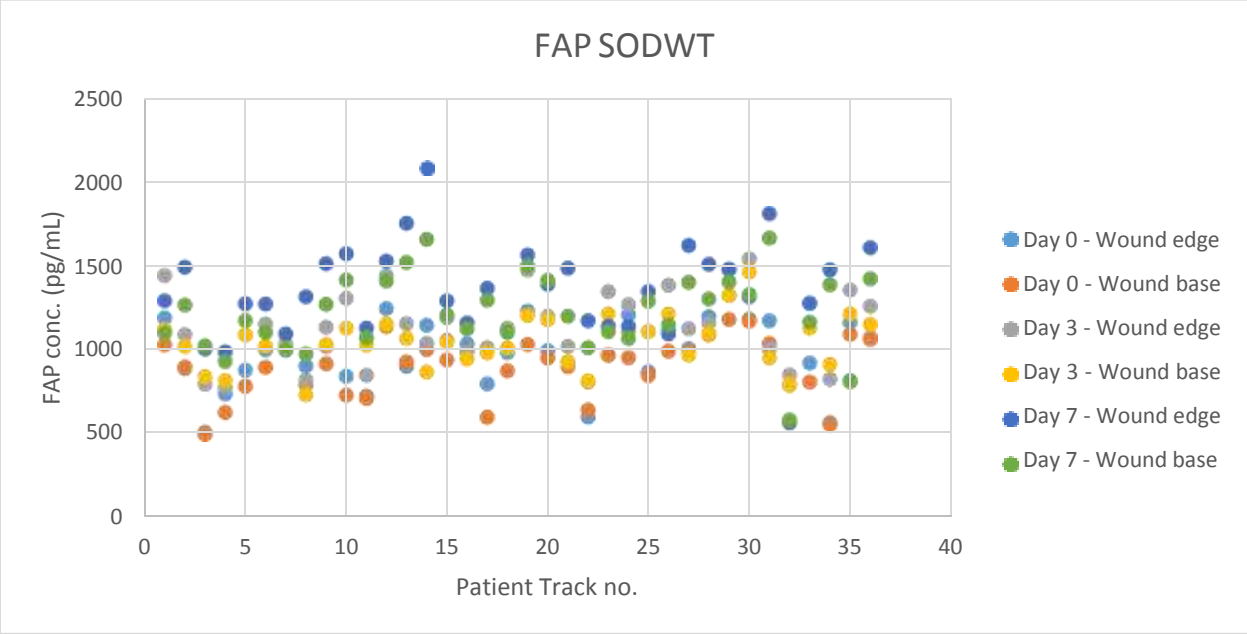

Figure 96: Scatter graph illustrating the distribution of FAP in the group managed by SODWT

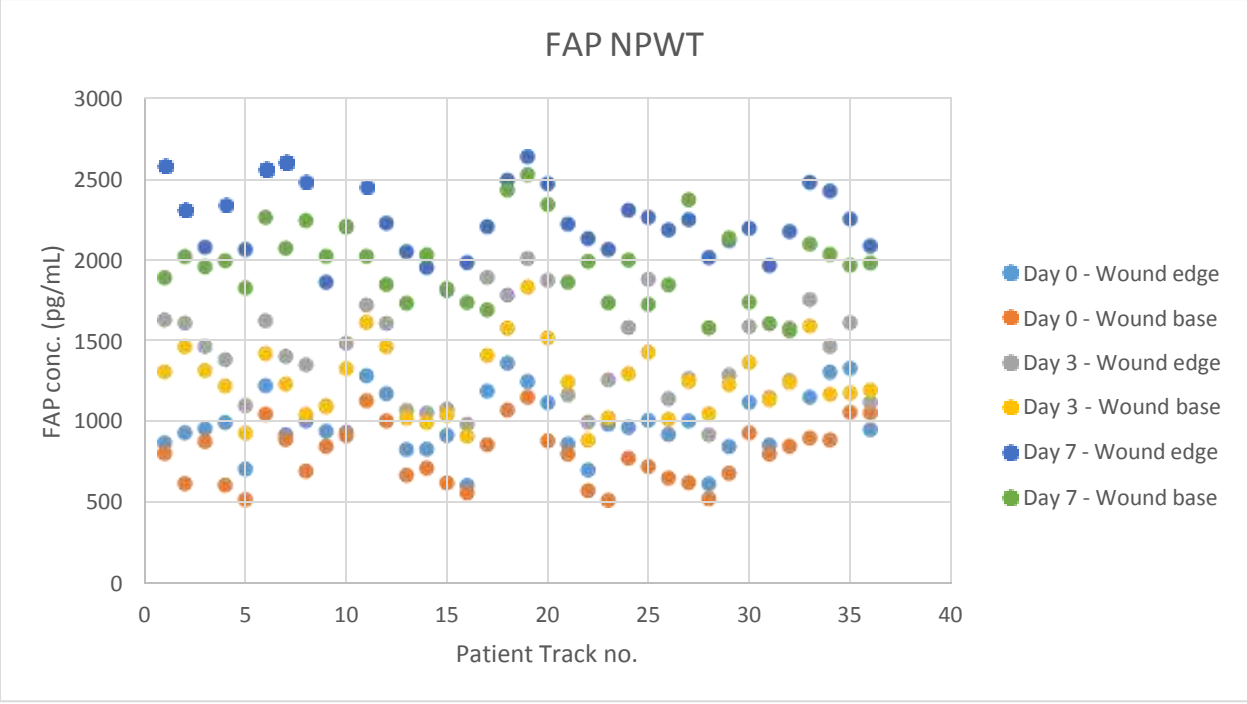

Figure 70: Scatter graph illustrating the distribution of FAP in the group managed by NPWT

4.2.1.1. QUANTITATIVE CHANGES OF HIF-1α IN WOUNDS MANAGED WITH NPWT COMPARED TO WOUNDS MANAGED WITH SODWT

The quantitative values of HIF-1α obtained during baseline day 0 analyses showed no statistical significant difference between the two groups (NPWT and SODWT). This was observed on the levels of HIF-1α both at the wound edges and wound bed between the two groups. When the paired samples t-test was performed on the quantitative levels of HIF-1α at the wound edges between NPWT group and SODWT group, the *p* value obtained was 0.883. The same paired sample t-test was performed on HIF-1α levels at wound bases between NPWT group and SODWT group and the *p* value obtained was 0.636 (*p* values > 0.05).

However, both groups showed statistically significant differences in the quantitative levels of HIF-1α when compared between wound edges and wound bases in between individual groups (*p* value < 0.001). The HIF-1α concentration levels were found to be increased more at the wound edges than the wound bases.

Assessment of HIF-1α quantitative levels on day 3 after NPWT and SODWT revealed a statistically significant increase in the concentration of HIF-1α both at the wound edges and wound bases. Furthermore, the increased in the concentration of HIF-1α in wounds managed with NPWT was statistically significant more than the increase occasioned in wounds under SODWT management (*p* value < 0.001). The same phenomenon was observed on day 7 assessment of concentration of HIF-1α whereby it was more in wound under NPWT management than those under SODWT (*p* value of < 0.001).

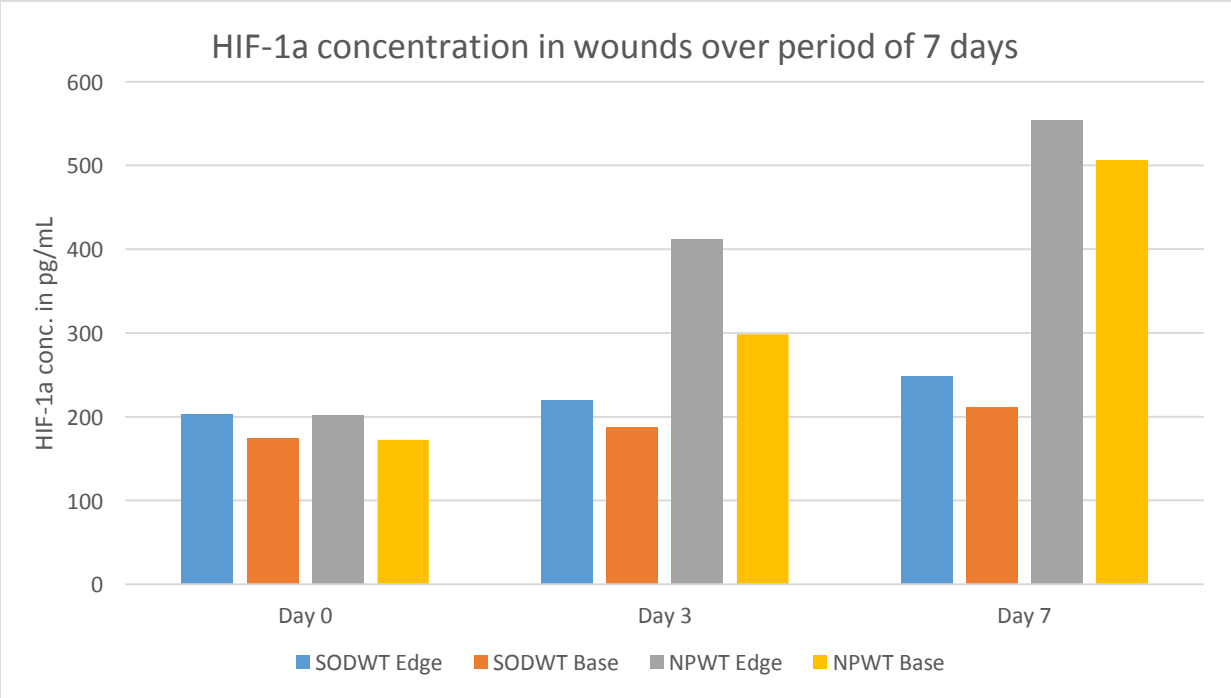

Figure 81: Bar chart summarizing the mean values of HIF-1a conc in the two groups noted over different days

HIF-1α:- Paired Samples Test						
		95% Confidence Interval of the Difference		t	df	*P* value
		Lower	Upper			
Pair 1	SODWT-day0E vs day3E	-22.18534	-11.59381	-6.475	35	.000
Pair 2	SODWT-day0E vs day7E	-52.96599	-38.78415	-13.134	35	.000
Pair 3	SODWT-day3E vs day7E	-36.42946	-21.54153	-7.905	35	.000
Pair 4	SODWT-day0B vs day3B	-20.33549	-5.22300	-3.433	35	.002
Pair 5	SODWT-day0B vs day7B	-43.90753	-28.88089	-9.834	35	.000
Pair 6	SODWT-day3B vs day7B	-29.46603	-17.76391	-8.194	35	.000
Pair 7	NPWT-day0E vs day3E	-235.5050	-185.1768	-16.969	35	.000
Pair 8	NPWT-day0E vs day7E	-392.3821	-313.4477	-18.153	35	.000
Pair 9	NPWT-day3E vs day7E	-174.2626	-110.8853	-9.134	35	.000
Pair 10	NPWT-day0B vs day3B	-147.5559	-105.3346	-12.160	35	.000
Pair 11	NPWT-day0B vs day7B	-360.9240	-306.6198	-24.955	35	.000
Pair 12	NPWT-day3B vs day7B	-236.7234	-177.9297	-14.318	35	.000
Comparing SODWT vs NPWT						
Pair 13	SODWT-day0E vs NPWT-day0E	-15.88965	18.38260	.148	35	.883
Pair 14	SODWT-day0B vs NPWT-day0B	-8.55248	13.81505	.478	35	.636
Pair 15	SODWT-day3E vs NPWT-day3E	-219.9748	-164.4346	-14.051	35	.000
Pair 16	SODWT-day3B vs NPWT-day3B	-131.2787	-90.79088	-11.135	35	.000
Pair 17	SODWT-day7E vs NPWT-day7E	-344.2384	-267.3483	-16.148	35	.000
Pair 18	SODWT-day7B vs NPWT-day7B	-322.0122	-267.4806	-21.946	35	.000

Table 1: Summary of p values in HIF-1a evaluation

4.2.1.2. QUANTITATIVE CHANGES OF FAP IN WOUNDS MANAGED WITH NPWT COMPARED TO WOUNDS MANAGED WITH SODWT

The quantitative values of FAP obtained during baseline day 0 analysis showed no statistical significant difference between the two groups (NPWT and SODWT). This was observed on the levels of FAP both at the wound edges and wound bed between the two groups. When the paired samples t-test was performed on the quantitative levels of FAP at the wound edges between NPWT group and SODWT group, the *p* value obtained was 0.630 (*p* value > 0.005). The same paired sample t-test was performed on FAP levels at wound bases between NPWT group and SODWT group and the p value obtained was 0.056 (*p* values > 0.05).

However, both groups showed statistically significant differences in the quantitative levels of FAP when compared between wound edges and wound bases in between individual groups (*p* value < 0.05). The FAP concentration levels were found to be increased more at the wound edges than the wound bases.

Assessment of FAP quantitative concentration levels on day 3 after NPWT and SODWT revealed a statistically significant increase in the concentration of FAP both at the wound edges and wound bases. Furthermore, the increased in the concentration of FAP in wounds managed with NPWT was statistically significant more than the increase occasioned in wounds under SODWT management (*p* value < 0.05). The same phenomenon was observed on day 7 assessment of concentration of FAP whereby it was more in wound under NPWT management than those under SODWT (*p* value of < 0.05).

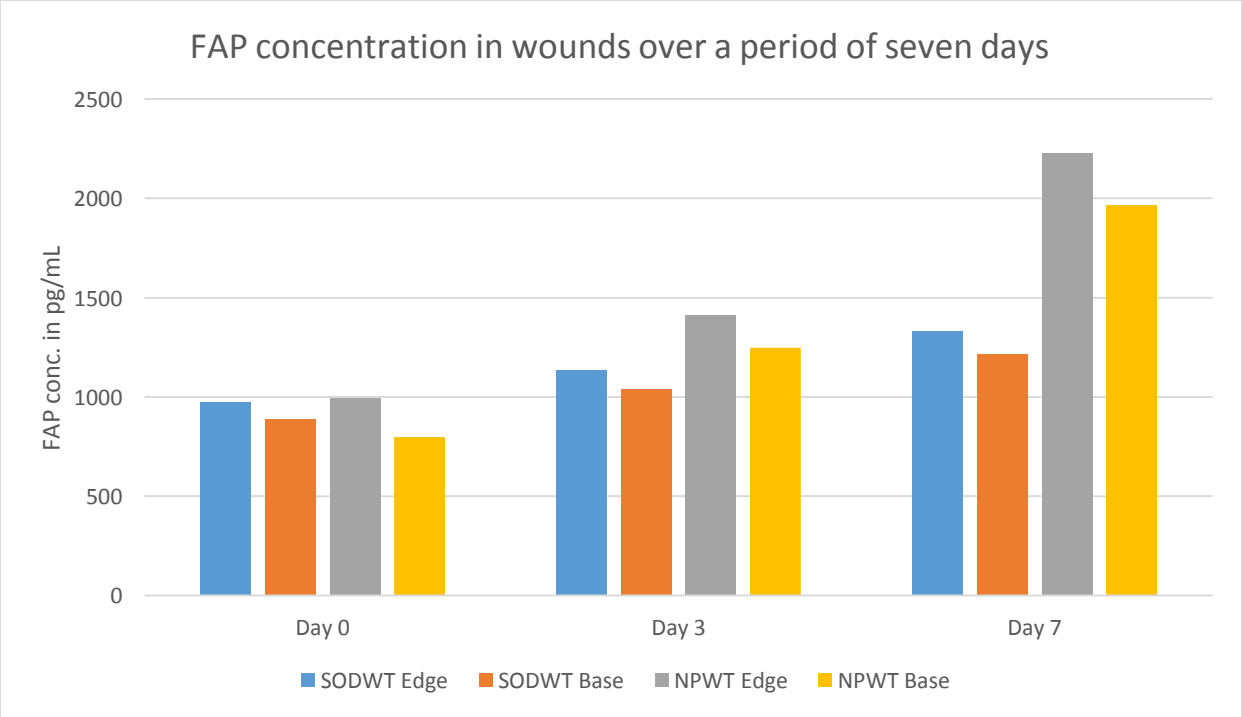

Figure 92: Bar chart summarizing the mean conc. of FAP of the two groups noted over different days

FAP:- Paired Samples Test						
		95% Confidence Interval of the difference		**t**	**df**	**P value**
		Lower	**Upper**			
Pair 1	SODWT-day0E vs day3E	-208.63508	-113.53487	-6.877	35	.000
Pair 2	SODWT-day0E vs day7E	-459.32174	-259.45570	-7.301	35	.000
Pair 3	SODWT-day3E vs day7E	-308.19267	-88.41482	-3.663	35	.001

Pair 4	SODWT-day0B vs day3B	-200.50808	-106.63180	-6.642	35	.000
Pair 5	SODWT-day0B vs day7B	-403.10133	-248.60263	-8.563	35	.000
Pair 6	SODWT-day3B vs day7B	-251.55720	-93.00688	-4.412	35	.000
Pair 7	NPWT-day0E vs day3E	-483.46542	-352.49385	-12.958	35	.000
Pair 8	NPWT-day0E vs day7E	-1300.3058	-1169.29750	-38.269	35	.000
Pair 9	NPWT-day3E vs day7E	-887.71016	-745.93393	-23.392	35	.000
Pair 10	NPWT-day0B vs day3B	-502.58921	-396.42233	-17.191	35	.000
Pair 11	NPWT-day0B vs day7B	-1245.9874	-1087.65021	-29.921	35	.000
Pair 12	NPWT-day3B vs day7B	-797.26463	-637.36152	-18.214	35	.000
Comparing controls vs experimental						
Pair 13	SODWT-day0E vs NPWT-day0E	-124.48302	76.36251	-.486	35	.630
Pair 14	SODWT-day0B vs NPWT-day0B	3.60656	175.49716	2.115	35	.056
Pair 15	SODWT-day3E vs NPWT-day3E	-394.52919	-167.38063	-5.022	35	.000
Pair 16	SODWT-day3B vs NPWT-day3B	-285.38325	-127.38469	-5.304	35	.000
Pair 17	SODWT-day7E vs NPWT-day7E	-1033.6178	-765.32853	-13.612	35	.000
Pair 18	SODWT-day7B vs NPWT-day7B	-857.08928	-645.74073	-14.435	35	.000

Table 2: Summary of p values in FAP evaluation

4.2.1.3. RATE OF CHANGE OF QUANTITATIVE CONCENTRATION OF HIF-1α IN WOUNDS MANAGED BY NPWT COMPARED TO SODWT

This study showed that there was an increase in concentration in time of HIF-1α from baseline day 0 to day 7 on both wound edge and wound base. This trend was noted in both wounds managed by NPWT and SODWT (Table 5, figure 16).

The increase in the NPWT groups was however more marked than the slight one noted among the SODWT groups.

It is also worth mentioning that in all days observed, the NPWT groups were noted to have higher concentration when compared to the SODWT groups.

	HIF-1α-Edge-SODWT	**HIF-1α-Base-SODWT**	**HIF-1α-Edge-NPWT**	**HIF-1α-Base-NPWT**
Baseline Day 0	202.52	174.76	201.28	172.13
Day 3	219.41 *8% increase from baseline	187.54 *6% increase from baseline	411.61 *104% increase from baseline	298.58 *73% increase from baseline
Day 7	248.40	211.16	554.19	505.91

	*13% increase from day 3 *22.56% increase from baseline	*13% increase from day 3 *20.82% increase from baseline	*35% increase from day 3 *175.46% increase from baseline	*69% increase from day 3 *193.91% increase from baseline

Table 3: Rates of increase of HIF-1a over 7 days

4.2.1.4. RATE OF CHANGE OF QUANTITATIVE CONCENTRATION OF FAP IN WOUNDS MANAGED BY NPWT COMPARED TO SODWT

This study showed that there was an increase in concentration in time of FAP from baseline day 0 to day 7 on both wound edge and wound base. This trend was noted in both wounds managed by NPWT and SODWT (Table 6, figure 17).

The increase in the NPWT groups was however more marked than the slight one noted among the SODWT groups.

It is also worth mentioning that in all days observed, the NPWT groups were noted to have higher concentration when compared to the SODWT groups.

	FAP-Edge-SODWT	**FAP-Base-SODWT**	**FAP-Edge-NPWT**	**FAP-Base-NPWT**
Baseline Day 0	970.60	887.76	994.66	798.21
Day 3	1131.68 *17% increase from baseline	1041.33 *17% increase from baseline	1412.64 *42% increase from baseline	1247.71 *56% increase from day baseline
Day 7	1329.99 *18% increase from day 3 *37.03% increase from baseline	1213.99 *17% increase from day 3 *36.75% increase from baseline	2229.46 *58% increase from day 3 *124.14% increase from baseline	1965.02 *58% increase from day 3 *146.18% increase from baseline

Table 4: Rates of increase of FAP over 7 days

4.2.1.5. EFFECT OF NPWT ON QUANTITATIVE CONCENTRATION OF HIF-1α COMPARED TO EFFECT ON QUANTITATIVE CONCENTRATION OF FAP

NPWT was noted to increase the quantitative concentration of HIF-1α more than its effect of increasing quantitative concentration of FAP.

Statistically significant differences were noted on the mean concentration between the NPWT groups of the FAP and HIF groups on all the days (p value < 0.05); (Table 7).

Paired Samples Test NPWT effect on HIF vs FAP						
		95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
		Lower	Upper			
Pair 1	HIF-day0E vs FAP-day0E	-863.85714	-722.90599	-22.854	35	.000
Pair 2	HIF-day0B vs FAP-day0B	-688.18023	-563.96953	-20.465	35	.000
Pair 3	HIF-day3E vs FAP-day3E	-1115.3047	-886.73577	-17.782	35	.000
Pair 4	HIF-day3B vs FAP-day3B	-1029.8440	-868.42655	-23.874	35	.000
Pair 5	HIF-day7E vs FAP-day7E	-1763.6525	-1586.8839	-38.479	35	.000
Pair 6	HIF-day7B vs FAP-day7B	-1551.6913	-1366.5518	-31.999	35	.000

Table 5: Summary of p values between HIF-1a and FAP

CHAPTER FIVE:

DISCUSSION

5.1.0. INTRODUCTION

The findings of this study are adding up to the emerging cumulative evidence that NPWT induces wound hypoxia when applied for wound management. This hypoxic cellular environment recruits hypoxic sensitive molecules that foster the quick increased granulation tissue formation observed in these wounds.

5.1.1. EFFECT OF NPWT ON HIF-1α

The expression of HIF-1α in wounds at baseline wound evaluation in an indication that these factors are crucial and play a major physiological role in wound healing. The integrity of the wound edge is vital in wound healing. The wound edge sustains the connection between injured tissues and healthy surrounding tissues and it serves as a conduit through which inflammatory molecules and cells, cytokines, nutrients and other important biological elements gain access to the wound to initiate the process of wound healing (72). Therefore, the wound edge is where wound healing commences.

NPWT has been shown to be an effective mechanical adjunct in wound management whereby wounds subjected to NPWT have been demonstrated to accelerate process of healing through enhanced granulation tissue formation. Our study demonstrated the quick onset of mechanism of action of NPWT with maximum activity witness in the initial 72 hours after application of NPWT.

In an animal RCT study carried out by Paolo Erba and colleagues, they demonstrated accumulation of HIF-1α at the wound edges but a reduction at wound bases when the wounds were subjected to NPWT (73). They attributed the reduction of HIF-1α to increased perfusion at the wound base and the increased concentration of HIF-1α at the edges to the hypoxic effects of NPWT. In comparison to their comparative group managed by SODWT, they demonstrated the wounds to have a higher concentration of HIF-1α (73). This is partially comparable to the findings of our study.

However, studies by Kairinos and in South Korea demonstrated reduced blood flow and partial pressure of oxygen in wounds subjected to NPWT (74). Indirectly, these findings were comparable to our findings of accumulation of these two hypoxia sensitive elements both at the edge and the base in wounds subjected to NPWT. Therefore our study is in support of Kairinos finding where he demonstrated reduced blood flow in wounds subjected to NPWT. In effect, this hypoxic wound environment then becomes the drive through which angiogenesis and vasculogenesis related peptides are recruited to foster granulation tissue formation.

There's also a sharp contrast to study by Grimm where he demonstrated a reduction of HIF-1α in wounds subjected to NPWT (75). This could be attributed to the different etiologies of wounds evaluated in our studies.

5.2.1. EFFECTS OF NPWT ON FAP

The finding of FAP expression in wound at baseline evaluation is also an indication that this molecule plays a major role in the process of wound healing. It has been demonstrated to accumulate in healing wounds.

In a study by Zou Baojia and colleagues on the expression of FAP in hepato-cellular carcinoma (HCC) cells, they found out that expression of FAP and HIF-1α in HCC were significantly correlated whereby both factors were increased in concentration (76). However it was not clear whether they were dependent or independent of each other.

There is a possibility of FAP being dependent on HIF-1α considering the former is a serine protease and the latter a transcriptional factor.

Throughout extensive literature search and review, no published data has demonstrated the effect of NPWT on quantitative concentration of FAP in wounds so as to make comparison with.

5.3.1. CONCLUSION

Having demonstrated a statistically significant increase in the quantitative concentration of the hypoxia sensitive elements HIF-1α and FAP in wounds managed with NPWT compared to wounds managed with SODWT (p value < 0.05), we reject the null hypothesis that Negative Pressure Wound Therapy is not more effective than Standard Occlusive Dressing Wound Therapy in inducing wound hypoxia during traumatic wound healing process.

We adopt the alternative hypothesis that Negative Pressure Wound Therapy is more effective than Standard Occlusive Dressing Wound Therapy in inducing wound hypoxia during the healing of traumatic wound.

Therefore, hypoxia induction should be recognized as a mechanism of action or a pathway through which NPWT exerts its effects of accelerating wound healing by enhancing granulation tissue formation.

5.4.1. RECOMMENDATIONS

I. More research into the hypoxia pathway by blocking HIF-1α and FAP pathways with inhibitory molecules and the downstream effects of NPWT deduced.
II. More studies to be done to establish the independence or dependence of FAP on HIF-1α.
III. More research on the effect of NPWT on other hypoxia sensitive factors.
IV. More studies on wound of other etiologies and determine whether similar response can be evoked.

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APPENDIX A: RAW DATA COLLECTION SHEET

Biopsy date – Day 0 (DD/MM/YY)_____/____20____

Day 3 (DD/MM/YY)_____/____20____

Day 7 (DD/MM/YY)_____/____20____

Research Track Number _____

Wound Details

Date of injury (DD/MM/YYYY)_____/_____/_____

Anatomical location of wound _____

Type of Wound (mechanism of injury) _____

Size of wound (cm^2) _____

Comorbidities _____

Any other information _____

_____

Mode of Wound Management (tick as appropriate)

- ○ NPWT
- ○ Conventional Standard Occlusive Wound Dressing

Biopsy ELISA Assay results

DAY	**HIF-1α LEVELS (pg/g tissue)**		**FAP LEVELS (pg/g tissue)**	
Wound site	***Edge***	***Base***	***Edge***	***Base***
0				
3				
7				

APPENDIX B: CONSENT FORM

ENGLISH VERSION

Topic: The Effectiveness of Negative Pressure Wound Therapy (NPWT) in inducing hypoxia compared to Standard Occlusive Dressing Wound Therapy (SODWT) in traumatic wound in wound healing.

This form is to ask for consent from patients who present to Kenyatta National Hospital (KNH) with wounds and while undergoing management either through NPWT or conventional standard occlusive wound dressing, obtain tissue biopsies for analysis of the process of wound healing and hypoxia.

Principal investigator: Dr. Matwa Christopher, Ondieki.

Institution: School of Medicine, Department of surgery – University of Nairobi.

Supervisors: Dr. Nangole Ferdinand and Prof. Khainga Stanley.

This informed consent has three parts:

A. Information sheet (to share information about the research with you)

B. Certificate of Consent (for signatures if you agree to take part)

C. Statement by the researcher

Part A: INFORMATION SHEET

Introduction:

I am known by the name Dr. Matwa Christopher, Ondieki. Currently I am a surgical post-graduate student pursuing a master's degree in plastic, reconstruction and aesthetic surgery at the University of Nairobi, School of Medicine.

As part of the requirements for conferment of the degree, I am carrying out a study on the ***effects of negative pressure wound therapy on wound hypoxia***.

Sampling procedure:

This study is based on patients admitted with traumatic wounds for wound care at the Kenyatta National and Referral Hospital. Through random sampling, I will pick patients consenting to participate in the study and randomly subject them through Negative Pressure Wound Therapy

(NPWT) and Standard Occlusive Dressing Wound Therapy (SODWT) with the aim of comparing the outcomes of the two kinds of wound management. It entails taking a small amount of wound tissue through punch tissue biopsy using punch biopsy tool. The procedures will be done under local anesthesia therefore should be painless.

The biopsies will be taken to KAVI laboratories for analysis of levels of HIF-1α and FAP in order to correlation wound hypoxia to wound healing.

I am inviting you to willingly take part in this study.

Benefits of the Study:

The results obtained aim to contribute academically to existing knowledge on the mechanism of action of NPWT. Currently, there exists gaps and no conclusion in regards to mechanism of action through which NPWT causes hastened wound healing.

Costs and Potential Harm:

It will cost you as a participant nothing in consumables used in NPWT and SODWT, however other hospital charges will apply.

Potential harm include pain and bleeding during the procedure. However, the use of local anesthesia should alleviate the pain and since it is a small amount of tissue biopsied on the skin, bleeding should stop with pressure application.

There will be no financial grant to you as a participant*

If you decline to participate in the study, be assured that your decision will not jeopardize the required care for you as a patient.

Obligation on you:

If you agree to participate in this study, you will be asked to provide a few personal information in regards to yourself relating to when you got injured, the mode of injury, part of the body that got injured, progression of the injury (wound) and any other underlying pre-existing medical conditions.

Confidentiality:

All the information gathered will be taken in confidence and no one will see it except my assistant, my supervisors and I, all who are duty-bound to ensure confidentiality.

Your name or identity will not appear in any research document. The information about you will be identified by a unique research number and only we, the researchers, can relate the number to you. Your information will only be used for this study and will not be shared with anyone else unless authorized by the Kenyatta National Hospital/University of Nairobi - Ethics and Research Committee (KNH/UoN-ERC).

Study Credibility and Legitimacy:

This study was approved by my two supervisors. It was appraised and approved by the Chairman of the Department of Surgery, School of Medicine at the University of Nairobi. It was then submitted to KNH/UoN-ERC which reviewed and approved it to be done for a duration of six months. KNH/UoN-ERC is the regulatory body in the hospital whose work is to make sure research process is safe for the participants and that you are protected from harm.

Relevant contacts:

You can ask questions or seek clarifications about the study any time you wish to. If need be, you may also talk to anyone you are comfortable with about the research before deciding.

If you have any query about the research you want addressed by another person other than the researchers, please feel free to contact the following who will address your concerns:

Secretary, KNH/UoN-ERC

P.O. Box 20723 – 00202, KNH; Nairobi.

Tel: +254 20 272 6300 ext. 44355

Email: KNHplan@Ken.Healthnet.org or uonknh_erc@uonbi.ac.ke

Twitter: @UONKNH_ERC (https://twitter.com/UONKNH_ERC)

Facebook: https://www.facebook.com/uonknh.erc

Dr. Matwa Christopher, Ondieki (principal researcher)

Department of Plastic Surgery, School of Medicine; University of Nairobi.

P.O. Box 478 – 00515, Buruburu; Nairobi.

Cell: +254 727 453 071

Email: chrimommv@yahoo.com or chrimommv@gmail.com

Dr. Nangole Ferdinand (supervisor)

Department of Plastic Surgery, School of Medicine; University of Nairobi.

P.O. Box 19676 – 00202, UoN; Nairobi.

Tel: +254 20 272 6300

Cell: +254 714 342 214

Email: nangole2212@gmail.com

Prof. Khainga Stanley (supervisor)

Chairperson - Department of Plastic Surgery, School of Medicine; University of Nairobi.

P.O. Box 19676 – 00202, UoN; Nairobi.

Tel: +254 20 272 6300

Cell: +254 723 436 408

Email: skhainga@yahoo.com

PART B: CONSENT CERTIFICATE

This document becomes confidential once signed.

Research track number _____

I freely give consent to take part in the study conducted by Dr. Matwa Christopher, Ondieki the nature of which has been explained to me by him/his research assistant. I have been informed and have understood that my participation is voluntary and understand that I am free to withdraw from it any time I wish and this will not in any way alter the care given to me as a patient. The results of the study may or may not benefit me as a patient directly but may benefit similar future patients and academic prospects with regards to existing knowledge on the **Effects of NPWT on Wound Hypoxia and healing**.

Signed consent _____

[**Guardian/Next of kin for minors (< 18 years old)**]

Signature/NA_____

Date (DD/MM/YY)_____/_____20_____

Thumb print if illiterate

Statement by a witness if participant is illiterate

I have witnessed the accurate reading of the consent form to the participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness _____

Signature of witness_____Date (DD/MM/YY)_____/____20_____

PART C: STATEMENT BY THE RESEARCHER

I have clearly read out the information sheet to the participant, and to the best of my ability made sure that the participant understood the following:

- All information gathered will be treated with confidentiality.
- Refusal to participate or withdrawal from the study will not compromise the quality of care and treatment given to the patient.

The results of this study might be published in a reputable journal to enhance the knowledge of the **EFFECTS OF NEGATIVE PRESSURE WOUND THERAPY ON WOUND HYPOXIA AND HEALING.**

Also, I confirm that the participant was given opportunity to seek clarification about his concerns in the study, and all the queries clarified to the best of my ability.

I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this Informed Consent Form has been provided to the participant and duly signed by the participant.

Name of researcher taking consent _____

Signature of the researcher_____Date (DD/MM/YY)_____/____20___

TOLEO LA KISWAHILI

Mada: Uchanganuzi wa viwango vya oksijeni na hali ya kuponya katika vidonda kuhusiana na mbinu ya 'Negative Pressure Wound Therapy (NPWT)' ikilinganishwa na mbinu ya kufunga vidonda kwa njia ya kawaida.

Fomu hii ni ya kuomba idhini kutoka kwako kama mgonjwa ambaye amefika katika Hospitali ya kitaifa ya Kenyatta na kidonda/ vidonda, kwamba wakati unaendelea kupata matibabu ya NPWT au kufungwa na vinyago vya vidonda tuweze kutoa sehemu kidogo ya kidonda na kuipeleka kwa utafiti wa kuchanganua hali ya kuponya na oksijeni.

Mtafiti mkuu: Dkt. Matwa Christopher, Ondieki.

Chuo: Shule ya Matibabu, Kitengo cha Upasuaji – Chuo Kikuu cha Nairobi.

Wahadhiri wasimamizi: Dkt. Nangole Ferdinand na Prof. Khainga Stanley

Makubaliano haya yana sehemu tatu:

A. Maelezo kuhusu utafiti huu.

B. Cheti cha Kibali (kitakacho tiwa sahihi na wahusika wanaokubali kujumuishwa utafitini)

C. Ithibati ya mtafiti

SEHEMU A: MAELEZO

Utangulizi:

Kwa jina najulikana kama Dkt. Matwa Christopher, Ondieki nami ni mwanafunzi wa shahada ya upasuaji wa plastiki katika Chuo Kikuu cha Nairobi.

Baadhi ya matakwa ili kutunukiwa hiyo shahada ni kufanya utafiti ambao nafanya na mada ni **Uchanganuzi wa viwango vya oksijeni na katika vidonda kuhusiana na mbinu ya 'NPWT.'**

Utafiti huu naufanya kwa wagonjwa ambao wamelazwa ili kupokea matababu ya vidonda. Natarajia kubaini matokeo baina ya NPWT na vinyago vya vidonda. Katika huu utafiti, nitachukua sampuli kidogo ya kidonda nikitumia sindano maalum. Nitadunga dawa ya kutoa makali kwa hivyo hautahisi uchungu.

Hiyo sampuli ya kidonda iliyotolewa itapelekwa katika mahabara za KAVI ili kufanyiwa huo utafiti.

Ninakukaribisha kwa hiari kushiriki katika utafiti huu.

Faida ya Utafiti huu

Matokeo ya utafiti huu yatachangia pakubwa kuelewa jinsi mbinu ya NPWT inaleta haraka kwa uponyaji wa vidonda. Kwa sasa kuna uchanganuzi haba na hali tatanishi.

Gharama na madhara za utafiti

Uchungu na ufiaji damu ni baadhi ya madhara yanayoweza sababishwa kwa huu utafiti. Uzuri ni kwamba tutatumia dawa ya kutoa makali ya uchungu na japo damu imwagike, kuweka msukumo kwa kidonda itazuia.

Natoa hakikisho kwamba hata kama hutaki kushiriki kwenye utafiti huu, wewe kama mgonjwa hutakashifiwa na utapata matibabu yanayostahili. Utafiti huu haupanii kuleta madhara aina yoyote au kukuathiriwa. **Hautatozwa fedha za ziada kwa minajili ya utafiti huu wala hakuna fedha utatunukiwa kwako wewe kama mhusika**.

Jukumu lako katika utafiti

Habari zote zitakazo kusanywa kwa ajili ya utafiti zitabanwa na watafiti na hazitatolewa ovyo. Jina au kitambulisho chako kama mgonjwa haitanakiliwa popote ila tu utapewa nambari maalum ya utafiti. Watafiti watatumia mbinu fiche itakayo kutambulisha kwao.

Habari zako kama mgonjwa zitatumiwa tu kwa ajili ya utafiti huu na hazitatolewa kwa yeyote pasipo na idhini ya Kamati ya Maadili ya Utafiti wa Hospitali Kuu ya Kenyatta na ile ya Chuo Kikuu Cha Nairobi (kwa ufupi KNH/UoN-ERC).

Uhalali wa utafiti huu

Utafiti huu umekubaliwa na wahadhiri wasimamizi wangu, ukapigwa msasa na Mwenyekiti wa kitengo cha upasuaji wa chuo kikuu cha Nairobi ambaye aliuwasilisha kwa Kamati ya Maadili ya Utafiti wa Hospitali Kuu ya Kenyatta na ile ya Chuo Kikuu Cha Nairobi (KNH/UoN-ERC) ambayo iliidhinisha uweze kufanywa kwa muda wa miezi sita. Kamati hii ndio ihakikishayo usalama wa wanaohusishwa kwa utafiti na kwamba hawadhuriwi kwa vyovyote vile.

Jukwaa la malalamishi na habari Zaidi

Waweza kutuuliza maswali yoyote wakati wowote au umuulize yeyote utakaye kuhusu mchakato wa utafiti huu kabla au hata baada ya kukubali kuhusishwa.

Iwapo una swali lolote kuhusu utafiti huu ambao waona heri lishughulikiwe na mtu mwingine isipokuwa watafiti, waweza kuwasiliana na wafuatao ambao wako tayari kuushughulikia ipasavyo:

Katibu, KNH/UoN-ERC

S.L.P. 20723 – 00202, KNH; Nairobi.

Simu: +254 20 272 6300 mlengo 44355

Barua pepe: KNHplan@Ken.Healthnet.org ama uonknh_erc@uonbi.ac.ke

Twitter: @UONKNH_ERC (https://twitter.com/UONKNH_ERC)

Facebook: https://www.facebook.com/uonknh.erc

Dkt. Matwa Christopher, Ondieki (mtafiti mkuu)

Kitengo ya Upasuaji wa plastiki, Chuo Kikuu cha Nairobi.

S.L.P. 478 – 00515, Buruburu; Nairobi.

Rununu: +254 727 435 071

Barua pepe: chrimommv@yahoo.com ama chrimommv@gmail.com

Dkt. Nangole Ferdinand (mhadhiri msaidizi)

Kitengo cha upasuaji wa plastiki, Chuo Kikuu cha Nairobi.

S.L.P. 19676 – 00202, UoN; Nairobi.

Simu: +254 20 272 6300

Rununu: +254 714 342 214

Barua pepe: nangole2212@gmail.com

Prof. Khainga Stanley (mhadhiri msaidizi)

Mwenyekiti - Kitengo cha upasuaji wa plastiki, chuo Kikuu cha Nairobi.

S.L.P. 19676 – 00202, UoN; Nairobi.

Simu: +254 20 272 6300

Barua pepe: skhainga@yahoo.com

SEHEMU B: CHETI CHA KIBALI

Hii fomu inakuwa siri baada ya kutiwa sahihi.

Nambari maalum _____

Mimi ninakubali kwa hiari kuhusishwa kwa utafiti unaoendelezwa na Dkt. Matwa Christopher, Ondieki kuambatana na maelezo ambayo yeye mwenyewe/ msaidizi wake amenipa. Ninaelewa kwamba nimehusishwa kwa hiari na kwamba niko huru kujiondoa wakati wowote nitakao hata bila sababu, na hii haitaathiri kwa namna yoyote matibabu kwangu ipaswavyo. Aidha naelewa kwamba matokeo ya utafiti huu huenda usinifaidi binafsi lakini huenda ukawa wa manufaa siku zijazo kwa waathiriwa wengine kama nilivyo. Kuna uwezekano utafiti huu utaongeza maarifa kwa taaluma ya utabibu kuhusu **Uchanganuzi wa viwango vya oksijeni na hali ya kuponya katika vidonda kuhusiana na mbinu ya NPWT.**

Sahihi/ kibali halisi _____

[**Mlezi wa mtoto aliye chini ya miaka 18**]

Sahihi/ haitajiki _____

Tarehe (SS/mm/MM)_____/_____20_____

Alama ya kidole kwa asiye na elimu

Taarifa ya shahidi ya makubaliano na mhusika asiyejua kusoma

Nimeshuhudia mgonjwa akisomewa kwa njia inayoeleweka kwa rahisi, naye akapewa fursa nzuri ya kuulaza maswali. Nina dhibitisha mhusika alipeana kibali kwa hiari yake mwenyewe

Jina la shahidi _____

Sahihi ya shahidi_____Tarehe (SS/mm/MM)_____/_____20_____

SEHEMU C: TAARIFA YA MTAFITI

Nimesomea mhusika na kadiri ya uwezo wangu kumuelewesha yafuatayo:

- Habari zozote zitokazo kwake zitawekwa siri.
- Kukataa kupeana kibali cha kuhusishwa kwa utafiti huu haitaathiri matibabu anayostahili.
- Matokeo ya utafiti huu kwa jumla utachapishwa katika jarida la kisayansi au utabibu ama upasuaji kuweza kuchangia maarifa kuhusu **Uchanganuzi wa viwango vya oksijeni na hali ya kuponya katika vidonda kuhusiana na mbinu ya NPWT.**

Nimehakikisha kwamba mhusika amepewa fursa kamili ya kuuliza maswali kuhusu kuhusika kwake kwa utafiti huu na kwamba kwa kadiri ya uwezo wangu nimemuelewesha ipasavyo.

Nina hakika kwamba mhusika hajalazimishwa kupeana kibali kuhusika kwenye utafiti huu bali amekubali kwa hiari yake.

Nakala ya kibali hiki kimewasilishwa kwa mhusika naye akatia sahihi ipasayvo.

Jina la mtafiti anayechukua kibali cha mhusika _____

Sahihi ya huyo matafiti_____Tarehe (SS/mm/MM)_____/_____20_____

APPENDIX C: KNH-UoN ERC APPROVAL

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
Tel:(254-020) 2726300 Ext 44355

KNH-UON ERC
Email: uonknh_erc@uonbi.ac.ke
Website: http://www.erc.uonbi.ac.ke
Facebook: https://www.facebook.com/uonknh.erc
Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERC

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/403

16th November 2020

Dr. Matwa Christopher Ondieki
Reg. No.H58/88012/2016
Dept.of Surgery
School of Medicine
College of Health Sciences
University of Nairobi

Dear Dr. Ondieki

RESEARCH PROPOSAL – THE EFFECTIVENESS OF NEGATIVE PRESSURE WOUND THERAPY IN INDUCING HYPOXIA COMPARED TO STANDARD OCCLUSIVE DRESSING WOUND THERAPY IN TRAUMATIC WOUNDS IN WOUND HEALING (P299/05/2020)

This is to inform you that the KNH- UoN Ethics & Research Committee (KNH- UoN ERC) has reviewed and **approved** your above research proposal. The approval period is 16th November 2020 – 15th November 2021.

This approval is subject to compliance with the following requirements:

a. Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b. All changes (amendments, deviations, violations etc.) are submitted for review and approval by KNH-UoN ERC before implementation.
c. Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UoN ERC within 72 hours of notification.
d. Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e. Clearance for export of biological specimens must be obtained from KNH- UoN ERC for each batch of shipment.
f. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
g. Submission of an *executive summary* report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/ or plagiarism.

For more details consult the KNH- UoN ERC website**http://www.erc.uonbi.ac.ke**

Yours sincerely,

PROF. M. L. CHINDIA
SECRETARY, KNH-UoN ERC

c.c. The Principal, College of Health Sciences, UoN
The Senior Director, CS, KNH
The Chairperson, KNH- UoN ERC
The Assistant Director, Health Information Dept, KNH
The Dean, School of Medicine, UoN
The Chair, Dept. of Surgery, UoN
Supervisors: Dr. Ferdinand W. Nangole, Dept.of Surgery, UoN
Prof. Stanley O.Khainga, Dept.of Surgery, UoN

APPENDIX D: KNH MEDICAL RESEARCH APPROVAL

DR. MATWA CHRISTOPHER O.

RESIDENT PLASTIC, RECONSTRUCTION AND AESTHETIC SURGEON,

UNIVERSITY OF NAIROBI.

P.O BOX 478 – 00515, NAIROBI.

CELL: +254 727 435071 EMAIL: chrimommv@yahoo.com

19TH NOVEMBER, 2020.

Please register this study
Dr Ngumbau
23/11/2020

THE DEPUTY DIRECTOR,

MEDICAL RESEARCH,

KENYATTA NATIONAL HOSPITAL.

Dear Sir/Madam,

RE: REQUEST TO COLLECT DATA FOR MMed (PRAS) DISSERTATION RESEARCH

I am a resident Plastic, Reconstruction and Aesthetic Surgeon in my fifth year now and as part of the requirements to be conferred the Master's Degree in my field of study, a dissertation is mandatory.

The objective of my dissertation is to establish whether Negative Pressure Wound Therapy (NPWT) induces wound hypoxia as a mechanism of action. It will involve subjecting some patients with traumatic wounds to NPWT and comparing the levels of Hypoxia Inducible Factor 1-alpha (HIF-1a) & Fibroblast Activation Proteins (FAP) to patients with traumatic wounds dressed with standard occlusive dressings.

Data collection will involve wound tissue biopsy before, in the middle and end of the either therapy which lasts seven days. The tissue biopsies will be transported to KAVI laboratories (UoN) for processing and analysis.

I will involve a nurse namely Mr. Ringera who is vast in wound and stoma care. He is an employee of KNH and manages both in and out-patients with wounds.

As precautions during this covid-19 pandemic, standard PPEs for myself, research assistant and patient will be used at the three occasions of contact. Only in-patients will be included in this study therefore monitoring will be effectively done. After consenting to the study, local anesthesia will be used to reduce pain and control bleeding during biopsies.

Also find attached the dissertation proposal abstract and approval of the same from KNH-UoN ERC.

Kindly regard my application and looking forward to your feedback.

Regards

Dr. Matwa Christopher

KNH/R&P/FORM/01

KENYATTA NATIONAL HOSPITAL
P.O. Box 20723-00202 Nairobi

Tel.: 2726300/2726450/2726565
Research & Programs: Ext. 44705
Fax: 2725272
Email: knhresearch@gmail.com

Study Registration Certificate

1. Name of the Principal Investigator/Researcher
DR MATWA CHRISTOPHER

2. Email address: chrismmw@yahoo.com Tel No. 0727435071

3. Contact person (if different from PI) N/A

4. Email address: N/A Tel No.: N/A

5. Study Title
THE EFFECTIVENESS OF NEGATIVE PRESSURE WOUND THERAPY IN INDUCING HYPOXIA COMPARED TO STANDARD OCCLUSIVE DRESSING WOUND THERAPY IN TRAUMATIC WOUNDS IN WOUND HEALING

6. Department where the study will be conducted PLASTIC & RECONSTRUCTION SURGERY
(Please attach copy of Abstract)

7. Endorsed by KNH Head of Department where study will be conducted.

Name: DR BENJAMIN WAIBIRE Signature [signature] Date 01/12/2020

8. KNH UoN Ethics Research Committee approved study number P299/06/2020
(Please attach copy of ERC approval)

9. I DR. MATWA CHRISTOPHER commit to submit a report of my study findings to the Department where the study will be conducted and to the Department of Medical Research.

Signature [signature] Date 01/12/2020

10. Study Registration number (Dept/Number/Year) SURG/NATIONAL/131/2020
(To be completed by Medical Research Department)

11. Research and Program Stamp

KENYATTA NATIONAL HOSPITAL
01 DEC 2020
P.O. Box 20723 - 00202 KNH
Resource Centre

All studies conducted at Kenyatta National Hospital **must** be registered with the Department of Medical Research and investigators **must commit** to share results with the hospital.

APPENDIX E: HIF-1α ASSAY PROCEDURE

Sample Preparation

A. Sample Handling

- Serum; EDTA, heparin plasma; and culture lysates may be tested in this ELISA.
- 50µL per well of serum, plasma or culture lysate is required.
- Store samples at 2-8°C and assay within 24 hours. For long-term storage, aliquot and freeze samples at -70°C.
- Avoid repeated freeze-thaw cycles when storing samples.
- Test samples and standards must be assayed in duplicate or triplicate each time the ELISA is performed.
- Mix samples by gently inverting tubes.
- If samples are clotted, grossly hemolyzed, lipemic or microbially contaminated, or if there is any question about the integrity of a sample, make a note on the template and interpret the results with caution.

B. Sample Dilution

- If the HIF-1A concentration from the sample will exceed the highest point of the standard curve (i.e., 20,000pg/mL), prepare one or more five-fold dilutions of the test sample. When testing culture lysates, prepare the serial dilutions using the culture medium. When testing serum or plasma, prepare the serial dilutions using the provided Standard Diluent. For example, a five-fold dilution is prepared by adding 0.1mL (100µL) of test sample to 0.4mL (400µL) of appropriate diluent. Mix samples thoroughly between dilutions before assaying.

Reagent Preparation

For procedural differences when using partial plates, look for (PP) throughout these instructions.

Wash Buffer

1. Label a new glass or plastic 2L container “Wash Buffer.” The 30X Wash Buffer may have a cloudy appearance.

2. Add entire contents of the 30X Wash Buffer (50mL) bottle to a 2L container and dilute to a final volume of 1.5L with ultrapure water. Mix thoroughly.

(PP) When using partial plates, store the reconstituted Wash Buffer at 2-8°C.

Note: Wash Buffer must be at room temperature before use in the assay. Do not use Wash Buffer if it becomes visibly contaminated during storage.

Standards

(PP) Reconstitute with Standard Diluent and use one vial of the lyophilized Standard per partial plate.

1. Prepare Standards just before use and use within one hour of reconstitution. Do not store reconstituted standards.

2. When testing culture lysate samples, reconstitute Standard with Standard Diluent. Reconstitution volume is stated on the Standard vial label. The Standard will dissolve in ~1 minute. Mix by gently inverting vial. Use the sample culture medium to prepare standard curve dilutions.

When testing serum or plasma samples, reconstitute Standard with Standard Diluent. Reconstitution volume is stated on the Standard vial label. The Standard will dissolve in ~1 minute. Mix by gently inverting vial. Use the Standard Diluent provided to prepare the standard curve serial dilutions.

When testing serum, plasma and cell culture lysate samples on the same plate, validate the media to establish if the same standard curve can be used for the different sample types. Prepare a standard curve (including a zero/blank) using culture medium to reconstitute and dilute the Standard. Use medium containing serum or other protein to maximize stability of the HIF-1A. Perform this curve in parallel with a standard curve reconstituted in Standard Diluent and diluted in the Standard Diluent provided. If the OD values of the two curves are within 10% of the mean for both curves, then the assay can be performed with Standard Diluent, whether you are testing culture lysate, plasma or serum samples.

3. Label eight tubes, one for each standard curve point: 20,000, 8,000, 3,200, 1,280, 512, 204.8, 81.92, and 0pg/mL. Prepare a 1:2 dilution of the reconstituted standard and then 1:2.5 serial dilutions for the remaining standards as follows:

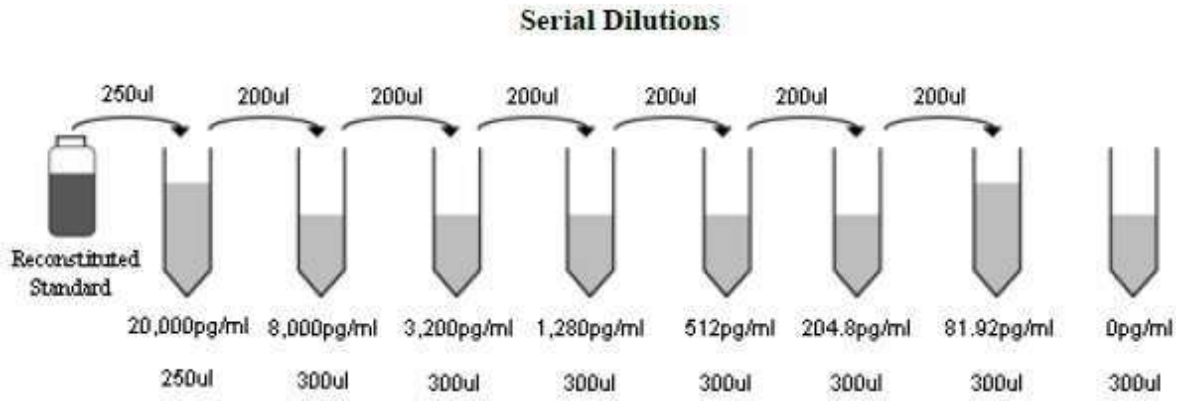
4. Pipette 250µL of appropriate diluent into the first tube (i.e., 20,000pg/ml).

5. Pipette 300µL of appropriate diluent into all of the remaining tubes.

6. Pipette 250µL of the reconstituted standard into the first tube (i.e., 20,000pg/mL) and mix.

7. Pipette 200µL of this dilution into the second tube (i.e., 8,000pg/mL) and mix.

8. Repeat the serial dilutions (using 200µL) five more times to complete the standard curve points. These concentrations, 20,000, 8,000, 3,200, 1,280, 512, 204.8, 81.92, and 0pg/mL are the standard curve.



Assay Procedure

A. Calibrator and Sample Incubation

 (PP) Determine the number of strips required. Leave these strips in the plate frame. Tightly seal remaining unused strips in the provided foil pouch with desiccant and store at 2-8°C. After completing the assay, retain the plate frame for second partial plate. When using the second partial plate, place strips securely in the plate frame.

 Use the Plate Template provided to record the locations of the zero standard (blank or negative control), HIF-1A standards and samples. Perform seven standard points and one blank in duplicate with each series of unknown samples.

 If using a multichannel pipettor, use a new reagent reservoir to add the Biotinylated Antibody Reagent. Remove from the vial only the amount required for the number of strips being used.

1. Add 50µL of diluted standards and test samples to each well. Mix well by gently tapping the plate several times.

2. Carefully cover the plate with an adhesive plate cover. Ensure all edges and strips are tightly sealed by running your thumb over the edges and down each strip. Incubate for 2 hours at room temperature (20-25°C) on a plate shaker.

3. Carefully remove the adhesive plate cover. Wash plate THREE times with Wash Buffer as described in the Plate Washing Section (Section B).

B. Plate Washing

1. Gently squeeze the long side of the plate frame before washing to ensure all strips securely remain in the frame.

2. Empty plate contents. Use a squirt bottle to vigorously fill each well completely with Wash Buffer, then empty plate contents. Repeat procedure two additional times for a total of THREE washes. Blot plate onto paper towels or other absorbent material.

Note: For automated washing, aspirate all wells and wash THREE times with Wash Buffer, overfilling wells with Wash Buffer. Blot plate onto paper towels or other absorbent material.

C. Biotinylated Antibody Reagent Incubation

1. Add 50µl of Biotinylated Antibody Reagent to each well.

2. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate the plate for 1 hour at room temperature (20-25°C) on a plate shaker.

3. Carefully remove the adhesive plate cover, discard the plate contents and wash THREE times as described in the

4. Plate Washing Section.

D. Streptavidin-HRP Solution Preparation and Incubation

□ Prepare Streptavidin-HRP Solution just before use. Do not prepare more solution than required.

□ Do not store the prepared Streptavidin-HRP Solution.

□ Use a 15mL plastic tube to prepare the Streptavidin-HRP Solution.

□ Use a new reagent reservoir and pipette tips when adding the prepared Streptavidin-HRP Solution.

1. Briefly centrifuge the Streptavidin-HRP Concentrate to force entire vial contents to the bottom.

2. (PP) Use only the Streptavidin-HRP Solution amount required for the number of strips being used. For each strip, mix 2.5µL of Streptavidin-HRP Concentrate with 1mL of HRP Dilution Buffer. Store Streptavidin-HRP Concentrate reserved for additional strips at 2-8°C.

3. For one complete 96-well plate, add 30µL of Streptavidin-HRP Concentrate to 12mL of HRP Dilution Buffer and mix gently.

4. Add 50µL of prepared Streptavidin-HRP Solution to each well.

5. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate the plate for 30 minutes at room temperature (20-25°C) on a plate shaker.

6. Carefully remove the adhesive plate cover, discard the plate contents and wash THREE times as described in the Plate Washing Section.

E. Substrate Incubation and Stop Solution Addition

Use new disposable reagent reservoirs when adding the TMB Substrate Solution and Stop Solution.

From the bottle, dispense ONLY the required amount of 100µL per well for the number of wells being used. Do not use a glass pipette to measure the TMB Substrate Solution.

(PP) Do not combine leftover substrate with that reserved for the second partial plate. Take care not to contaminate the remaining TMB Substrate.

1. Pipette 100µL of TMB Substrate Solution into each well.

2. Allow color reaction to develop at room temperature in the dark for 30 minutes. Do not cover plate with aluminum foil or a plate sealer. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.

3. After 30 minutes, stop the reaction by adding 100µL of Stop Solution to each well.

F. Absorbance Measurement

Evaluate the plate within 30 minutes of stopping the reaction. Measure absorbance on an ELISA plate reader set at 450nm and 550nm. Subtract 550nm values from 450nm values to correct for

optical imperfections in the microplate. If an absorbance at 550nm is not available, measure the absorbance at 450nm only.

Note: When the 550nm measurement is omitted, absorbance values will be higher.

G. Calculation of Results

 The standard curve is used to determine HIF-1A amount in an unknown sample. Generate the standard curve by plotting the average absorbance obtained for each Standard concentration on the vertical (Y) axis vs. the corresponding HIF-1A concentration (pg/mL) on the horizontal (X) axis.

 Calculate results using graph paper or curve-fitting statistical software. Determine the HIF-1A amount in each sample by interpolating from the absorbance value (Y-axis) to HIF-1A concentration (X- axis) using the standard curve.

 If the test sample was diluted, multiply the interpolated value obtained from the standard curve by the dilution factor to calculate pg/mL of HIF-1A in the sample.

 Absorbance values obtained for duplicates should be within 10% of the mean value. Carefully consider duplicate values that differ from the mean by greater than 10%.

APPENDIX F: FAP ASSAY PROCEDURE

Procedural guidelines

Review the Procedural guidelines and Plate washing directions in the ELISA Technical Guide at thermofisher.com for details prior to starting the procedure.

Reagents are lot-specific. Do not mix or interchange different reagent lots from various kit lots.

Prepare 1X Wash Buffer

1. Allow Wash Buffer Concentrate (20X) to reach room temperature and mix to redissolve any precipitated salts.

2. Dilute 20 mL of the Wash Buffer Concentrate into 380 mL of deionized or distilled water. Label as 1X Wash Buffer.

3. Store the concentrate and 1X Wash Buffer in the refrigerator. Use the diluted buffer within one month.

Prepare diluent

Assay Diluent B should be diluted 5-fold with deionized or distilled water before use.

Prepare biotin conjugate

1. Briefly spin down the biotin conjugate before use.

2. Add 100 µL of 1X Assay Diluent B into the vial to prepare a biotin conjugate concentrate.

3. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days).

4. The biotin conjugate concentrate should be diluted 80-fold with 1X Assay Diluent B and used in step 2 of ELISA procedure.

Sample preparation guidelines

Collect samples in pyrogen/endotoxin-free tubes.

Freeze samples after collection if samples will not be tested immediately. Avoid multiple freeze-thaw cycles of frozen samples.

Thaw completely and mix well (do not vortex) prior to analysis.

Avoid the use of hemolyzed or lipemic sera. If large amounts of particulate matter are present in the sample, centrifuge or filter sample prior to analysis.

Pre-dilute samples

Assay Diluent A should be used for dilution of serum and plasma samples. 1X Assay Diluent B should be used for dilution of cell culture supernatant samples.

Dilute serum and plasma 200-fold.

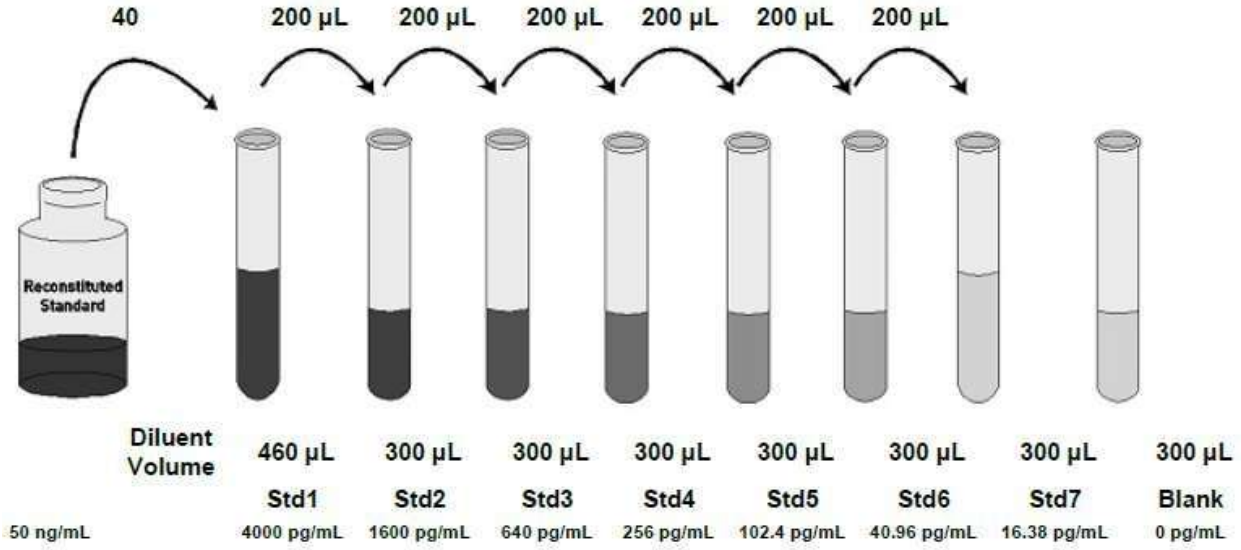
Because conditions may vary, it is recommended that each investigator determine the optimal dilution to be used for each application.

Dilute standards

Note: Use glass or plastic tubes for diluting standards.

1. Briefly spin down a vial of lyophilized standard.

2. Add 400 µL Assay Diluent A (for serum/plasma samples) or 1X Assay Diluent B (for cell culture supernatants) into the lyophilized standard vial to prepare a 50 ng/mL standard. Dissolve the powder thoroughly by gentle mixing. Add 40 µL FAP standard (50 ng/mL) from the vial of reconstituted standard, into a tube with 460 µL Assay Diluent (A or B) to prepare a 4,000 pg/mL standard solution. Pipette 300 µL Assay Diluent (A or B) into each tube. Use the 4,000 pg/mL standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Assay Diluent (A or B) serves as the zero standard (0 pg/mL).



Prepare 1X Streptavidin-HRP solution

Note: Prepare the Streptavidin-HRP within 15 minutes of usage.

1. Briefly spin the Streptavidin-HRP and pipette up and down to mix gently before use, as precipitates may form during storage.

2. Dilute Streptavidin-HRP 200-fold with 1X Assay Diluent B.

3. Do not store diluted solution for future use.

Perform ELISA (Total assay time: 4 hours and 45 minutes)

Allow all reagents to reach room temperature before use. Mix all liquid reagents prior to use.

IMPORTANT! Perform a standard curve with each assay.

Determine the number of 8-well strips required for the assay. Insert the strips in the frames for use. Re-bag any unused strips and frames, and store at 2 to 8°C for future use.

1. a. Bind antigen a. For the standard curve, add 100 µL of standards to the appropriate wells (see Dilute standards). For samples, add 100 µL of diluted samples (see Dilute samples) to the wells.

b. Cover wells and incubate for 2.5 hours at room temperature or overnight at 4°C with gentle shaking.

c. Discard the solution and wash 4 times with 1X Wash Buffer. Wash by filling each well with Wash Buffer (300 µL) using a multi-channel Pipette or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

2. a. Add biotin conjugate a. Add 100 µL of prepared biotin conjugate (see Prepare biotin conjugate) to each well.

b. Incubate for 1 hour at room temperature with gentle shaking.

c. Discard the solution. Repeat the wash as in step 3.

3. a. Add Streptavidin-HRP a. Add 100 µL of prepared Streptavidin-HRP solution (see Prepare Streptavidin-HRP solution) to each well.

b. Incubate for 45 minutes at room temperature with gentle shaking.

c. Discard the solution. Repeat the wash as in step 3.

4. a. Add TMB substrate a. Add 100 µL of TMB Substrate to each well. The substrate will begin to turn blue.

b. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

5 Add stop solution Add 50 µL of Stop Solution to each well. Tap the side of the plate gently to mix. The solution in the well changes from blue to yellow.

Read the plate and generate the standard curve

1. Read the absorbance at 450 nm. Read the plate within 30 minutes after adding the Stop Solution.

2. Use curve-fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit. Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls prior to plotting.

3. Read the concentrations for unknown samples and control from the standard curve. Multiple value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution.