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ORIGINAL ARTICLE

Proposed Mechanism of Action of Topically Applied Autologous Blood Clot Tissue

A Quintessential Cellular and Tissue Based Therapy

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Background: Chronic wounds, especially in patients with diabetes, often represent clinical challenges. Recently the use of a topically applied blood clot has garnered significant interest. This stromal matrix contains viable cells that are autologous, biocompatible, biological and consistent with a metabolically active scaffold. It has been shown to be safe,

effective, and cost efficient. However, the mechanism of action of this modality remains elusive. The objective of this manuscript is to identify a potential mechanism of action of an autologous blood clot.

Methods: Review of clinical and scientific literature hypothesizes on how autologous blood clots may stimulate healing and facilitate the movement of critical substrates while lowering bioburden and fostering angiogenesis.

Results: Blood serves as a carrier for many components: red blood cells, white blood cells, platelets, proteins, clotting factors, minerals, electrolytes, and dissolved gasses. In response to tissue injury, the hemostatic mechanism employs a host of vascular and extravascular responses initiating primary, secondary, and tertiary hemostasis. The scaffold created by the autologous blood clot tissue provides a medium in which the body can transform the wound from a non-healing chronic condition into a healing “acute” condition. The autologous blood clot tissue also creates a protective setting for the body to utilize its own mechanisms to promote wound healing in an organized manner. This transient scaffold recruits surrounding fibroblasts and promotes cell ingrowth to foster granulation tissue remodeling. Cells in this matrix not only sense soluble factors, but also their physical environments. This well-orchestrated mechanism includes signals from soluble molecules, from the substrate/matrix to which the cell is adherent, from the mechanical or physical forces acting on it, and from contact with other cells. Topically applied autologous blood clot tissue can lower bacterial bioburden while stimulating angiogenesis and fostering the movement of keratinocytes and fibroblasts.

Conclusions: Topically applied autologous blood clot tissue represents a formidable cellular and tissue based therapy that has been shown to be safe and effective. Although the central component of this therapy is blood, the autologous clot tissue creates a scaffold that performs as a biologic delivery system that functions to control the release of growth factors and cytokines over several days

Autologous therapies have gained in popularity especially involving the treatment of foot ulcers in patients with diabetes (DFUs). Advantages include biocompatibility, minimization of risks associated with disease transmission observed with grafts and cell allografts, and the minimal chance of rejection. There are several technologies currently in the wound healing space that meet these criteria ranging from bioengineered skin equivalents, platelet-rich plasma, cellular therapies and molecular biology. [1] However, the use of autologous blood clot tissue has garnered significant interest. Unlike other products, autologous blood represents an unlimited resource which can be prepared and utilized immediately as a point of care therapy. Autologous blood tissue possesses several key characteristics of an ideal wound dressing. These include adherence, water vapor transport, elasticity, creation of bacterial barrier, absence of toxicity and antigenicity, antisepsis, hemostatic activity, ease of application and removal, minimal storage requirements, and reasonable expense. [2, 3]

According to guidance from ASTM International, cellular and/or tissue-based products (CTPs) for skin wounds are defined primarily by their composition and comprise cells and/or the

extracellular components of tissue. CTPs may contain viable or nonviable cells, tissues, proteins, and other materials for which there is a rationale for benefit beyond that achievable with conventional wound coverings. This definition includes ulcers associated with underlying chronic conditions. Grounded upon evidence-based prospective controlled trials, an autologous blood clot tissue appears to meet the requirements as a living cell biologic. This matrix contains viable cells that are autologous, biocompatible, biological and consistent with a metabolically active scaffold. Its stromal construct creates mechanical stability. [4] It is further proposed that this stroma may replace the extracellular matrix and facilitate the movement of cells such as keratinocytes, fibroblasts and endothelial cells among others. The rational for this manuscript is to review current in-vitro and clinical data to formulate a potential hypothesis for the mechanism of action of autologous blood clot tissue.

Key Methods

A search of EMBASE and PubMed including Medline, Scopus and CINAHL was conducted from database inception through 20 June 2020. Four reviewers (RS, GS, CW, and AJ) independently searched the electronic databases. Key words included 'Cellular and tissue-based products, topical blood clot tissue, wound healing cascade, clotting cascade and diabetic foot ulcers'. Reference lists of all retrieved papers, clinical guidelines and review articles were manually

searched for additional studies and original research was included with no limitations on sample size of reviewed and selected relevant studies and bench research.

Clinical Evidence

Serena et al (2019) analyzed 24 full thickness dermal wounds in a porcine model. Eighteen wounds received whole blood tissue and 6 were treated with saline soaked gauze (the control) for 18 days. Microscopic evaluation revealed that the whole blood tissue product was associated with partial to complete wound reepithelization, while the control garnered minimal reepithelization. By day 18, the mean reduction in wound area was 66% (SD: 6.4) for the wounds treated with the whole blood tissue clot versus 41% (SD: 3.8) in the control group ($P<0.0001$). [5] Snyder et al (2018) studied the safety and efficacy of an autologous blood clot product in the management of Texas 1A or 2A neuropathic diabetic foot ulcers in a prospective, multicenter, open label pilot study. Twenty patients were enrolled; 20 were analyzed in the intent-to-treat (ITT) population and 18 in the per-protocol (PP) group. The proportion of wounds healed in the ITT and PP populations were 13 out of 20 (65%) and 13 out of 18 (72.2%), respectively. Percent area reduction (PAR) for the ITT population at 4 and 12 weeks was 61.6% and 67.1%, respectively; the PARs for the PP population were 60.3% and 76.2% at 4 and 12 weeks, respectively. Mean times to wound healing were 59 days and 56 days in the ITT and PP populations, respectively. This study demonstrated that the blood clot tissue product was safe and efficacious for treating DFUs. [6] A case from this study is seen below. (Figure 7)

Donner et al. (2019) analyzed three prospective, open label, clinical trials utilizing the autologous blood clot tissue RD1 (RedDress Ltd.) in chronic wounds. These analyses demonstrated the efficacy of healing acute and chronic ulcers utilizing autologous blood clot tissue both in vitro and in vivo. [7]

Potential Mechanisms of Action

Despite clinical success, mechanism of action of a topically applied autologous blood clot tissue remains somewhat elusive. However, a review of the wound healing cascade and literature regarding the components of blood clot tissue and their function in wound healing could shed light on this complicated biochemical cascade. Blood serves as the basic necessity for health by moving nutrients and waste around the body, regulating the bodies pH levels, and preventing infections. Blood also serves as a medium for carrying an array of components: red blood cells, white blood cells, platelets, proteins, clotting factors, minerals, electrolytes, and dissolved gasses. [8] An understanding of hemostasis, its components, and the intercorrelation between them remains pivotal in understanding how autologous blood tissue may function when applied topically.

Human skin represents a unique paradigm for organ homeostasis that enables researchers to study putative repair mechanisms for regenerative medicine. [9] In response to tissue injury, the hemostatic mechanism employs a plethora of vascular and extravascular responses initiating hemostasis. Hemostasis can be categorized into three different stages:

primary, secondary, and tertiary. [8] Primary hemostasis involves the formation of the platelet plug at the site of tissue injury. Secondary hemostasis involves consolidating the formed platelet plug with a fibrin mesh via the coagulation cascade creating a scaffold. (Figure 2) Tertiary hemostasis retracts and tightens the newly created scaffold and develops a more organized structure bringing the two ends of the wound together. [8] This could explain how a topically applied autologous blood clot tissue may function in a clinical setting.

A Closer Look at Platelets

Platelets are anucleated, metabolically active, multipurpose cells which arise as fragmentations from the larger nucleated megakaryocytes cortical cytoplasm found in the bone marrow. [10,11,12] Although platelets play the central role of regulating hemostasis, thrombosis, and maintaining the vasculature patency, it is important to further explore the collaborative role platelets have in immune responses, healing, and in the diverse inflammatory processes that influence normal leukocyte biology and inflammatory process. [10,11,12] When activated, platelets secrete a number of growth factors such as platelet derived factors (PDGF) and epidermal growth factors (EGF) among others. These growth factors then stimulate wound healing by promoting fibroblasts to produce collagen, glycosaminoglycans, and proteoglycans. [12, 13,14]

The platelet inflammatory axis consists of five functions including microbial capturing thrombi, the release of pro-inflammatory mediators, the ability to engulf microbes, neutrophil NETosis, and leukocyte migration (Figure 4). [11] While platelets capture and sequester pathogens via

expression of Toll like receptors (TLRs) they also participate in the formation of the Neutrophilic Extracellular Traps (NETs) by adhering to neutrophils. [11,12] Neutrophils then aid in bacterial clearance. [11] Finally, platelets aid in wound healing by providing a scaffold along with fibrin and an array of growth factors within their granules. [8, 12] Platelets are the primary source of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and Epidermal Growth Factor (EGF).[11,12] EGF plays a crucial role wound healing, specifically in re-epithelialization and dermal maturation, via stimulation of three important biological actions in tissue repair; cytoprotecting, mitogenesis, and migration. EGF is also the key signaling molecule in stimulating epithelial cell motility mandating its presence for re-epithelialization. In dermal wound healing, EGF aids in stimulation, proliferation, and migration of keratinocyte, endothelial cells, and fibroblast which all facilitate dermal regeneration. [13] This further reinforces the importance of topical autologous blood clot tissue application in facilitating wound healing.

The Role of fibrin

As primary hemostasis occurs, secondary hemostasis runs parallel, ultimately resulting in the conversion of soluble fibrinogen into insoluble fibrin fibers via the coagulation cascade. This results in a provisional fibrin matrix which serves to not only prevent hemorrhage, but also to provide a provisional extracellular matrix. This facilitates tissue repair, leukocyte cell adhesions,

and endothelial cell migration during angiogenesis. In addition to its role as a provisional matrix, fibrin actively recruits cells to trigger fibrin-mediated responses, such as cell adhesion, migration, proliferation, and tubule formation. Hence wound healing outcomes depend largely on fibrin and its structures in terms of fiber thickness, the number of branch points, the porosity, and the permeability. [14]

Fiber sealants, a mixture of fibrinogen and thrombin, have been investigated and have shown to be a good treatment option in terms of a matrix to promote wound healing and a vehicle to deliver and bind to leukocytes, keratinocytes, fibroblasts, and endothelial cells. It is important to mention that keratinocytes cannot attach directly to fibrin, due to the lack of integrin αvβ3. However, they can bind to fibrin scaffolds that contain fibronectin. Leukocytes eliminate contaminating bacteria via generation of oxygen radical species and enzymatic digestion during phagocytosis. When they work synchronously with Factor XIII and macrophages, the latter release cytokines in the matrix which further stimulates fibroplasia and angiogenesis. Factor XIII also promotes an increase in tensile strength and stability of the matrix. [14] Therefore, topical autologous blood tissue may play a role in decreasing excessive bacterial burden while creating a stable matrix and fostering the formation of new blood vessels.

The Phases of Wound Healing

It is well known that normal wound healing proceeds through four main segments including hemostasis, inflammation, repair and remodeling. This dynamic represents a well-orchestrated

cascade of overlapping phases occurring in tandem. [31,32] (Figure 1) The first phase, hemostasis, includes the formation of the fibrin clot tissue that prevents hemorrhage and provides a "provisional wound matrix" that is critical for formation of an initial scaffold. Fibroblasts and vascular endothelial cells express fibrin receptors on their plasma membrane, so they are able to migrate into fibrin matrix. In contrast, epidermal cells do NOT express fibrin receptors so they cannot migrate across a fibrin clot until it is "modified" with key extracellular proteins including fibronectin which is present in plasma at relatively low levels as soluble fibronectin and is synthesized and secreted by fibroblasts. When platelets contact the fibrin matrix, they release the contents of their alpha granules that are filled with growth factors and cytokines that are critical to initiate chemotaxis of inflammatory cells from intact capillaries adjacent to injury. The first inflammatory cells to move from capillaries into wound bed are neutrophils within 24 hours followed by M1 macrophages that arrive ~24 hours after injury. They are essential to engulf and kill contaminating bacteria, fungi and yeast. The M1 macrophages also synthesize and secrete more chemotactic cytokines that replace those that were initially released by the platelets.

The released growth factors are chemotactic for fibroblasts and stimulate migration of fibroblasts into provisional fibrin matrix. The fibroblasts begin to proliferate and synthesize additional growth factors and cytokines, which replace the growth factors and cytokines that were initially released by platelets. The cytokines released by platelets also contain factors that stimulate migration of stem cells from bone marrow and stem cell niches close to the wound site. [15, 16] These include all three dimer forms of platelet-derived growth factor (PDGF-AA,

PDGF-AB, and PDGF-BB), fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), transforming growth factor beta 1 (TGF1) and vascular endothelial growth factor (VEGF). [16, 17] Plasma also contains antimicrobial peptides such as LL-37 and plasma gelsolin (pGSN) that are important components of the innate immune system and aids in activating macrophages while localizing the inflammatory response. Guo (2010) postulates that wound healing is stimulated by cell-to-cell signaling within the local wound milieu. [18] This includes release of growth factors, particularly VEGF, TGF, & PDGF, from the macrophages and secondary platelet plug complex within the whole-blood autograft. These factors mobilize monocyte migration to the wound, stimulate differentiation into macrophage subtypes, and promote angiogenesis. [19, 30]

However, chronic wounds fail to respond to the normal healing cascade and are often mitigated by underlying comorbidities such as diabetes. This results in the production of senescent cells, hyperproliferative wound edges, and increased numbers of inflammatory proteases. This paradigm shift leads to a paucity of growth factors, poorly functioning receptor sites and an increase in bacterial burden.[32] The utilization of topical autologous blood clot tissue along with wound bed preparation, could reverse this pattern. In effect, the whole blood tissue autograft is thought to recreate the acute phase of healing. [20]

The Extracellular Matrix

The extracellular matrix (ECM) is the largest structure in the skin and is composed of multiple noncellular scaffold proteins(i.e.: collagen, elastin, fibronectin and proteoglycans),

glycosaminoglycans, polysaccharides and water that facilitates bidirectional communication between cells and their biochemical/biophysical .microenvironment. [9] Counterintuitively, the ECM is a dynamic structure that not only provides physical infrastructure but acts as a dynamic regulator of cellular activity between itself and other cells in the wound such as fibroblasts and endothelial cells. This communication, called dynamic reciprocity, remains essential to support tissue repair. [21] Chronic wounds are 'stuck' in the inflammatory phase of wound healing; left unchecked excessive amounts of matrix metalloproteases 2,8 and 9 and human neutrophil elastase (serine elastase) can completely destroy the ECM thus 'short circuiting' the wound healing process. [22, 23] Matrices such as an autologous blood clot tissue may replace the ECM, thus reestablishing this communication.

The Role of Macrophages

Macrophages are essential elements of an autologous blood clot tissue and possess phagocytic and immune capabilities that could mitigate wound infection. They are an essential part of wound healing and their functions may be underestimated. Macrophages participate in host defense, inflammation management, growth factor production, phagocytosis, and cellular proliferation. One of the most important roles of macrophages in stimulating healing of chronic wounds is restoration of tissues in damaged areas. During the inflammatory phase of wound healing, recruitment of macrophages occurs at the wound site. The characteristics of the wound environment generate polarization of the macrophages in order to diversify their phenotypes into 2 categories: classic/pro-inflammatory and alternative/anti-inflammatory.

Differentiated wound macrophages are not a homogeneous population of cells but exist as multiple phenotypes that can be broadly classified as M1 and M2 phenotypes that evolve as a wound matures. [24, 25] Classically activated M1 macrophages produce pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-6 (IL-6), and other mediators that facilitate the initial stages of wound healing. The M1 cells are highly phagocytic, enabling them to phagocytose neutrophils that have undergone apoptosis while removing contaminating pathogens (bacteria) or debris (denatured ECM). In contrast, activated M2 macrophages are typically anti-inflammatory and produce anti-inflammatory cytokines (IL-10, IL-1RA) and pro-angiogenic growth factors (PDGF, VEGF, EGF). [27, 31, 32]

During normal healing, M1 macrophages infiltrate the wound after an injury to clean the damaged tissue of bacteria, foreign debris, and dead cells. In acute wounds, as the tissue starts to repair, macrophage plasticity commences, changing the M1 pro-inflammatory macrophage into an M2 anti-inflammatory macrophage (Figure 6). During the remodeling phase, migration and proliferation of fibroblasts, keratinocytes, and endothelial cells is initiated to assist in restoring the dermis, epidermis, and vasculature. Macrophages assist in the mechanism of revascularization by being positioned near newly formed blood vessels and by assisting in stabilizing and fusing the blood vessels.[26] Macrophages also release matrix metalloproteinases (MMPs) which aid in breaking down the extracellular matrix (ECM) for proper remodeling to take place. The macrophages then go through apoptosis in order for maturation of the skin to occur.

In chronic wounds, M1 macrophages reside in the wound, are unable to engage in plasticity, and cannot transition into M2 leading to further tissue damage. The dysregulation of macrophage transformation causes a reduction in the ability to phagocytize dead neutrophils. The apoptosed neutrophils then begin to accumulate in the wound and cause further inflammation to the area. For example, diabetic patients have macrophages with reduced apoptotic clearance activity because of the effects of hyperglycemia and advanced glycation end products.[26]

Neutrophil clearance by macrophages expedites phenotypic change of the M1 macrophage to an M2 which results in the cessation of the inflammatory phase of healing. Another inducer of macrophage phenotypic change is differential iron regulation. M1 macrophages store the majority of iron intracellularly in the form of ferritin, whereas, M2 macrophages which release the iron into the extracellular environment through the transmembrane channel, ferroportin. [27] Sindrilaru et. al (2011) identified the role of high intracellular iron stores in maintaining M1 macrophages in chronic wounds, particularly chronic venous ulcers. The source of iron was hemoglobin from erythrocytes that escape from damaged blood vessels and enter the wound environment.[26, 33] This represents yet another potential area where topically applied autologous blood could be of benefit.

The mechanism creating chronic wounds has been evaluated in animal and human models. For example, diabetic wounds in both humans and mice were found to have macrophages with an inflammatory phenotype that secreted IL-1b, which was discovered for the first time to be part of a pro-inflammatory positive feedback loop that blocked the

activation of the M2 phenotype."[27] It has been noted that in both venous and diabetic subjects, the wound peripheries reveal significantly greater numbers of macrophages in chronic wounds verses those that are acute. Macrophages that are found in acute wounds tend to be hybrids containing both M1 and M2 activation phenotypes. This aspect is key in enabling their plasticity, allowing for the ability to shift between functions specific to each phenotype during the healing process. It is noted that both undifferentiated macrophages as well as M2 macrophages participate together to achieve tissue repair. If this mechanism is disrupted in any way, inflammation continues, and the wound healing process fails fostering a worsening prognosis. It has been hypothesized that modalities such as topically applied autologous blood clot tissue may have the capacity to abate the M1 macrophage phenotype. This dynamic could simultaneously foster the phenotypic switch into M2 macrophages thus facilitating wound healing.[27]

Additionally, these cells appear to work in tandem with fibroblasts during matrix remodeling. Animal experiments proffer the notion that macrophages may also foster cytokine production, matrix elaboration and matrix breakdown during different stages of wound repair. [9] In vitro studies by Macrae and colleagues (2018) utilizing a murine model revealed that the highly permeable fibrin network in blood creates a film which forms at the air liquid interface. Functionally, this film retains blood cells and has a protective effect against penetration by bacterial pathogens. [28] Furthermore, a study by Chen et al (2019) revealed that coagulation factors VII. IX and X, three proteins with a well- established role in the initiation of the clotting cascade, may have a significant antibacterial effect against gram-negative bacteria. This in-vitro

research revealed efficacy against historically drug-resistant strains including Pseudomonas aeruginosa and Acinetobacter baumannii. [29] The findings could elucidate an additional therapy for combating 'superbugs' in chronic wounds. These characteristics remain essential components of the CTP model and specifically, topically applied autologous blood clot tissue. It is hypothesized that a fundamental difference between topically applied autologous blood tissue and platelet rich plasma (PRP) stems from the principle that the autologous blood clot tissue firmly adheres to the wound edge and pulls the edges together, causing the wound to contract. [10]. (Figs. 5,6) The autologous blood clot tissue also provides a durable scaffold in which growth factors are not easily destroyed and the growth factor receptors are not damaged as opposed to PRP which is in a gel-like form. [8, 11, 12, 13, 14]

Conclusions

Chronic wounds, particularly in patients with diabetes, create wound healing challenges. Therefore, it is frequently necessary to incorporate advanced cell and tissue-based products (CTP's) into the treatment algorithm. Topically applied autologous blood clot tissue represents a formidable CTP that has been shown to be safe and effective. Although the central component of this therapy is blood, the autologous clot tissue creates a scaffold that performs as a biologic delivery system that functions to control the release of growth factors and cytokines over several days. In response to tissue injury, the hemostatic mechanism employs a host of vascular and extravascular responses initiating primary, secondary, and tertiary hemostasis. These hemostatic responses involve a variety of intricate components all of which

ultimately result in a provisional scaffold preventing hemorrhage and microbial invasion, aiding in wound contraction and healing, and ultimately contracting the wound edges together for repair. Two key elements which set forth the groundwork of the scaffold via conformational change and cleavage are activated platelets and fibrin, respectively. This represents a similar construct to the 'brick and mortar' system where the platelets serve as the 'brick' and the fibrin mesh as the 'mortar'. Aside from the central role platelets display with hemostasis, they additionally serve to provide immunity to the wound site, aid in the inflammatory process, provide the essential growth factors and clotting factors required for wound healing and repair via their granules, and are essential for wound healing. Similarly, apart from the primary role the fibrin matrix serves by providing a temporary extra cellular matrix, it aids in tissue repair, leukocyte adhesions, endothelial cell migration during angiogenesis, and actively recruits cells to trigger fibrin-mediated responses migration, proliferation, and tubule formation. The scaffold created by the autologous blood clot tissue provides an environment that is favorable for dynamic macrophage plasticity to take place. Through proper cytokines and mediators, M1 macrophages can transform from their pro-inflammatory phenotype into M2 the anti-inflammatory phenotype. This scaffold provides a medium in which the body can transform the wound from a non-healing chronic condition into a healing "acute" condition.

The autologous blood clot tissue also creates a protective setting for the body to utilize its own mechanisms to promote wound healing in an organized manner. Structural integrity of the scaffold is important to ensure lack of disruption of the wound bed and to prevent further injury that may elicit an M1 macrophage pro-inflammatory response. Additionally, this

transient scaffold recruits surrounding fibroblasts and promotes cell ingrowth to foster granulation tissue remodeling. Cells in this matrix not only sense soluble factors, but also their physical environments. This well-orchestrated mechanism includes signals from soluble molecules, from the substrate/matrix to which the cell is adherent, from the mechanical or physical forces acting on it, and from contact with other cells. Topically applied autologous blood clot tissue can lower bacterial bioburden while stimulating angiogenesis and fostering the movement of keratinocytes and fibroblasts. These functions are created through replacement, repair, and regeneration, and appear to be consistent with a definition of CTP. However, additional research is required to further elucidate the hypothesized mechanisms of action proffered in this manuscript.

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Figure 1: The well-orchestrated phases of normal wound healing. Used with permission from Surgical Materials Testing Laboratory.

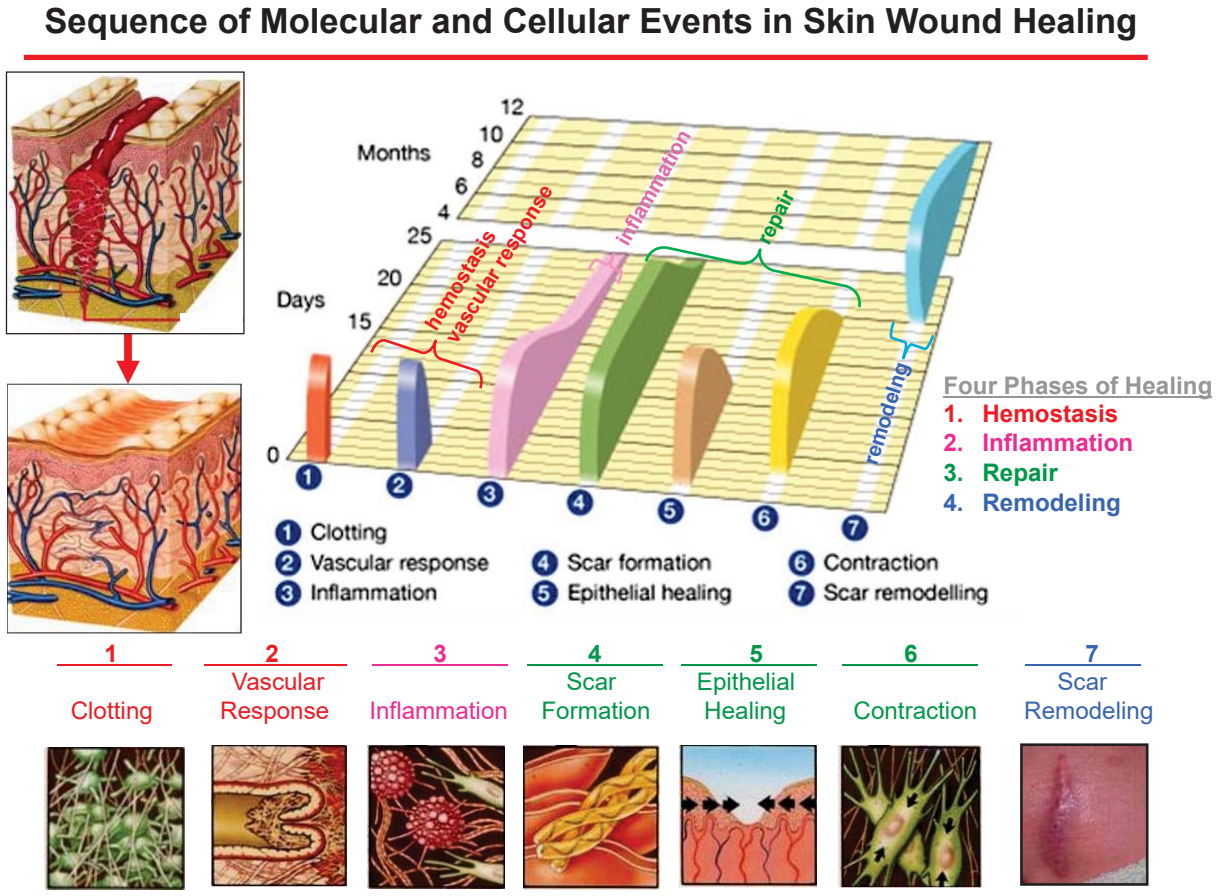

Figure 2: Electron microscopy of stromal matrix in topical autologous blood clot tissue.

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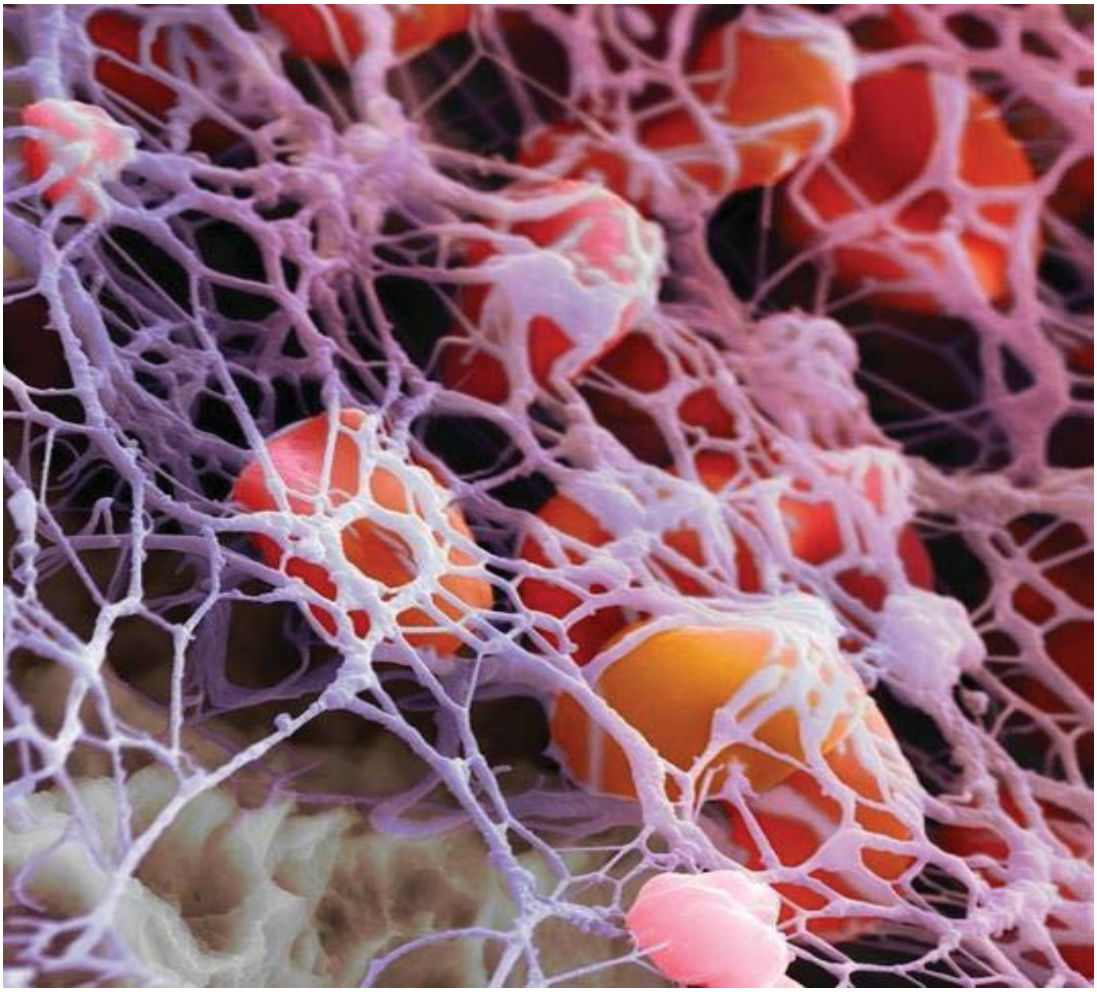

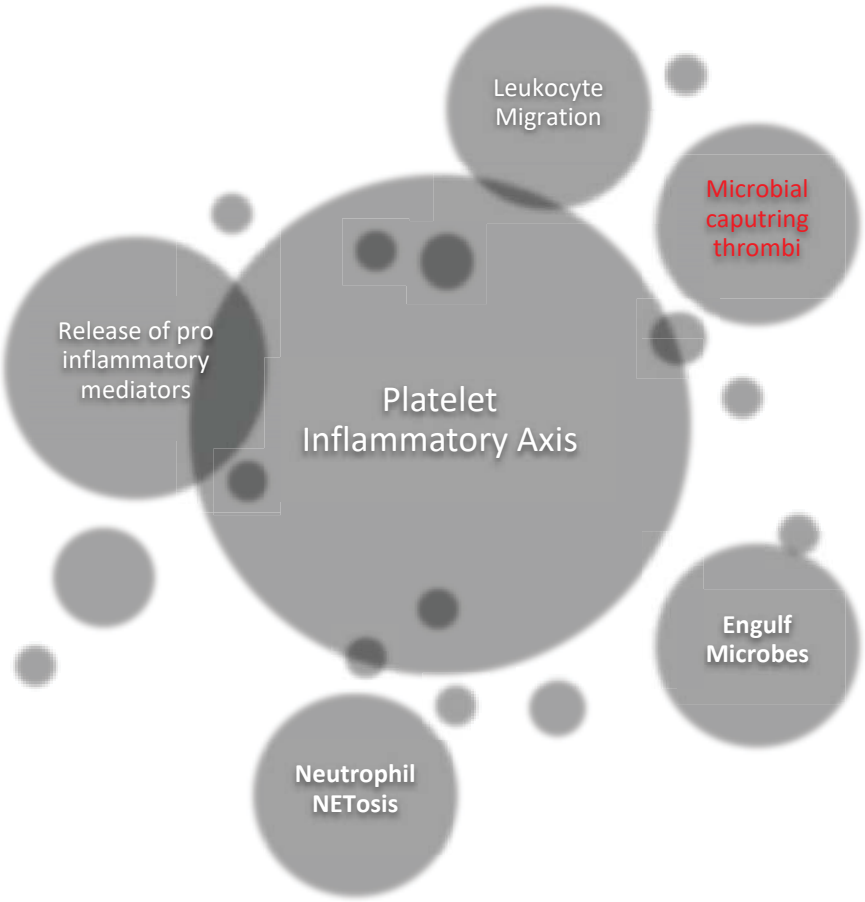

Figure 3: The Platelet Inflammatory Axis

Figure 4: M1 & M2 phenotypes

Figure 5: Application of topically applied blood clot tissue to a plantar foot ulcer in a patient with diabetes

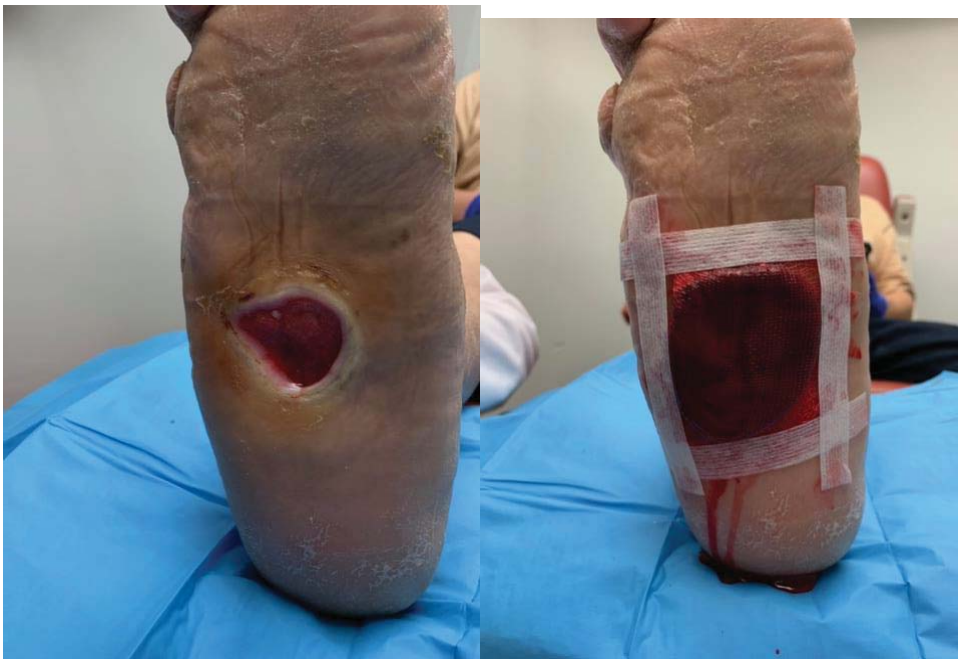

Figure 6: Application of topically applied blood clot to a plantar heel ulcer in a patient with diabetes

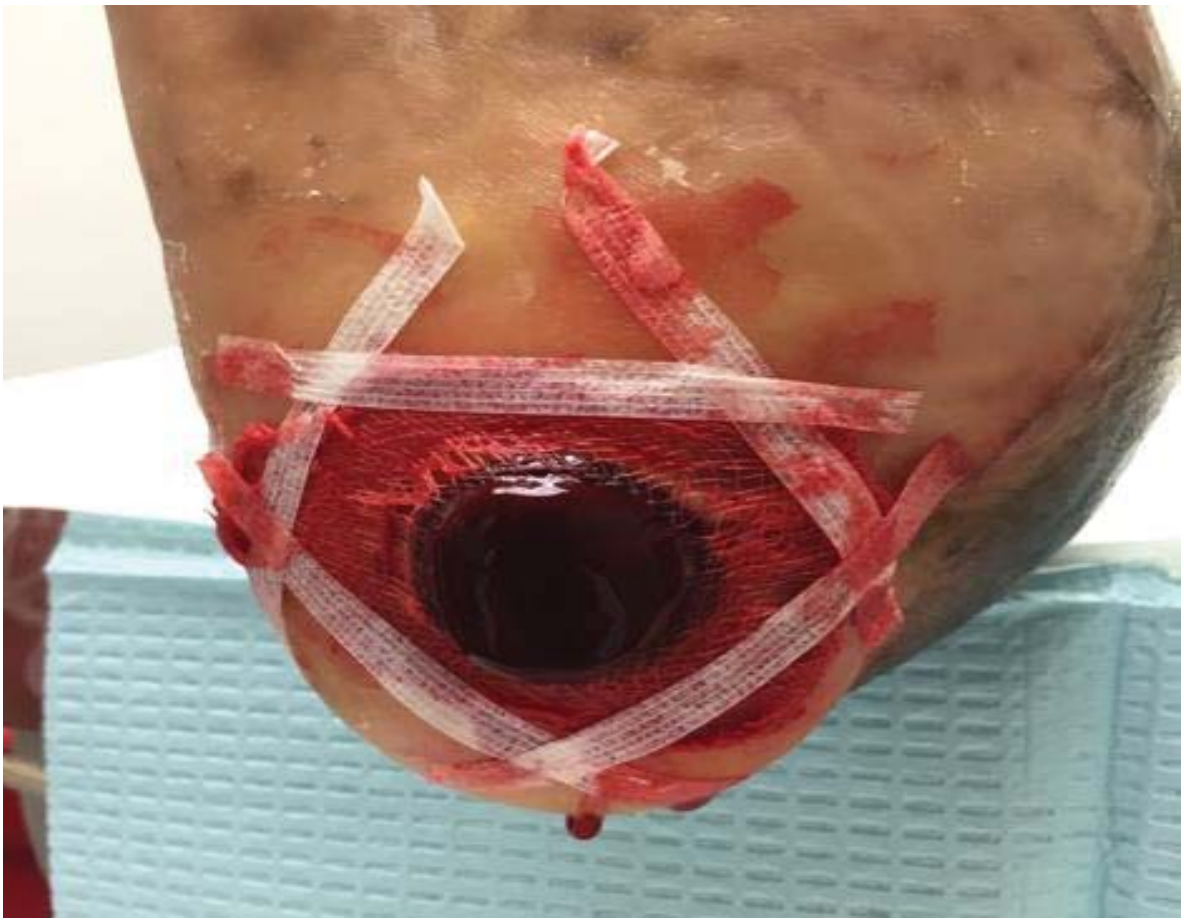

Figure 7: Case Report

A 72-year-old male presented with a plantar diabetic foot ulcer Texas grade IA. Symptoms had been present for 6 weeks and failed to respond to topical therapies (Impregnated Gauze, antiseptics, weekly sharp debridement) and an offloading boot.

Past Medical History includedType II diabetes Mellitus, Diabetic Neuropathy, Hypertension, Peripheral Arterial Disease, Charcot Marie Tooth, and Hypercholesterolemia.

Ulcer Baseline size: 3.5 cm
Healing: Complete healing in 7 weeks, 7 blood clot tissue (RD1) applications

Baseline Week 7