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Extracellular Microvesicles as a Novel Biomarker for Wound Healing

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Extracellular Microvesicles as a Novel Biomarker for Wound Healing

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY **WALID MARI** entitled **EXTRACELLULAR MICROVESICLES AS A NOVEL BIOMARKER FOR WOUND HEALING** BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract:

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Introduction: Wound healing is a sophisticated dynamic process that involves complex coordination among a variety of resident cells in a suitable extracellular environment. Chronic wounds are defined as wounds that fail to heal within a period of 3 months. Negative pressure wound therapy (NPWT) is a closed suction drainage system is used to enable mass transport of fluid from the body as an adjunct to surgical procedures. (Oasis-ultra) is a triple layer extracellular matrix containing different types of glycosaminoglycans (GAGs) like heparin sulfate, hyaluronic acid and chondroitin sulfate. In addition, it has adhesion molecule such as fibronectin and laminin along with various growth factors. Extracellular vesicles, (EVs); microvesicles and exosomes are a diverse family of membrane bound vesicles laden with various proteins, nucleic acids, and lipids that cells release to the extracellular environment. EVs play a crucial role in cell to cell communication, as they carry signaling molecules, like lipids, proteins, mRNAs, and miRNAs. We hypothesize that there is a correlation between the concentration of EMVs and the percentage of wound healing in treated chronic wounds and the healing percentage will be enhanced by the combination of Oasis ultra and NPWT. A prospective, multi-centered, randomized, single-blinded clinical trial that permitted by the Ethics Committee of the Copernicus was conducted to study whether the combination of Oasis ultra and NPWT will enhance the healing of chronic wounds when compared with only NPWT, to measure the concentration of EMVs in the collected wound fluids and correlate it with the percentage of wound healing and to analyze the extracellular

microvesicles composition to look for growth factors, chemokines and cytokines that play role in the wound healing

Material and Methods: Wound fluid samples obtained from 14 patients with stage IV trunk pressure ulcers. The patients were divided in two groups (7 in each group, n=7): control group on negative pressure wound therapy (NPWT) alone & study group with NPWT plus Oasis Ultra dressing. NPWT was replaced two times a week, and Oasis Ultra was applied once weekly for all subjects. Wound size was measured every week and healing percentage was calculated for the whole 12 weeks of the study. A canister of NPWT device (wound VAC) was collected from the patients every four weeks of the study and brought to the lab where a hole was made in the canister by using a drill to drain the fluids. Protease inhibitor added to the fluid before the fluid stored in a -80°C freezer. EMVs were isolated using Differential Ultracentrifugation methods, and EMVs concentration in wound fluid was measured by Nanoparticle Tracking Analysis machine (Nanosight). EMVs were analyzed for cytokines and growth factors using BioPlex Pro cytokine assays after determining protein concentration using Bradford assay.

Results: Our data showed that overall healing percentage in the study group after 12 weeks of study was ~ **89%** as compared with the control group, which was ~ **52% ($P < 0.05$)**. Further, to examine the correlation between EMVs and wound healing percentage, simple linear regression was conducted using SPSS and Prism pad soft wares. The data showed that there is a strong positive correlation between EMVs concentrations in wound fluid and the healing percentage, the R-square was **0.66,** and **($P \leq 0.05$).** The study group shows high levels of intravesicular concentration of pro-healing cytokines and low levels pro-inflammatory cytokines; however, control group shows high levels of intravesicular

concentration of pro-inflammatory cytokines and low levels of pro-healing cytokine

Conclusion: Our clinical trial demonstrates that using Oasis-Ultra combined with NPWT hasten the healing percentage in stage IV pressure ulcers. There is a correlation between EMVs concentration in wound fluid and the healing percentage of the wound. EMVs composition could determine the outcomes of the wound healing. The EMVs particles in wound fluid could serve as a biomarker of the wound healing.

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Author

Walid Mari.

Introduction:

Wound healing is a dynamic process in which tissue repair itself. Moreover, all tissues in the human body have the ability of healing, either by regeneration or repair mechanisms. Regeneration is a process that is simple in concept, i.e., to replace the damaged tissue with identical cells. On the contrary, repair is the main mechanism by which injured tissues is replaced by connective tissue, which leads to scar formation [3]. The wound healing process progresses through interactive and overlapping phases, i.e., hemostasis, inflammation, proliferation, and remolding. During this process, local cells, inflammatory mediators, and extracellular matrix actively participate in the four phases of healing [1]. Furthermore, overlaid on the healing process is a series of precisely controlled processes corresponding to different cell types in the wound throughout each specific phase of healing. Tissue damage initiates the healing process that continues as a series of time-dependent phases: (i) homeostasis and coagulation phase starts immediately after injury; (ii) inflammatory phase, which starts shortly after that; (iii) proliferation phase occurs within days of injury; and (iv) remolding phase where the scar tissue formed and may remain up two years [2]. Immediately after injury, the hemostasis phase is initiated and is characterized by transient vasoconstriction at the wound site, due to a temporary neuronal reflex. This transient vasoconstriction decreases or stops the blood flow in post capillary venules to allow blood cells to marginate from the center of the blood flow. At this point, the cells are close to the endothelial wall of the blood vessel and migrate to the site of injury. In addition, hemostatic clotting forms by activation of the coagulation cascade that is activated by intrinsic and extrinsic pathways leading to platelet aggregation and formation of a hemostatic plug. This hemostatic plug provides a

matrix for invading cells to migrate to the area of injury and start the healing process. Platelets release cytokines and growth factors to recruit immune cells, endothelial cells and fibroblast that act to start the process of healing.

Inflammatory phase:

The inflammatory phase starts within hours after the hemostatic phase and lasts up to 7 days [8]. The inflammatory phase can be subdivided into an early inflammatory phase and a late inflammatory phase (Velnar et al., 2009). The early inflammatory phase activates the complement cascade and initiates a series of molecular events causing infiltration of the wound by leukocytes to fight infection [2]. In the early inflammatory phase, the endothelial cells upregulate selectin molecules (P selectin, E selectin) that cause leukocyte rolling over the wall of blood vessels, and cellular adhesion molecules (ICAM, VCAM) to interact with integrin on the leukocyte leading to firm adhesion of the leukocyte to the wall of blood vessels [9]. Leukocytes like neutrophils and macrophages migrate to the site of injury simultaneously, yet the neutrophil comes first due to their large quantity in the blood [11]. Neutrophils start to be attracted to the wound location within 24 to 36 hours after injury by a variety of chemo-attractant agents like IL8, C3a, C5a, bacterial product (formylmethionyl peptides), and platelet product [9]. Leukocytes migrate to the site of injury using diapedesis, which is a process by which leukocyte squeeze between the endothelial cells to reach to injury site [2]. Neutrophils fight bacterial infection by using oxygen-dependent killing through the release reactive oxygen species (ROS) and release proteases to cleanse wound of cell debris. Once neutrophils finish their task of removing all bacteria, its activity decreases. Ultimately, before going to the next stage of healing, the neutrophils must be removed from the wound. The

dominant cell in the late inflammatory stage is the macrophage, which arrives at the wound site 48 to 72 hours after injury. The macrophage is a blood monocyte that undergo to phenotypic changes upon its arrival to the wound site. Macrophages are attracted to the wound site by chemokine agents, including C3a, C5a, cytokines like PDGF, TGF-β, and platelet factor IV, in addition to elastin, and collagen products. Macrophages can work in low pH and have a longer lifespan than neutrophils [2]. Macrophages are the key regulator cell that switch the healing process from inflammation to subsequent stages of healing. Macrophages can release anti-inflammatory cytokines such IL10 and TGF-β to promote resolution and healing or release pro-inflammatory mediators such as IL8 to continue acute inflammation by recruiting more neutrophil [9]. The last cells to come to injury site are lymphocytes, which come to the wound site within 72 hours after the injury attracted by IL1, Immunoglobulin G (IgG) product, and complement product.

Proliferative phase:

At the end of the inflammatory phase, the proliferative phase begins and proliferation becomes a dominant process. Proliferation is characterized by re-epithelization of the wound bed, angiogenesis to reestablish the wound vascularity, and granulation tissue formation [14]. Re-epithelialization starts hours to a day after an injury and involves migration and proliferation of keratinocytes that are derived from epithelial stem cells of the skin. There are two main populations of the epithelial stem cells in the skin. First to proliferate are the inter-follicular stem cells that are found in the basal layer of the inter-follicular epidermis (IFE), and the hair follicle (HF) bulge epithelial stem cells that are found in the outer root sheath (ORS) of the permanent part of the hair follicle where the erector Pili muscle insert [15]. These stem cells start their proliferation

within two to three days of the injury. Afterward, migration is elicited by loss of contact inhibition and physical pressure at the cellular adhesion molecules (hemi-desmosomes and desmosomes), which trigger the downstream signal to allow calcium to induce restructuring of the cytoskeleton for migration. Simultaneously, collagenase and elastase release keratinocytes from their original locations. When the cell comes into contact with each other and a new adhesion molecules formed, the migration process stops. Furthermore, the keratinocytes release protein to rebuild the basement membrane. The re-epithelialization process is triggered by many wound-associated signals, e.g., nitric oxide (NO), which is produced by macrophages during early stages of wound healing [16], growth factors, including epidermal growth factors (EGF) KGF, IGF-1, and nerve growth factor (NGF) that are released from multiple cell types in the wounds.

Angiogenesis is a process by which new blood vessel formation is established to supply the damaged area with critical oxygen and nutrients that are required during wound healing. Angiogenesis is started by growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and the serine protease thrombin. The remaining endothelial cells of the existing vessels proliferate and migrate toward the angiogenic stimulus. These cells initially form the lumen of the vessels and then differentiate into venules and arteries and mature by involving smooth muscles and pericytes. Further, endothelial cells derived from the bone marrow also form blood vessels de novo [16]. In the proliferative phase, the provisional matrix made during hemostasis is changed by new granulation tissue. The new granulation tissue consists of the enormous amount of blood vessels, macrophages,

granulocytes, fibroblasts, and collagen bundles to retrieve the structure and function and the injured skin to a certain limit.

The fibroblast is the major role player of the proliferative phase and it plays a critical role in the process of granulation tissue formation. In response to cytokines and growth factors like PDGF, transforming growth factor (TGF-□) and bFGF that are produced by platelets and macrophages, respectively, fibroblasts migrate from the adjacent dermis to the wound. Furthermore, fibroblasts may also originate from bone marrow–derived mesenchymal progenitor cells called fibrocytes. These cells circulate in the blood and migrate to the site of a skin injury to induce healing by secreting cytokines, growth factors and chemokines, enhance angiogenesis, and serve as antigen presenting cells. Once in the provisional matrix of the wound, fibroblasts proliferate and secrete proteases, e.g., matrix metalloproteases (MMPs) to breakdown the provisional matrix and on the same time deposit collagen and extracellular matrix (ECM) elements like, hyaluronic acid, glycosaminoglycans, proteoglycans, and fibronectin to form granulation tissue which seal the wound cavity and serve as a scaffold for cell migration and adhesion during the wound healing process [16].

Remodeling Phase:

The remodeling process is initiated toward the end of granulation tissue development. Fibroblasts are induced by mechanical tension and cytokines like TGF-β to differentiate into myofibroblasts, which contracts the wound through the expression of smooth muscle actin (SMA). After the wound healing process is accomplished, myofibroblasts undergo apoptosis. During this phase, collagen type I, which has high tensile strength, replaces type III collagen in the extracellular matrix (ECM). Finally, the

number of newly formed vessels and blood flow decreases, and mature environment that lack of cells and blood supply is established. Ultimately, Skin appendages like sweat glands and hair follicles cannot be retrieved after extreme damage; however, the skin can regain up to 80% of the original tensile strength [16]

Chronic wounds and Pressure Ulcers:

Chronic wounds are defined as wounds that fail to heal within a period of 3 months. Additionally, chronic wounds represent a significant health care problem since they affect more than 1% of the western population. Chronic wounds also contribute to an enormous financial burden on the health care system, resulting in annually additional health care costs of more than $25 billion in the United States [22]. Common chronic wounds categories are pressure ulcers, diabetic foot ulcers, venous ulcers, and ischemic ulcers [19]. Per the National Pressure Ulcer Advisory Panel (NPUAP) “a pressure injury is localized damage to the skin and underlying soft tissue usually over a bony prominence or related to a medical or another device”. The damage could be presented as unbroken skin or wide-open ulcer and might be painful. The injury arises due to sever or continued pressure or combined shear and pressure. Soft tissue may exhibit different tolerance to pressure and shear and that depends up tissue perfusion, microclimate, nutritional status of the patients, comorbidities, and the condition of the soft tissue [20].

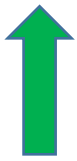
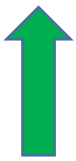
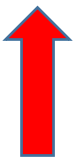
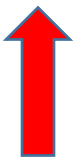
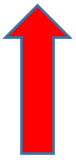
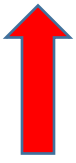
Pressure ulcers are classified into stages as follows: Stage 1 Pressure ulcer: Non-blanchable erythema of intact skin. Stage 2 Pressure Injury: Partial-thickness skin loss with exposed dermis. Stage 3 Pressure Injury: Full-thickness skin loss. Stage 4 Pressure Injury: Full-thickness skin and tissue loss with exposed or directly palpable fascia, muscle, tendon, ligament, cartilage or bone in the ulcer. Unstageable Pressure Injury:

Obscured full-thickness skin and tissue loss. Deep Tissue Pressure Injury: Persistent non-blanchable deep red, maroon or purple discoloration (NPUAP, April 2016) [20]. Pressure ulcers occur in different body areas include trunk, limbs, and buttocks; however, sacrum and heel are the most vulnerable areas to pressure since they have a thin layer of soft tissue and more prone to develop pressure ulcers [18]. These ulcers, as a chronic wound are thought to be 'stuck' in the inflammatory phase of wound healing. The current concept is that a local increase in the level of proteases like MMPs in the wound bed. The hypoxic microenvironment breaks down important growth factors and thus prevents the wound from continuing to the next phase of healing (proliferative phase) to form granulation tissue and new provisional matrix for remolding and healing [19].

Highly addressed growth factors and interleukins in wound healing:

Complex induction of signals via cytokines, growth factors, and chemokines to coordinate cellular process is imperative for the successful wound healing process. Those biologically active polypeptides impact wound healing through affecting target cell differentiation, metabolism, and growth by paracrine, autocrine, endocrine or juxtacrine mechanisms. Binding these cytokines with its corresponding receptors stimulate molecular pathways that regulate cell cycle, motility, and differentiation.

Table 1: Highly addressed cytokines in wound healing.

Cytokines	Cells	Function	Level in Acute Wound	Levels in Chronic Wounds
PDGF	Platelets Macrophages Endothelial cells Keratinocytes Fibroblasts.	1. Inflammation 2. formation of granulation tissue 3. Re-epithelialization 4. Matrix formation 5. Remodeling.		
FGF-2 or basic FGF	Mast Cells Keratinocytes Fibroblasts Smooth muscle cells Chondrocytes Endothelial cells	1. Granulation tissue formation 2. Re-epithelialization 3. Matrix formation 4. Remodeling		
VEGF	Neutrophils Platelets Macrophages Smooth muscle cells Fibroblasts Endothelial cells	formation of granulation tissue		
IL-1	Monocytes Macrophages Neutrophils Keratinocytes	1. Inflammation 2. Re-epithelialization		
IL-6	Monocytes Macrophages Neutrophils Keratinocytes	1. Inflammation 2. Re-epithelialization		
TNF-a	Monocytes Macrophages Neutrophils Keratinocytes	1. Inflammation 2. Re-epithelialization		

Adapted from Barrientos, 2008 [31].

Platelet derived growth factor (PDGF):

Platelet Derived growth factor-(PDGF) is one of the growth factors that plays a crucial role in wound healing, where it enhances cell proliferation, angiogenesis and chemotaxis response. PDGF is classified based on its structure as a disulfide dimeric protein comprised of different polypeptide chains (A, B, C, and D). PDGF conveys its signals through tyrosine kinase receptors, e.g., PDGF-aR or PDGF-bR, and the downstream extracellular signal kinase (ERK) pathway [21]. It is released from platelets, fibroblast, endothelial cells, and macrophages. PDGF signaling plays a crucial role in both cell migration and proliferation. In fact, scientific evidence shows that PDGFR-a signaling pathway can oversee fibroblast translocation throughout this process. PDGF plays an important role in each stage of healing. Platelets store PDGF in their alpha granule and during injury release it in the wound fluid. In the wound, PDGF serves as a strong chemo-attractant for both fibroblast, smooth muscle cells, neutrophils and monocytes [31]. PDGF induces macrophages to release growth factors such as TGF-b, that along with PDGF enhances macrophage mediated tissue debridement and formation of granulation tissue. Along with VEGF-A, PDGF-BB is the gene product that regulate hypoxia contribute to angiogenesis [26]. Moreover, in vitro PDGF shows ability to stimulate formation of new blood vessels through enhancing VEGF expression synergistically with hypoxia (ischemic injury). PDGF upregulate the synthesis and production of IGF-1, which has been shown to induce migration of keratinocytes. PDGF plays a crucial role in re-epithelialization and thrombospondin-1 that delays proteolytic response and induce proliferative response in wound healing [31]. Further, PDGF also stimulates fibroblast proliferation and controls

collagen production, which are important during the proliferative phase of wound healing [26]. PDGF stimulate pericyte production of extracellular matrix components such as collagen, fibronectin, and proteoglycans. PDGF upregulates MMP enzymes and helps to degrade old collagen which is important during tissue remodeling. PDGF levels is increased in acute wound and decreased in chronic wound. Indeed, it has been shown that PDGF levels vulnerable to the chronic wound proteolytic media and its breakdown can be prevented by MMPs inhibitors [31]. Clearly, PDGF plays a critical role in all phases of wound healing through stimulating phase specific wound healing cells to communicate and release their pro-healing cytokines to heal wounds.

Basic fibroblast growth factor (bFGF):

Basic fibroblast growth factor (bFGF) is a member of a family that contains a structurally related polypeptide found in a diversity of organisms like drosophila, nematode, mouse and human [32,33]. There are 22 isoforms that comprise the FGF family in vertebrates. They share 13-71% of their amino acid sequence and have molecular weights that range from 13 to 17 kDa. Further, FGF1-4 has a strong affinity for heparan sulfate, which is needed to activate FGF cell surface receptor [34]. FGF produces a cellular response through binding to a family of four tyrosine kinase receptors which has an extracellular single transmembrane, ligand binding domain, and a cytoplasmic domain that contains a tyrosine kinase enzyme and regulatory sequences. The extracellular ligand binding domain contain immunoglobulin like domains that serves as a binding site for heparin. [33]. The FGF signaling pathway starts with binding FGF ligand to FGFRs followed by dimerization process in which a complex of two FGFs, two heparin sulfate, and two FGFRs formed. Furthermore, each FGF binds to two receptors

which contact each other by D2 domains to facilitate phosphorylation of each receptor by tyrosine kinase domain. The best understood FGF downstream signaling pathways are the phosphoinositide 3 (PI3) kinase/ AKT pathway, RAS/mitogen-activating protein (MAP) kinase pathway, and phospholipase C gamma (PLCg) pathway. However, the major downstream pathway is RAS/mitogen-activating protein (MAP) kinase pathway, which is implicated during cellular differentiation and proliferation [32]. FGFs play a role in different biological process including cellular embryogenesis, mitogenesis, differentiation, migration, proliferation, angiogenesis, and wound healing [32]. The bFGF plays a crucial role in wound healing. In fact, bFGF is a positive regulator and strong inducer of angiogenesis since it recruits inflammatory cells like neutrophils, monocytes, and macrophages, increase expression of different chemokines like CXCL1 and chemokine 2 and 7, and induces endothelial cells mitogenesis [36]. It also plays a critical role in re-epithelialization, formation of granulation tissue and tissue remodeling. Previous in vitro studies have shown that bFGF controls ECM deposition, increases migration of keratinocytes during re-epithelialization, and enhances fibroblast migration and trigger it to release collagenase. Previous in vitro studies have also shown that FGF 7-10 (keratinocyte growth factor) can induce keratinocyte proliferation and migration that is critical in re-epithelialization and that they participated in the detoxification process of reactive oxygen species (ROS). In acute wounds bFGF levels increase, yet their levels are decreased in chronic wounds. Further, clinical studies using FGF to treat pressure ulcers showed a trend toward earlier wound closure [31]. In conclusion, bFGF is a member of family that structurally related polypeptide found in variety of organisms including

human. The bFGF play important role in wound healing processes including angiogenesis with granulation tissue formation, re-epithelialization, and tissue remodeling.

Vascular Endothelial Growth Factor (VEGF):

Also, known as vascular permeability factor is a homodimeric glycoprotein that stimulate endothelial cell migration, proliferation and survival. In addition, VEGF has inflammatory properties through it is ability to induce microvascular permeability and leukocyte adhesion [30]. VEGF has five isoforms which resulted from alternative splicing of its mRNA, and are generally denoted as VEGF-A, VEGF-B, VEGF-C which secreted from macrophage, VEGF-D and placental growth factor (PIGF) [29]. The chain length changes VEGF from weakly acidic to a basic form, which increases the capability of the molecule to bind to heparin. The shorter variant is the main isoform which is soluble in extracellular space. VEGF is released from different kind of cells that contribute in wound healing: fibroblast, endothelial cells, smooth muscle cells, platelets, macrophage, and neutrophil. VEGF binds to receptors that are members of type III tyrosine kinase family, that contain seven immunoglobulin-like extracellular domains, a single transmembrane spanning domain, and intracellular tyrosine kinase domain. Both KDR and Flt-1receptors found on the endothelial surface of mature and immature blood vessels. KDR receptor mediate chemotactic and mitogenic activity of VEGF and proliferation, chemotaxis, and membrane ruffling in KDR expressing endothelial cells. However, Flt-1 receptor mediate vascular permeability and chemotactic response of macrophage and neutrophil [29]. Stimulation of angiogenesis is one of the roles that VEGF plays in the process of wound healing. Angiogenesis includes vasodilatation, basement membrane breakdown, and endothelial cell proliferation and migration. The levels of VEGF-A along with VEGFR are elevated in

acute wounds. Indeed, upon acute injury platelets secrete VEGF-A. Moreover, during wound healing process, macrophages release TNFα which increase the expression of VEGF-A in fibroblasts and keratinocytes. Furthermore, hypoxia is the main trigger for VEGF-A release in acute wound that results in angiogenesis to reestablish microcirculation to maintain tissue perfusion. In fact, hypoxia induce expression of VEGF in Fibroblasts, monocytes, keratinocytes, endothelial cells, and myocytes [31]. To sum up, VEGF is an important growth factor that promote wound healing through promoting angiogenesis to form granulation tissue.

Pro-inflammatory cytokines and chemokines:

Pro-inflammatory cytokines and chemokines are important players in the wound healing process. After injury, a huge number of cytokines including specifically Interleukin-1 **(**IL-1), Interleukin-6 (IL6), and Tumor necrotic factor alpha (TNFα) induce inflammation and are released from different inflammatory cells [37]. These cytokines are upregulated during inflammatory phase of wound healing. IL-1 is secreted by neutrophils, macrophages, monocytes, and keratinocytes. In wound healing keratinocytes produce IL-1 immediately and along with its action on target cells IL-1 also enhances keratinocytes migration and proliferation. It has been shown that IL-1 upregulates the expression of K6 and K16 in migrating keratinocyte [31]. In addition, IL-1 also stimulates fibroblast to release of FGF-7[37]. IL-6 is released by neutrophils and monocytes and has been shown to play role in starting healing response. Its levels increase in acute wounds and remain high in chronic wounds. It is a neutrophil chemoattractant and induces keratinocytes proliferation and mitogenesis. TNFα is produced by monocytes, macrophages, neutrophils, keratinocytes and can enhance the synthesis of FGF7, proposing potential its implication in promoting

re-epithelialization indirectly. Further, Exogenous TNFα has time and concentration dependent effect on the wound healing indicating that balancing pro-inflammatory signals regulating wound healing. At low concentration TNFα can promote healing through triggering inflammation and inducing macrophage to release growth factors. In contrast, at high concentration particularly for prolonged time TNFα can negatively affect wound healing. At high levels, TNFα increases the production of MMPs (MMP-1, MMP-2, MMP-3, MMP-9MMP-13, and MT1-MMP) and suppresses the production of ECM proteins and tissue inhibitor metalloproteinases (TIMPs). TNFα has been shown to increase the level of tissue collagenase, stromelysins, and gelatinase which their levels is elevated in chronic non-healed wounds. Chemokines are another contributor in wound healing, which induces migration of multiple cells to the wound site. Further, chemokines receptors (CXC receptors) on resident cells suggests that these chemokines participate in controlling of angiogenesis, re-epithelialization, and tissue remodeling. Macrophage chemoattractant protein (MCP1) is one of CC family ligands that works by binding to CXC receptors (G couple receptors). Its expression is upregulated in keratinocyte during wound healing. It is a chemoattractant for monocytes, macrophages, mast cells, and T cells. Persistent expression of MCP-1 in chronic wounds lead to prolonged recruitment of neutrophils and macrophages which lead to sustained inflammatory phase. Hence, chronic wounds stuck in inflammatory phase and do not heal.

Extracellular Microvesicles:

Extracellular vesicles, (EVs); microvesicles and exosomes are a diverse family of membrane bound vesicles laden with various protein, nucleic acids, and lipids that cells

release to the extracellular environment [12]. In 1967, Wolf observed EVs in the extracellular space [13]. In 1983, Pan and Johnstone described EVs as part of the disposal mechanism to get rid of the wasted materials from the cells [12]. Recently, the International Society for Extracellular Vesicles has suggested calling all types of vesicles that found in the extracellular space by the term of extracellular microvesicles (EVs). EVs are particles that varies in size 20nm- 2mm surrounded by phospholipid bilayer membrane and shed to the extracellular space through a specific release and formation mechanisms. EVs release and content depend on which cell they originated from and the stimulus of release, e.g., exposure to lipopolysaccharide (LPS), hypoxia, oxidative injury, exposure to shear force stress [23]. MVs formation is initiated from outward budding of cytoplasmic protrusions, then detached from the cell surface. This process is dependent on calcium influx, and cytoskeleton reorganization. The MVs release ensues in all cells in resting state or up on activation by physical or chemical stress like hypoxia, oxidative stress, or shear stress or soluble agonists [24]. Furthermore, MVs hold the signature of the cellular origin and function as a cargo for transporting cellular content to other cells, in which they induce change in the phenotype and behavior of the cells [23]. There are three different groups of EVs have been defined based on their physical features and mechanism of formation: 1- microvesicles (MVs), 2- exosomes, 3- apoptotic bodies. MVs sizes range between 100-1000nm, its density is 1.04-1.07 cell surface (g/ml), the mechanism of formation is by outward cell membrane blebbing, and composed RNA, miRNA, other non-coding RNA, cytoplasmic protein, and cell organelles. Further, its membrane is not permeable (PI negative), and cell surface markers are Annexin V positivity, Origin cell-specific markers. Moreover, there is no single unique marker to

recognize each group of EVs including MVs; however, there are proteins that have been used as markers and not specific to each EVs group. Those surface markers include, major histocompatibility complex (MHC) molecules, heat shock proteins; HSPs, Tsg101, tetraspanins like CD9, CD63, CD81 and CD82; 14-3-3 proteins, and the Endosomal Sorting Complex Required for Transport (ESCRT-3) binding protein Alix [27]. MVs can be characterized by using high sensitivity flow cytometry and imaging flow cytometer as small as 100 nm in diameter. However, a flow cytometer is unable to characterize smaller sizes like exosomes. Innovative instruments like dynamic light scattering and nanoparticle tracking analysis have shown ability to determine and characterize EVs with diameter smaller than 100 nm [23]. Various mechanisms whereby MVs stimulate the cells including direct interaction with cells by receptors and molecules are expressed on their surfaces that bind with ligands expressed on the recipient cells. After interacting with ligands, MVs could transport their content to the receiver cells [24]. Extracellular vesicles play a critical role in cell to cell communication, as they carry signaling molecules, like lipids, proteins, mRNAs, and miRNAs. In 1980, Poste et al. first show that plasma membrane vesicles from highly metastatic B16 cell line transfer plasma membrane content to a poorly metastatic B16 cell line, that increased its metastatic ability (Poste and Nicolson, 1980). In 1998, Zitvogel et al reported that dendritic cells derived EVs (exosomes) stimulate T-cell antitumor response in vivo (Zitvogel et al, 1998). In 2010, Camussi et al., showed that human liver stem cells derived microvesicles induce in vitro proliferation and apoptosis resistance in human and rat hepatocytes and in vivo MVs hasten the functional and morphological recovery of the liver in a model of 70 % hepatectomy in rats [24]. In 2012, Camussi et al., also showed administration of a single

dose of endothelial progenitor cells derived microvesicles (EPC- MVs) instantly after renal ischemia and reperfusion injury in rats was able to produce morphological and functional protection from acute kidney injury through inducing proliferation and reducing apoptosis of renal tubular cells [24]. In addition, EPC-MVs showed the ability to protect against chronic kidney disease by blocking glomerulosclerosis, capillary rarefaction, and tubule-interstitial fibrosis. Depletion of miRNA content in MVs decreased the renal protecting result of EPCs [24]. Further, EPC-MVs also decreased damage and improved reperfusion in murine model of hind limb ischemia. He et al. recently showed that MSc- derived MVs protect against renal injury in the mouse remnant kidney model (5/6 subtotal nephrectomy). In this model, single administration of MVs preserved the function of leftover kidney [24]. Reis et al. also reported that purified MVs reduce gentamicin induced acute kidney injury. When MVs incubated with RNase, the in vivo effects were reduced, indicating that the protective effects are mediated by RNAs that conveyed by MVs [24]. Ultimately, EVs are no longer considered as "cell dust", yet as efficient messengers in intercellular signaling, impacting both adjacent and distant cells (Camussi et al., 2010a; Loyer et al., 2014) [23].

Negative Pressure Wound Therapy:

Closed suction drainage system is used to enable mass transport of fluid from the body as adjunct to surgical procedures. For instance, a chest tube using this system to evacuate air from the pleural cavity. Moreover, closed suction is also used to drain stomach content, which facilitate small intestinal obstruction treatment. High suction can remove even tissue from the body like in liposuction. In 1990s, Argenta and Morykwas faced a huge number of complicated wounds, and they tried to come up with a better approach to

treat these complex wounds. The first idea was to develop a way to apply suction on the wound to pull the wound edges together; based on this idea, they designed several prototypes that expedited wound healing. The prototype that has had significant success in treating wounds was the one that has open pore polyethylene foam that positioned in the wound, shielded by semi-adhesive dressing, and linked by tube to a suction source. They named this technique as negative pressure wound therapy (NPWT). Alternative description that is more precisely define the physics of the method is micro-deformational wound therapy (MDWT). Argenta and Morykwas device has changed the course of treatment of many patients with complex wounds now a day, and has become the standard of care that used to heal these kinds of wounds in the USA. According to Orgill et al. (Orgill & Bayer, 2013, p.17) NPWT mechanisms of action divided into primary and secondary effects that affect the process of wound healing. The primary effects include tissue macro-deformation in which the wound edges pulled together by the open pore foam; however, that effect depends on the mobility of tissue surrounds the wound. Furthermore, microscopic deformation at the wound surface stretches the cells enhances cell proliferation and division [5]. In addition, NPWT can eliminate large amount of extracellular fluid and afford an isolated suitable moist environment to the wound to heal. The secondary effect includes promoting granulation tissue via up regulation of HIF-1alpha-VEGF pathway through micro-deformation effect, which promotes localized hypoxia close to the wound surface [4]. In addition to that, NPWT modulate inflammation, induce cellular proliferation, and change the bacterial levels in the wound. NPWT indicated for various type of wounds include acute, subacute, chronic, Traumatic, or dehisced wounds. NPWT also indicated for partial-thickness burns, ulcers like diabetic,

pressure or venous insufficiency ulcers, and flaps and grafts. NPWT is contraindicated in malignancy (since proliferation is induced by NPWT may enhance malignant growth), untreated osteomyelitis, non-enteric and unexplored fistulas, necrotic tissue with eschar, sensitivity to silver (GranuFoam Silver dressing only) [4].

Oasis Ultra:

Collagen is the most abundant protein in the human body, and it is a fundamental component in the extracellular matrix. Collagen is an obligatory protein in all stages of wound healing cascade. Fibroblast secrete collagen in the proliferative phase of wound healing to direct keratinocyte migration to the areas that need substitution of the injured epidermis. Collage-rich dressing is a dressing that induces the production and organization of collagen. Furthermore, it also helps in making an appropriate environment to enhance healing through recruiting cells like fibroblast and macrophage. In addition, this type of dressing is convenient in application, removal, and provide an absorptive moisture environment to promote healing. Collagen-rich biomaterial is often synthesized from avian, bovine, and porcine collagen. This kind of dressing made from oxidized regenerated cellulose plus plant based biomaterial to produce a dressing that inactivate the metalloprotease to prevent destruction of the provisional extracellular matrix [6]. Oasis-ultra is a triple layer extracellular matrix containing different types of glycosaminoglycans (GAGs) like heparin sulfate, hyaluronic acid and chondroitin sulfate. In addition, it has adhesion molecule such as fibronectin and laminin along with various growth factors like transforming growth factor (TGF-b), basic fibroblast growth factor (b-FGF) and vascular endothelial growth factor (VEGF) [7]. Oasis Ultra composition stimulates cell migration, reduces inflammation, provides moisture to the wound bed,

structural support, and enhance cellular proliferation and attachments. Oasis Ultra used for variety of wounds including chronic vascular ulcer, partial thickness burn, donor site, diabetic ulcer, preparation for skin graft, venous ulcer, partial and full thickness wound, surgical wound, traumatic wound, venous ulcer, and wound dressing [6].

Hypothesis:

We hypothesize that there is a correlation between the concentration of EMVs and the percentage of wound healing in treated chronic wounds and the healing percentage will be enhanced by the combination of Oasis ultra and NPWT.

Specific Aims:

1. To see whether the combination of Oasis ultra and NPWT will enhance the healing of chronic wounds when compared with only NPWT.
2. To measure the concentration of EMVs in the collected wound fluids and correlate it with the percentage of wound healing.
3. To analyze the extracellular microvesicles composition to look for growth factors, chemokines and cytokines that play role in the wound healing.

Material and Methods:

Experimental design:

The study was a prospective, multi-centered, randomized, single-blinded clinical trial that permitted by the Ethics Committee of the Copernicus. Written informed consent was attained from all patients. We discussed and answered patients' concerns before

obtaining the consent. Wound fluid samples obtained from 14 patients with stage IV trunk pressure ulcers. The patients were divided in two groups (7 in each group, n=7): control group on negative pressure wound therapy (NPWT) alone & study group with NPWT plus Oasis Ultra dressing. Further, NPWT was replaced two times a week, and Oasis Ultra was applied once weekly for all subjects. Wound size was measured every week for the whole 12 weeks of study.

Subject Selection:

Potential candidates evaluated by the study principal investigator for Inclusion/Exclusion criteria. Most the subjects enrolled at Sycamore Medical Center's Wound Center, yet subjects also enrolled at other participating sites.

Inclusion criteria for this study include:

1. Adults aged 18-89 who exhibit stage III or IV trunk pressure wounds with no signs of infection.

2. HbA1C < 8 (if patient is diabetic)

3. Adequate nutrition including albumin ≥ 2.0 and pre-albumin ≥ 12.5.

Exclusion criteria for this study include:

1. Wounds that cannot have a NPWT device properly applied due to location (too close to anus), diarrhea, peri-wound skin issues.

2. Patients with Infected wounds.

3. Patients with HbA1C >8, uncontrolled diabetes.

4. Malnourished patients.

5. Patients that are immunodeficient or immunocompromised.

6. Patients that have a religious or ethical aversion to porcine products.

7. Patients that have any allergy to porcine products.

8. Patients who are at High Risk of bleeding including:

   a. Patients who have weakened or friable blood vessels or organs in or around the

   b. wound as result of, but not limited to:

   c. Suturing of the blood vessel (native anastomoses or grafts) / organ

   d. Infection

   e. Trauma

   f. Radiation

   g. Patients without adequate wound hemostasis

   h. Patients who have been administered anticoagulants or platelet aggregation inhibitors

   i. Patients who do not have adequate tissue coverage over vascular structures

9. Patients who are do not resuscitate and/or do not intubate (DNR/DNI).

The patient's medical record will be referenced for all Inclusion and Exclusion criteria. Lab results for albumin and prealbumin up to 30 days prior to enrollment and HbA1C results up to100 days prior to enrollment will be considered in determining eligibility.

At approximately months 1, 2, and 3, wound specimen canisters will be collected and taken to the laboratory at Wright State University (WSU) department of Pharmacology and Toxicology for analysis of the drained fluids from all wounds. Specimen canisters will be secured in biohazard bags and transported to WSU by a member of the study team within 24 hours of collection.

For all wounds, fluid samples, canisters fluids will be obtained in a sterile fashion. Part of the fluid will be used for the study and the other part will be stored in liquid nitrogen at- 80C for potential later use, possibly unrelated to this study. If a subject refuse to allow their samples to be used outside of this study, that subject's extra fluid sample will be destroyed.

To maintain blinding for the assessment lab personnel, specimen canisters and stored specimens will be identified by a coded label. Pressure reduction beds and patient repositioning will be employed throughout the study as a standard of care for those patients.

Table 2: Demography of the patients who joined the clinical trial

Variable	control	study
Total number	7	7
Males	3	4
Female	5	2
Wound location	6 Sacrum, 1 left ischium	sacrum
Smoking	None	None

Sample preparation:

A canister of NPWT device (wound VAC) was collected from the patients every four weeks of the study and brought to the lab where a hole was made in the canister by using a drill to drain the fluids. The wound fluid was pull out from the canisters and aliquoted to 1.5 ml eppendorph tubes. Protease inhibitor added to the fluid before the fluid stored in a -80°C freezer.

Microvesicles isolation:

Microvesicles were isolated using Differential Ultracentrifugation method.

1. Patients sample mixed in 5 ml of 20 nm filtered (Whatman, Pittsburgh, PA) phosphate-buffered saline (PBS).
2. Samples transferred to a centrifuge tubes, 5 ml of Filtered PBS added to dilute the samples.
3. The wound fluid samples are centrifuged at 4 °C (500 × *g* for 10 min followed by 2,000 × *g* for 20 min) to remove intact cells and cell debris. New tubes used after every centrifugation stage.
4. The supernatant centrifuged at 10,000-× *g* for 2 hours to isolate MVs.
5. MVs pallets suspended in 1 ml of filtered PBS.
6. Take 5 microliter from the sample and dilute it in 695 µl Filtered PBS for analysis by using Nanoparticle Tracking Analysis machine (Nanosight).
7. Microvesicles concentration measured by Nanoparticle Tracking Analysis machine (Nanosight).

Nanoparticle tracking analysis (NTA):

Purified MVs samples were analyzed by NTA using NanoSight (NS300) with a 405-nm laser instrument (Malvern Instruments, United Kingdom). 5 microliters from the samples were diluted in 695 µl Filtered PBS and three 30 second videos were recorded using camera level 12–15 with frame rate 30 frame per second. The data was analyzed using NTA software 3.0 software (Malvern Instruments) which was optimized to first identify and then track each particle on a frame-by-frame basis [35]. The detection threshold optimized for each sample and screen gain at 10 to track as many particles as possible with minimal background [10].

Cytokine Analysis:

BioPlex Pro cytokine assays are multiplex immunoassays similar to sandwich ELISA assays that differ by using magnetic beads to couple a biomarker of interest. The antibodies are covalently linked to magnetic beads and bind to the biomarker of interest when exposed to the sample. The magnetic bead-cytokine complex washed repeatedly to remove excess protein and a detection antibody added that bound to the primary antibody, thus completing the sandwich complex. Streptavidin-phycoerythrin conjugate served as a fluorescent indicator.

1-The 96 wells plate from the Human Cytokine 27Plex kit was first ‘wetted’ with Assay buffer.

2-the beads solution was added to the respective wells and washed twice with wash buffer.

3- Samples and controls (50 □l @ ~ 3-10 □g/□l) were loaded into individual wells and analyzed in duplicate.

4- An aliquot of each sample assayed for protein content by the Bio-Rad method.

5- The plate was kept for incubation followed by 3 washes with buffer.

6- A detection antibody added to each of the wells for 30 minutes incubation at room temperature.

7- Streptavidin-phycoerythrin conjugate added to each of the wells and washed 3 times with wash buffer.

8- By using Bioplex 200 instrument the plate scanned at both a low PMT (normal) and high PMT, and statistics computed using suitable software and analyses.

Statistical analyses:

Analyses performed using Graph Pad Prism 7, Excel 2016, and SPSS version 13 software. Statistical comparisons were expressed as mean ± SEM for continuous variables of experiments conducted at least three times, where *n* is the human subject. The differences between the groups were done by multiple comparison and analyzed by ANOVA, Paired or non-Paired t test. Simple linear regression employed to study the correlation between the healing percentage and EMVs concentration. In all cases, $P \leq 0.05$ was considered statistically significant.

Results:

The total number of patients involved in the study during the period between August 2014 and June of 2016 was 94 patients. Thirty-nine subjects were consented, nineteen were withdrawn from the study for different reasons that the primary investigator determined and seventeen subjects finished the study successfully.

The effect of Oasis ultra on the healing percentage in both groups

The healing rate was studied over a 12week period with every week's measurement recorded. The wounds that received the standard of care (NPWT, wound VAC) only, showed an increase in the healing rate at the beginning of the study with a peak at 8 weeks, after which the healing rate declined for the rest of the study period (Figure 1). The healing rate of the wounds that received Oasis ultra in addition to the standard of care (NPWT, wound VAC) showed a steady increase over time until the end of the study period (Figure 1).

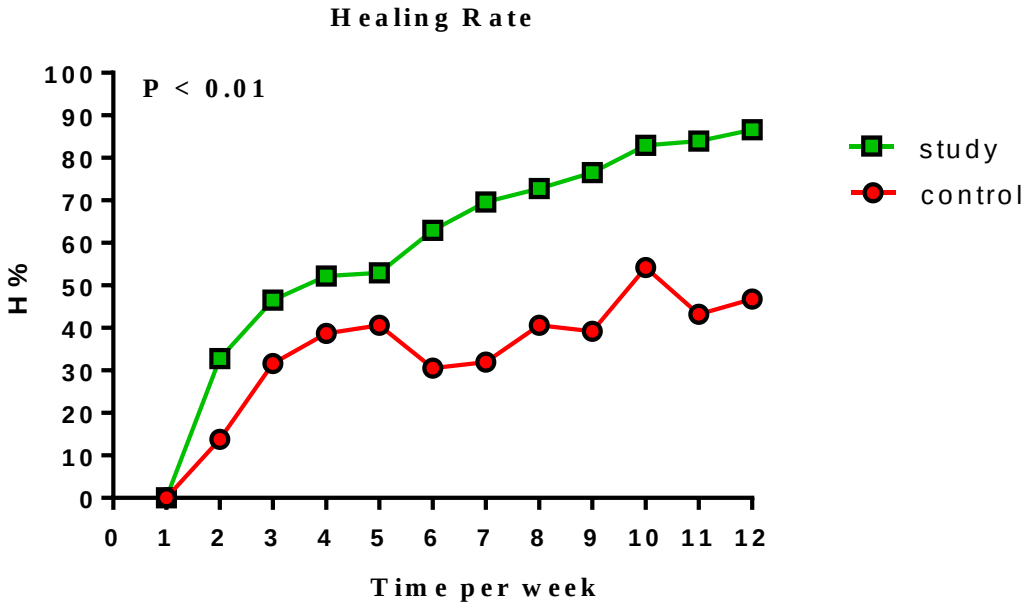


Figure 1: Percent Healing versus Time. Healing percentage calculated for every week by comparing wound volume of the current week with the previous week of the study.

The difference in healing between the two groups was analyzed by one-way ANOVA, (Table 1). The difference was significant at $p< 0.05$. Using these results a Box and Whisker Plot was performed to show the average of overall healing percentage after 12 weeks in both groups (Figure 2).

Table 3: Average healing percentage after 12 weeks of study both groups.			
Dependent variable: Healing percentage			
Groups	**Mean**	**Std. Deviation**	**N**
Control	52.3271	35.82278	7
Study	88.8386	9.741854	7
ANOVA: single factor Control vs study groups: F= 6.77, df (1, 12), ($P \leq 0.05$).			

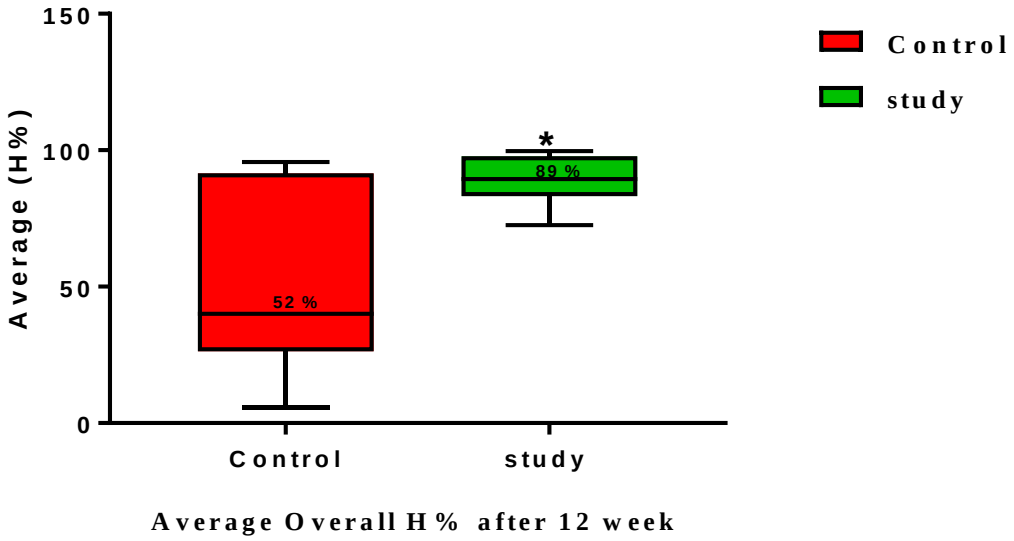


Figure 2: Average Percent Healing in the Control and Study groups. The box and whisker plot shows the difference in healing between the two groups.

The average healing percentage in the 2 groups at four, eight and twelve weeks was compared using one-way ANOVA. There was no significant difference in the healing percentage between the two groups at four-weeks, $p = 0.29$; eight-weeks $p = 0.41$. However, at twelve-weeks there was a significant difference between the two groups $p < 0.05$. Both group's healing percentage increased over time with the peak at 8 weeks. In the 12th week, the wounds treated with Oasis Ultra continued to show an increase in healing, while the wounds treated with the standard of care (NPWT) did not. These findings suggest that Oasis Ultra may be facilitating the progression through wound healing stages.

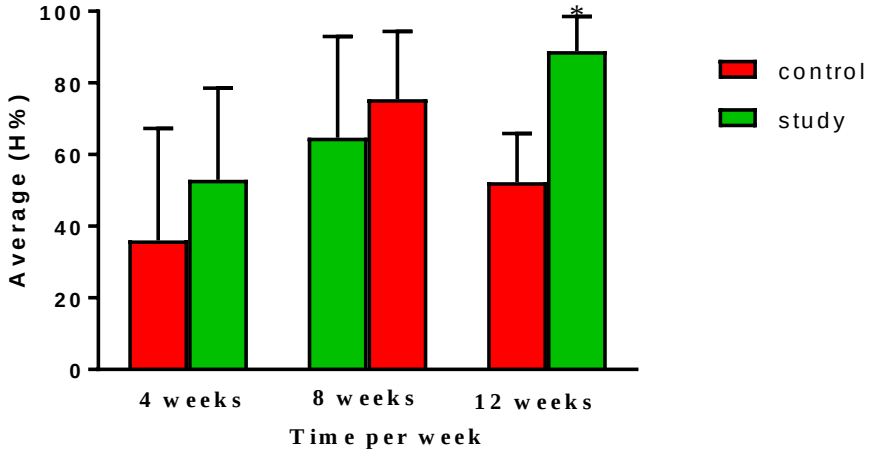


Figure 3: Overall healing percentage (H%) in both study and control groups at 4, 8, and 12 weeks.

The difference in healing percentage between the two-time points in the (4 weeks,12 weeks) study group was performed by Paired t test, (Table 3). The difference was significant, $p<0.05$. Using these results, a Box and Whisker Plot was performed to show the average healing percentage at 4 and 12 weeks in the study group (Figure 4). However, the difference in the average healing percentage between 4 and 12 weeks' time points was not significant ($P = 0.07$). Using this result, Box and Wesker Plot was performed to show the average of healing percentage at 4 and 12 weeks in study group (Figure 4).

Table 4: Average healing percentage at 4 and 12 weeks of study in study group			
Dependent variable: Healing percentage			
Study Group 4, 12 weeks	Mean of differences	SD of differences	Number of pairs
	-35.9	18.92	7
Paired t test 4 weeks, vs 12 weeks: t, df, t=5.022 df=6, ($P \leq 0.05$).			

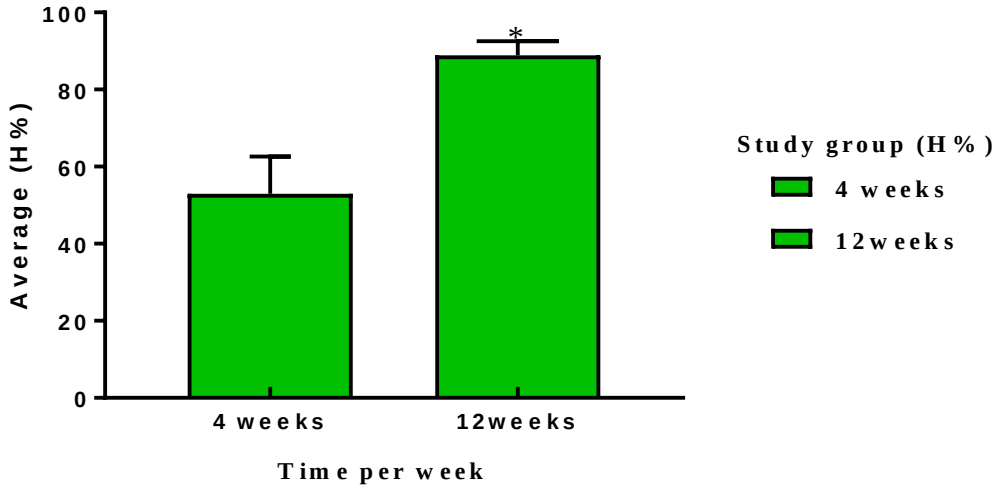


Figure 4: Average healing percentage. between 4 and 12 weeks' time point of the study group ($P \leq 0.05$).

Extracellular Microvesicles Correlation with Percent Healing

One of the goals of this project was to determine if microvesicles concentration would correlate with the percent healing or healing rate of wounds. To study this, we examine the number of Extracellular Microvesicles (EMVs) in the wound exudate at the different time points. This data was then compared with wound size of each patient and are presented as the Control group and Study group for clarity (Figure 5 & 6). Patients in the control group appeared to have less MVs concentration and relatively smaller size wounds in comparison with the study group patients who had larger wounds and more MVs. The assignment of each patient to the group was randomized, even though the wound sizes were more tightly grouped than would be thought by random selection.

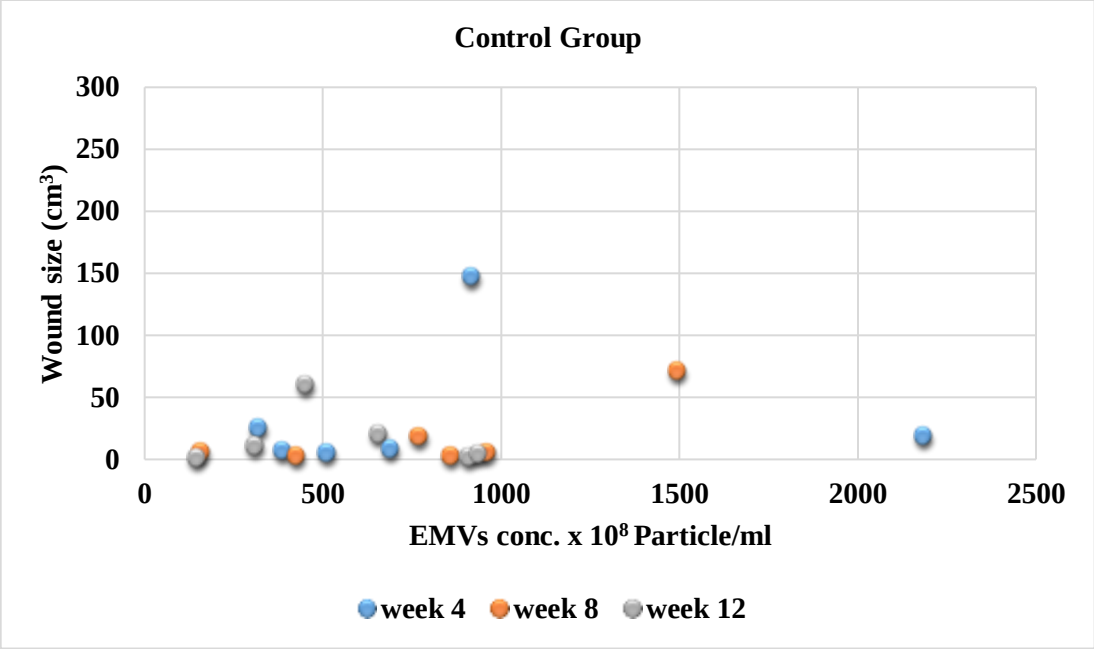


Figure 5: Control EMVs conc. at 4, 8, and 12 weeks. Each dot represents data of one patient.

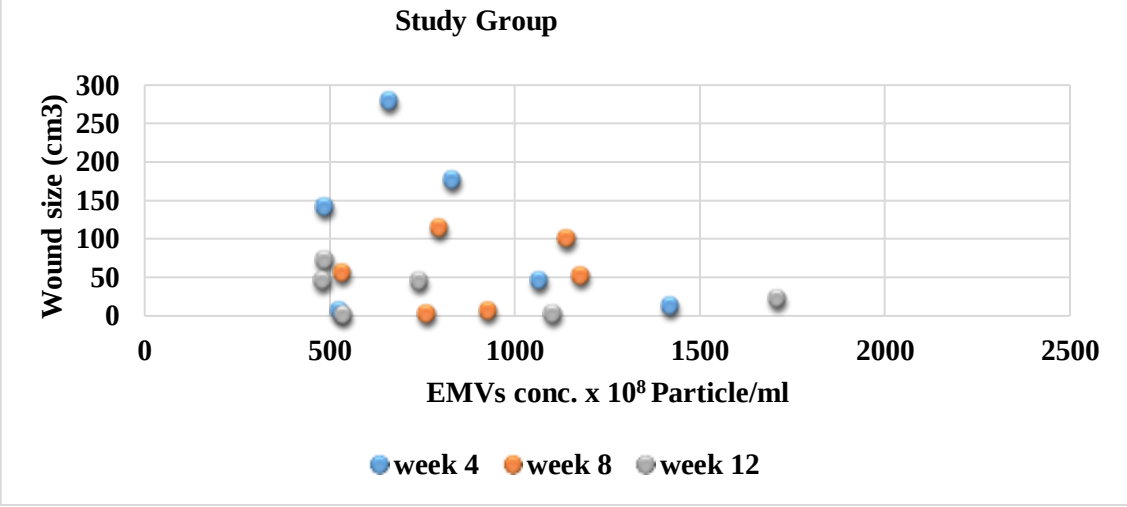


Figure 6: EMVs conc. vs Wound size in the study group at 4, 8, and 12 weeks. Each dot represents data of one patient.

To analyze this, further, the MVs concentration (EMVs conc. x 10^8 Particle/ml) was plotted against Percent Healing and the data for each time point analyzed by 'connecting the dots' and color coding (Figure 7A & B). From these plots, we determined that there was a higher EMV concentration that correlated with higher percent healing in the wounds treated with the combination of Oasis Ultra dressing and NPWT, as compared with the wounds that were treated with NPWT alone. These findings suggest that Oasis Ultra may provide an environment conducive to increased EMVs concentration.

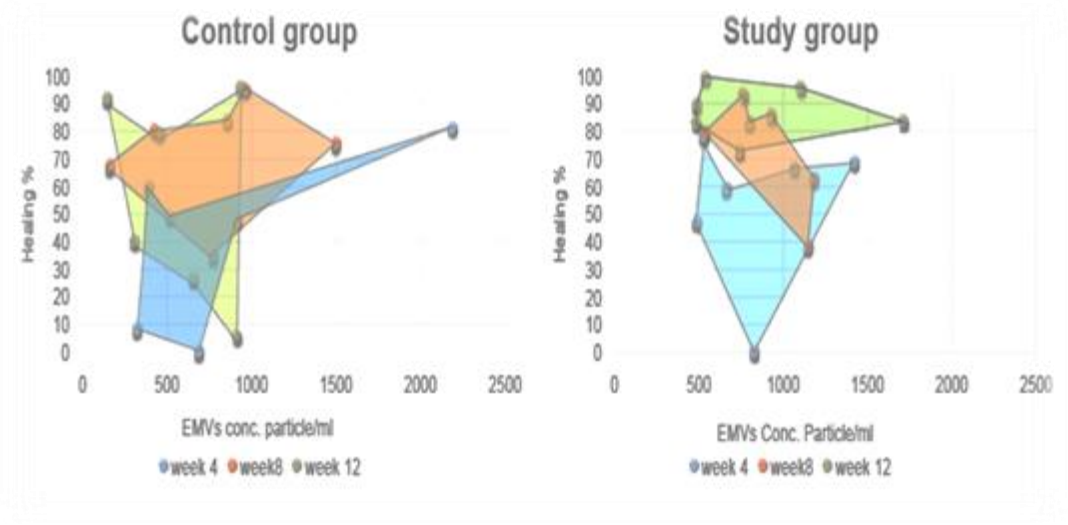


Figure 7 A & B: EMVs conc. vs Wound Healing percentage in the control and study groups at 4, 8, and 12 weeks. Each dot represents data of one patient.

To test if EMVs concentration in wound fluid could impact wound healing percentage regardless of the wound treatment, a simple linear regression was conducted to see if EMVs concentration could be a predictor for the healing percentage (Table 4). We observed that regardless of which treatment the patients get, there is positive correlation between EMVs concentration in wound fluid and healing percentage. In

another way, there is increase in healing percentage for every single unit change in the EMVs concentration (Figure 8). These finding also suggests that EMVs concentration in wound fluid could predict the healing rate and potentially could serve as biological biomarker for wound healing.

Table 5: the effect of EMVs Con. On H% treatment		
Standardized coefficient, Beta	R square	Number of patients
0.81	0.66	12
Simple Linear Regression All patients: F, (df, df) = 69.42, df =1,35 (P = 0.00)		

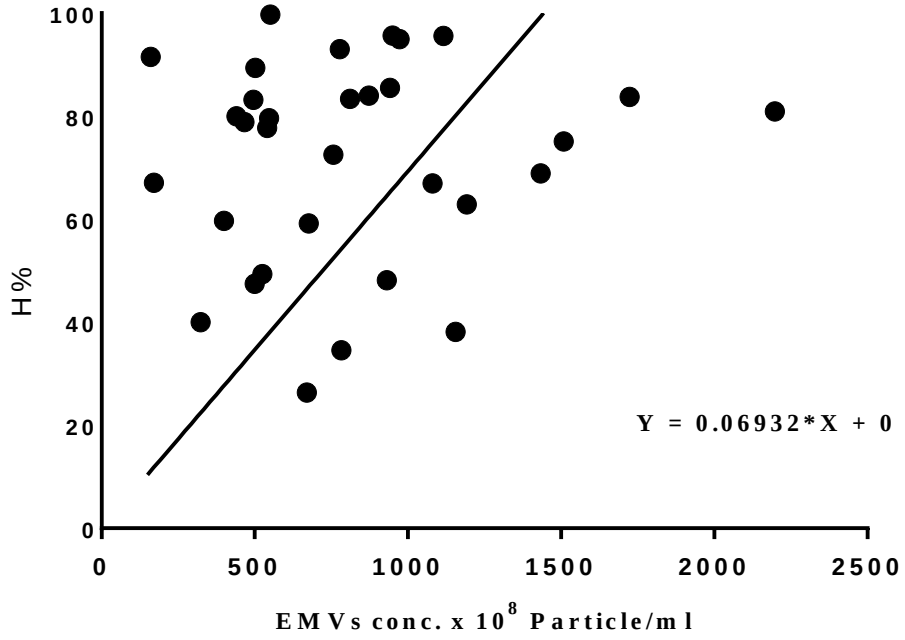


Figure 8: Analysis of Percent Healing versus EMV Concentration. Linear regression using SPSS Statistical Software version 13 was conducted to analyze the data of all patients regardless the treatment, and data showed that there is a positive correlation

between MVs concentration and wound-healing percentage, the R-square was 0.66, and ($P \leq 0.05$).

Extracellular Microvesicles (EMVs) Composition Analysis :

Based on the results so far, we analyzed the EMVs for the presence of cytokines, chemokines and growth factors using a Bio-Rad BioPlex (Luminex) instrument to run the 27 Bio-Plex Pro™ Human Cytokine, Chemokine, and Growth Factors Assay (Table 5). All data was presented as mean ± SEM. All cytokine concentrations were normalized to total protein concentrations that were measured by Bio-Rad (Bradford) protein assay.

Table 6: EMVs Cytokines analyzed by 27 Bio-Plex Pro Human Cytokine, Chemokine, and Growth Factors.

IL-1	**IL-10**	**IL9**	**G-CSF**
IL-6	**PDGF**	**IL13**	**MIP-1α**
IL-8	**VEGF**	**IL15**	**MIP-1β**
IL-12	**bFGF**	**IL17**	**G-CSF**
TNF-α	**IL-1 Ra**	**IL2**	**GM-CSF**
INF- □	**IL-4**	**Eotaxin**	
IP-10	**IL-5**	**GM-CSF**	
MCP-1(MCAF)	**IL7**	**RANTES (CCL5)**	

In our study, we found that the wound fluid concentration of EMVs was different among the two groups and the composition of cytokines in or on these vesicles was also different. We observed that the concentration of PDGF, bFGF, and VEGF in EMVs isolated from the wounds treated with the combination of Oasis ultra and NPWT appeared to increase during 4 weeks and to decrease gradually in the 8th and 12th week (Figure 12, 13, 14). However, from a statistical standpoint, there was no significant

difference within the groups at 4,8, and 12 weeks of the study, most probably due to the small sample size. Overall, the wounds who treated with NPWT alone appeared to show smaller concentration of these intravesicular growth factors at 4, 8, and 12 weeks compared with the wounds that received combined therapy of oasis ultra and NPWT. Further analysis with larger N is necessary to show significant differences in these growth factors.

There also appeared to be an increase in intravesicular IL-1 and IL-1RA antagonist levels in study group at 4, 8, 12 weeks compared with control group (Figure 12 & 13), although again, the data was not significant.

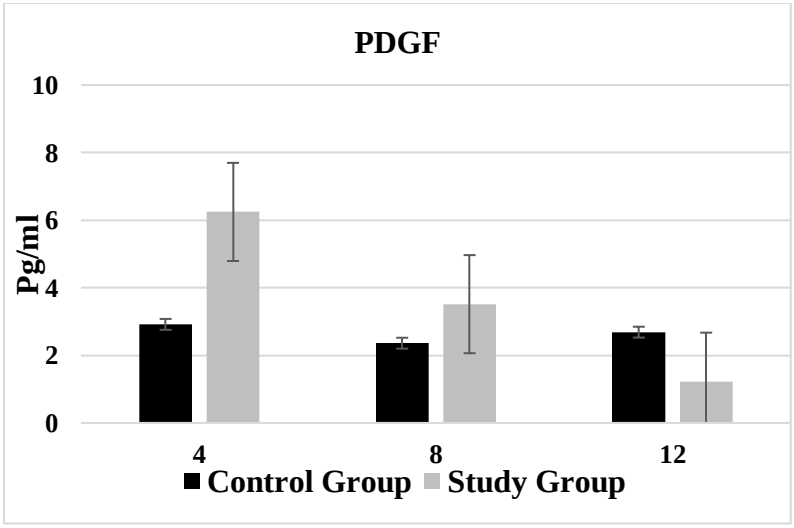


Figure 9: Intra-vesicular concentration of platelet derived growth factor PDGF (Pg/ml). At 4 week and decrease gradually at 8, and 12 weeks of the study group. However, in the control group, its concentration is small in all weeks of study. Statistical comparisons were expressed as mean ± SEM for continuous variables of experiments conducted, where *n* is the human subject.

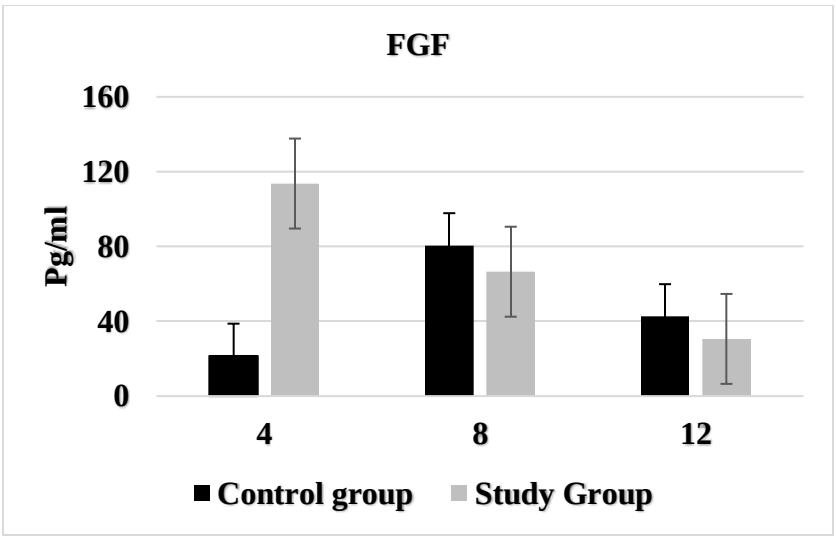


Figure 10: Intra-vesicular concentration of Fibroblast growth factor bFGF (pg/ml) at 4 week and decrease gradually at 8, and 12 weeks of the study group. However, in the control group, its concentration is small in all weeks of study. Statistical comparisons were expressed as mean ± SEM for continuous variables of experiments conducted, where *n* is the

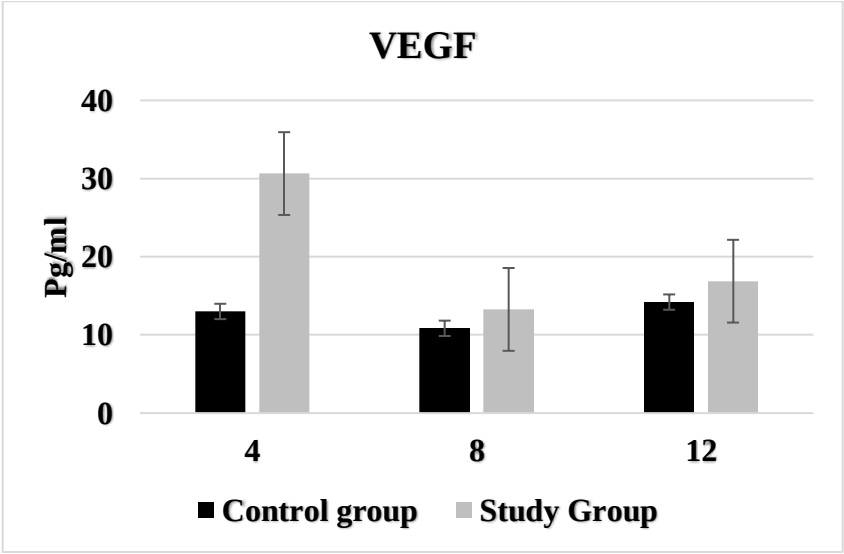


Figure 11: Intra-vesicular concentration of Vascular Endothelial Growth Factor(VEGF) (Pg/ml) at 4 week and decrease gradually at 8, and 12 weeks of the study group. However, in the control group, its concentration is small in all weeks of study. Statistical comparisons were expressed as mean ± SEM for continuous variables of experiments conducted, where *n* is the human subject.

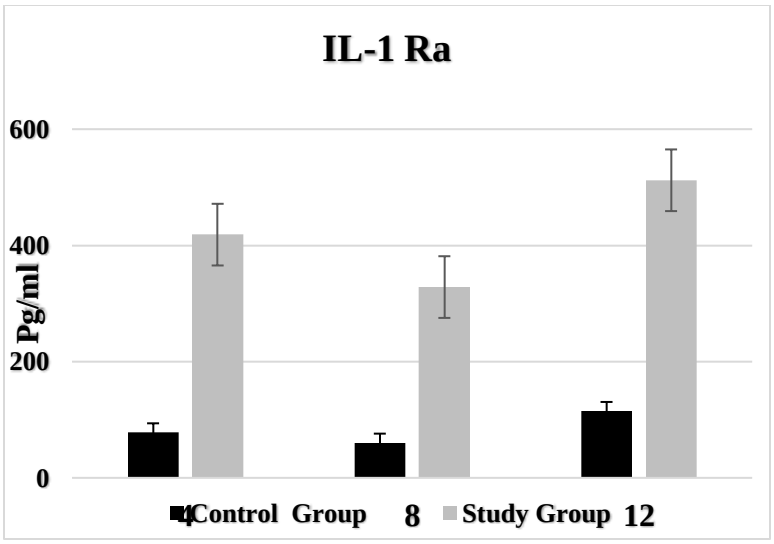


Figure 12: Intra-vesicular concentration of interleukin 1receptor antagonist IL-1Ra (Pg/ml). Vs time at 4, 8, and 12 weeks of the study both groups. Statistical comparisons were expressed as mean ± SEM for continuous variables of experiments conducted, where *n* is the human subject.

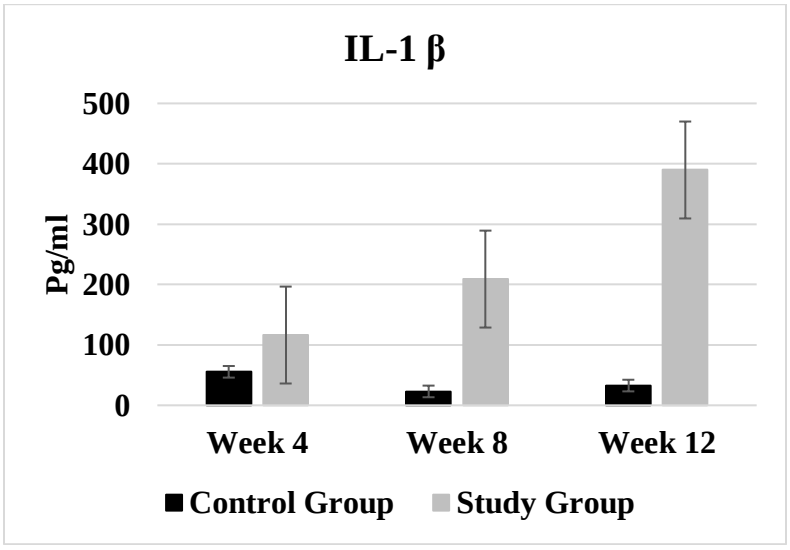


Figure 13: shows the intra-vesicular concentration of interleukin-1 beta IL-1β (Pg/ml) Vs time at 4, 8, and 12 weeks of the study both groups. Statistical comparisons were expressed as mean ± SEM for continuous variables of experiments conducted, where *n* is the human subject.

The intravesicular concentration of pro-inflammatory cytokines, i.e., IL-6, TNFα, IL-8, IL-12, INF-Ɣ, MCP, and IP10 appeared to increase at 4 weeks and remain elevated at 8 and 12 weeks in the control group as compared with study group (Figures 14-18).

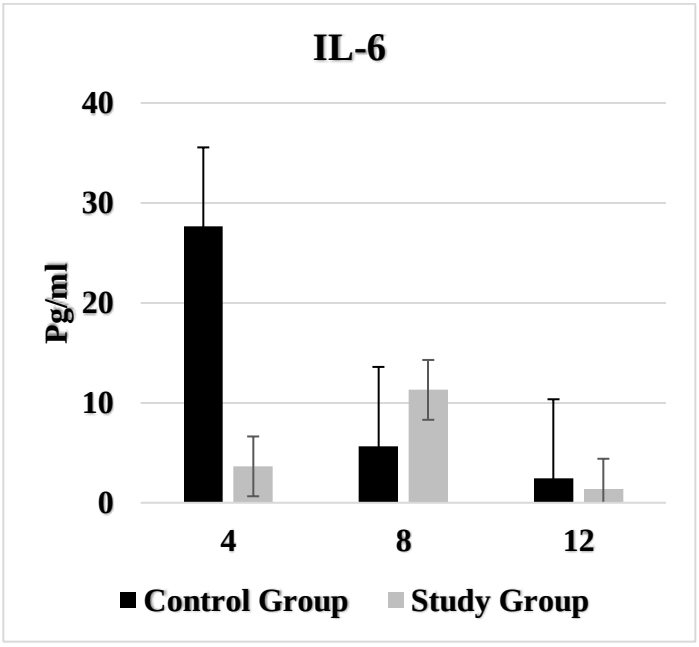


Figure 14: Intra-vesicular concentration of interleukin 6 IL-6 (Pg/ml) Vs time at 4, 8, and 12 weeks of the study both groups. Statistical comparisons were expressed as mean ± SEM for continuous variables of experiments conducted, where *n* is the human subject**.**

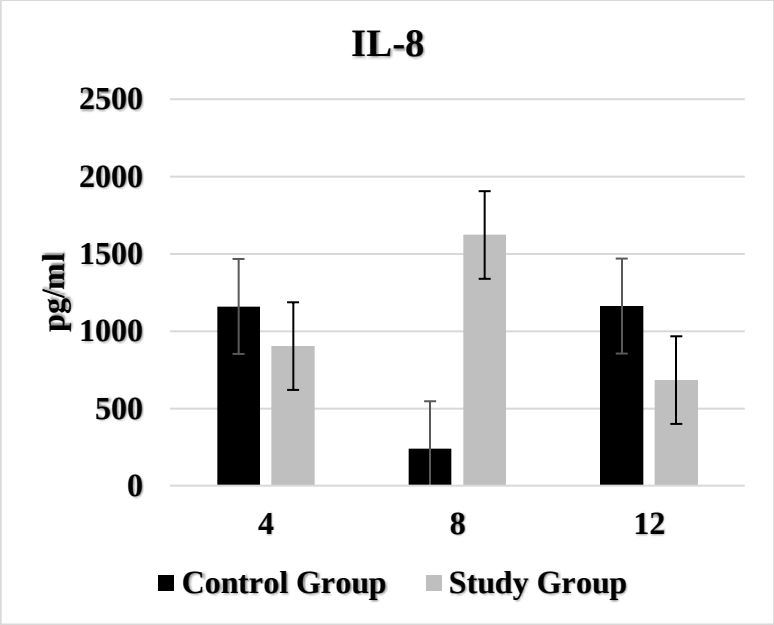


Figure 15: Intra-vesicular concentration of interleukin 8 IL-8 (Pg/ml) Vs time at 4, 8, and 12 weeks of the study both groups. Statistical comparisons were expressed as mean ± SEM for continuous variables of experiments conducted, where *n* is the human subject.

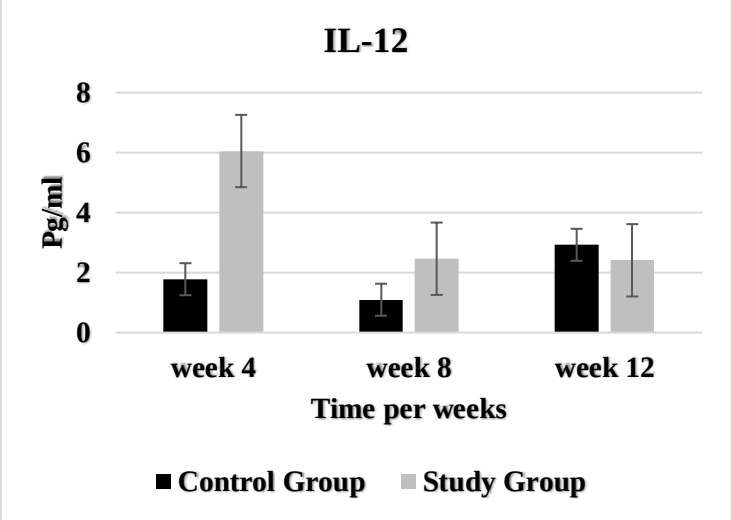


Figure 16: Intra-vesicular concentration of interleukin 12 IL-12 (Pg/ml) Vs time at 4, 8, and 12 weeks of the study both groups. Statistical comparisons were expressed as mean ± SEM for continuous variables of experiments conducted, where *n* is the human subject.

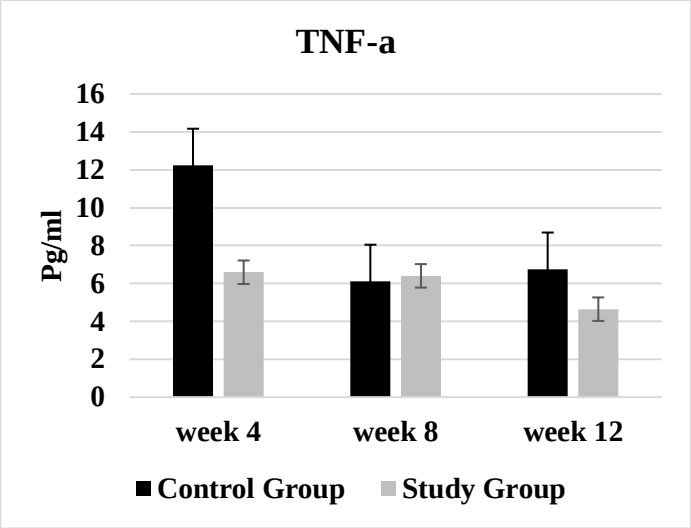


Figure 17: Intra-vesicular concentration of tumor necrotic factor alpha TNF-α (Pg/ml) Vs time at 4, 8, and 12 weeks of the study both groups. Statistical comparisons were expressed as mean ± SEM for continuous variables of experiments conducted, where *n* is the human subject.

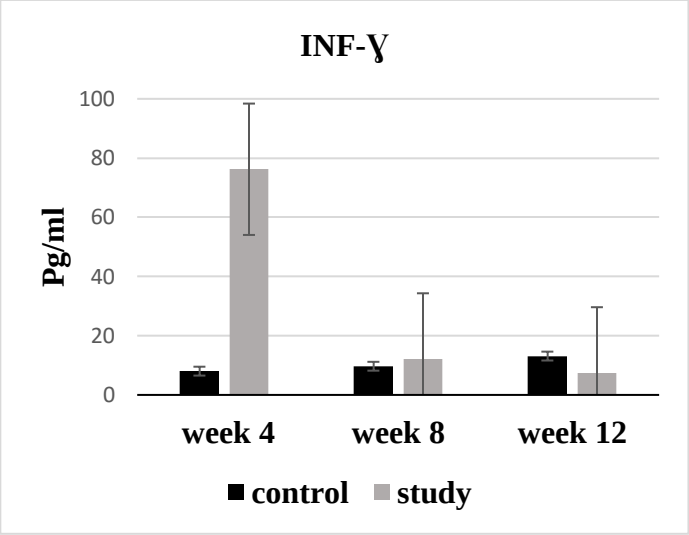


Figure 18 A & B: Intra-vesicular concentration of interferon gamma INF-Ɣ (Pg/ml) Vs time at 4, 8, and 12 weeks of the study in both groups. Statistical comparisons were expressed as mean ± SEM for continuous variables of experiments conducted, where *n* is the human subject.

Discussion and Conclusion:

Wound healing is a sophisticated dynamic process that involves complex coordination among a variety of resident cells in a suitable extracellular environment [39]. The wound healing process is initiated by tightly coordinated, interactive, and overlapping time-dependent stages. These stages include hemostatic, inflammatory, proliferative, and remodeling phases wherein resident cells, inflammatory mediators, and extracellular matrix actively participate in each phase of healing [1,3]. Highly coordinated signals through cytokines, growth factors, and chemokines are required for fruitful wound healing process. Those cytokines binding to their corresponding receptors and exert their actions through endocrine, autocrine or paracrine mechanism through triggering specific molecular cascade that yield a change in target cells metabolism, growth, and differentiation [31]. The goal of this project was to attempt to identify specific factors that could either influence wound healing, or act as an indicator of the wound healing process, both positively or negatively.

Our main objective of this project was to determine if wound healing could be enhanced by addition of a type of dressing, the Oasis Ultra, in conjunction with NPWT which was being used as the standard of treatment. The results clearly show that the Oasis Ultra did in fact significantly enhance wound healing, as measured by healing rate or average wound size. This is a substantial finding considering that the average size of the wounds in the Study group was significantly larger than the size of the wounds in the Control group. No other study has been conducted to compare the use of Oasis Ultra in combination with NPWT with wound NPWT alone. Thus, our results would suggest a positive shift in the treatment for large wounds such as these.

A second goal of the project was to determine if microvesicles were in higher abundance in the Oasis Ultra Study Group than in the wound NPWT Control group. Microvesicles are being identified in numerous studies as both an indicator of risk as well as having the ability to enhance or positively influence wound healing. We showed that the overall concentration of microvesicles increased over time in the wounds treated with Oasis Ultra versus NPWT alone. The results also showed an increased healing rate concomitant with an increased microvesicles concentration regardless of the treatment, but that it appeared to be increased with Oasis Ultra. The small sample size of this study made significance difficult for most of these assays.

The third goal of the project was to ascertain if the microvesicles contained specific cytokines, chemokines, or growth factors. This has not been determined previously, but would suggest that if they did, there would be a potential for some type of communication between the cytokine, chemokine, or growth factor and cells in the wound site itself. Cellular communication between cells is crucial during the healing process. Although cells communicate mainly through secretion of different kinds of proteins that bind to specific receptors on adjacent cells; the mode of intercellular communication via EMVs has been suggested to play a critical role in healing [40]. In our present study, we observed intravesicular cytokines and growth factors concentration attached to or inside the microvesicles. This would strongly suggest that there could be signaling events initiated by these types of proteins that could play a dynamic role throughout the healing process and the type of intravesicular cytokines could determine the outcomes of wound healing.

We were able to show a persistent increase in pro-inflammatory cytokines such as IL-6, TNFα, IL-8, IL-12, and INF-ɣ in the VAC-treated wounds that could negatively impact wound healing and render the wound to chronic state. These results are also consistent with our clinical findings that showed overall healing rate is higher in the study group subjects who have more EMVs. In contrast, Oasis Ultra Study Group EMVs have fewer pro-inflammatory cytokines which have negative impact on healing. Alternatively, pro-healing cytokines such as PDGF, bFGF, VEGF, and IL-1 R antagonist were found to be elevated in the Oasis Ultra Study Group and could hasten the healing rate. Ultimately, these findings suggest that intravesicular composition of EMVs could determine the outcomes of the wound healing. However, there is a need to increase the number of patients in the study that would provide more data to establish statistical significance.

The findings propose that a high demand on cytokines like PDGF, bFGF, VEGF, IL-1, and IL6 are needed to start the healing response and as soon as the wound progresses through phases of healing the demand of these cytokines decreased. These findings are consistent with our clinical findings that showed higher healing rate in the study group as compared with control group.

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The Role of Extracellular Microvesicles in Wound Healing

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Introduction: Extracellular Microvesicles (EMVs): EMVs are cellular membrane fragments ranging from 100 nm to 1µm and are shed from almost all cell types. EMVs mediate intercellular communication by transporting mRNA, miRNA, growth factors and other proteins between cells. SIS (small intestinal submucosa) is a three layers of bio absorbable extracellular matrix obtained from naturally derived, intact porcine small intestinal sub-mucosa (SIS *****). **Objective:** The aim of the study is to test correlation between the EMVs concentration and the rate of wound healing in pressure wounds, to analyze the EMV content, and to see whether the healing rate will be enhanced in SIS ***** group. **Material &Method:** Wound fluid samples obtained from 12 patients with stage IV trunk pressure ulcers. The patients were divided in two groups (6 in each group, n=6): control group on negative pressure wound therapy (NPWT) alone & study group with NPWT plus SIS dressing. EMVs were isolated using Ultracentrifugation method. EMVs concentration was measured by Nanoparticle Tracking Analysis machine (Nano sight). EMVs content was analyzed using 27 Bio-Plex Pro assay. **Result:** Using Statistical software (SPSS), version13.00, simple linear regression was conducted to analyze our data which showed there is a positive correlation between Extra cellular MVs concentration and the rate of wound healing in both groups, study group, R= 0.87, R^2 = 0.76 and P 0.05 and control group R = 0.78, R^2 = 0.61and P 0.05. The overall healing rate after 12 weeks of study was higher in the study group (87.3 %) in comparison with the control group (56.3 %). **Conclusion:** Cellular communication through EMVs plays a crucial role in wound healing. **SIS *** may enhance the cells' release of EMVs, which provide damaged cells with required material to recover and hasten the healing rate.
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Accepted Abstract in Symposium of Advanced Wound Care (SAWC), San Diego, California, Spring 2017.

Extracellular Microvesicles as a Novel Biomarker for Wound Healing

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Background: Extracellular Microvesicles (EMVs): are cellular membrane fragments ranging from 100 nm to 1µm and are shed from almost all cell types. EMVs mediate intercellular communication by transporting mRNA, miRNA, growth factors and other proteins between cells. SIS (small intestinal submucosa) is a three layers of bio absorbable extracellular matrix obtained from naturally derived, intact porcine small intestinal sub-mucosa (SIS *****). **Objective:** To test the hypothesis that there is a correlation between the concentration of EMVs and the percentage of wound healing in treated chronic wounds and the healing percentage will be enhanced by the combination of Oasis ultra and NPWT. **Material &Method:** Wound fluid samples obtained from 14 patients with stage IV trunk pressure ulcers. The patients were divided in two groups (7 in each group, n=7): control group treated with negative pressure wound therapy (NPWT) alone & study group treated with NPWT and SIS dressing. EMVs were isolated using Ultracentrifugation method. EMVs concentration was measured by Nanoparticle Tracking Analysis machine (Nano sight). EMVs content was analyzed using 27 Bio-Plex Pro assay. **Result & Conclusion:** The overall healing rate after 12 weeks of study was higher in the study group (89 %) in comparison with the control group (52 %). There is a positive correlation between EMVs concentration and the percentage of healing in both groups, $R^2 = 0.66$, $P \leq 0.05$. The control group shows high level of intravesicular concentration of pro-inflammatory cytokines and low pro-healing cytokines. However, study group shows high levels of intravesicular concentration of pro-healing cytokines and low pro-inflammatory cytokines. This finding suggests that the EMVs composition could determine the outcomes of the wound healing. The EMVs particles in wound fluid could serve as potential biomarker of the wound healing.

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