REVIEW ARTICLE

Mesenchymal Stem Cell–derived Extracellular Vesicles: An Emerging Therapeutic Strategy for Diabetic Wound Healing

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Abstract

One of the most serious side effects of diabetes is diabetic foot ulceration (DFU). It is a severe and extremely morbid illness that has been linked to higher mortality on its own. The development of effective wound therapeutics in the future may be influenced by our current and developing understanding of wound pathophysiology. By reestablishing cellular functioning, small extracellular vesicles (sEVs), a crucial medium for intercellular communications, exhibit encouraging therapeutic potential in the treatment of DFU. Mesenchymal stem cell (MSC) derived exosomes and engineered extracellular vesicles (EVs) have the potential to aid in the healing of wounds. Along with encouraging the growth and stimulation of endothelial cells, keratinocytes, and fibroblasts, they also have immunomodulatory and anti-inflammatory properties. They help prevent damaged cells from dying, revitalize senescent cells, and boost angiogenesis. MSC-EVs can be a safe, effective and ethical therapy for DFU by increasing M2 macrophages polarization, improving the proliferation, reducing scar, and improving angiogenesis.

KEYWORDS: mesenchymal stem cell, extracellular vesicle, diabetic wound, wound healing

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Introduction

The largest organ in the human body, the skin is susceptible to both acute and chronic lesions, including burns and diabetic ulcers. Nearly 6.7 million people worldwide recently suffer from chronic wounds.(1) Over the past few decades, diabetic wounds, a common representation of chronic wounds, have been identified as an important clinical concern that has plagued the medical community globally, particularly with the aging of society.(2) The hallmark clinical characteristics of diabetic wounds are generally acknowledged to be recurrent infection, an overabundance of inflammatory response, and poor vascular regeneration.(3) For many years, there has been a lot of interest in creating effective wound care, particularly for chronic wounds like diabetic foot ulcers (DFU). There are many different approaches and techniques for treating wounds today, but a cost-effective and safe methods are still needed.

For individuals with DFU, the outlook is dismal. Thirty-three percent of diabetic ulcers remain chronic sores because they do not heal.(4) The chronic, relapsing, and remitting nature of this illness is exemplified by the fact that 65% of ulcers that do achieve "healing" will re-ulcerate after three years. Patients with diabetes are up to 25 times more likely to lose their limb than people without the disease, and 20% of moderate to severe DFU result in some kind of amputation.(5) An odd ratio of 0.34 for foot amputation in DFU patients in Asia was determined.(6) After a significant amputation due to diabetes, the anticipated five-

year survival rate was 47%; for dialysis patients, this rate dropped to 17%.(7) Heart failure, myocardial infarction, stroke, and some types of cancer have survival rates that are on par with or worse than those of other serious illnesses. (8,9) In line with findings from prior investigations, a large population-based study discovered that individuals with a history of DFU had a twice as high chance of dying over a ten-year follow-up period as those without diabetes.(9-11) Patients with diabetes who have foot ulcers have a lower quality of life, are less able to function physically, are more likely to experience anxiety and sadness, and are at greater risk of dying.(12,13) Healthcare providers and caregivers need to consider the numerous repercussions an ulcer may have on a patient's health.

Diabetic wounds vary from normal healing processes, such as hemostasis, inflammation, proliferation, and remodeling, due to the elevated glucose and toxic advanced glycation end-products (AGEs).(14) In essence, the repair process is thought to be suspended when skin repair cells, including fibroblasts, immune cells, epidermal cells, and vascular endothelial cells, exhibit dysregulated behavior near wound beds.(15) For instance, excessive inflammatory cytokine secretion by overactivated immune cells hinders vascular maturation, re-epithelization, and collagen deposition.(16) Therefore, it is anticipated that the healing processes of diabetes wounds would be recovered from the pathogenesis by creating efficient techniques to regulate and restore normal cellular activities.(17)

Mesenchymal stem cell (MSC) transplantation has emerged as a novel wound treatment option in recent years. (18) Clinical studies have demonstrated the effectiveness of stem cell transplantation in the treatment of wounds, especially full-thickness or diabetic wounds.(19)Adipose tissue, dental pulp, placenta, amniotic fluid, and umbilical cord blood (UCB) are just a few of the various sources of MSCs, which are significant members of the stem cell family and share the traits of self-replication and multidirectional differentiation.(20) It has been shown that paracrine activities mediated by stem cell secretion factors are the main mechanism of action of stem cell treatment.(21,22) This approach does, however, have many drawbacks, including being costly and time-consuming and being fraught with ethical and legal issues. As a result, researchers are still working to develop a cell-free wound treatment.(23)

Exosomes, a type of nano-sized extracellular vesicles (EVs) have been found to have a significant paracrine function among specific secretory entities linked to stem cells. After being expelled from cells, the tiny vesicles created by reverse budding are known as EVs.(24) These vesicles are composed of lipids, proteins, and nucleic acids and frequently act as mediators of intercellular communication. These tiny EVs are currently the subject of research by numerous clinical investigators. EV-based cell-free therapy has already started to be used commercially. (25,26) Indeed, MSC-derived EVs have been exploited as cell-free alternatives to MSCs in a variety of disease models, such as cancer, musculoskeletal disease, nerve disease, lung disease, liver disease, kidney disease, cardiovascular disease, eye disease, skin disorders, and musculoskeletal disease.(27) To develop cell-free therapy to enhance the healing of both normal skin wounds and chronic refractory wounds, researchers have tried to investigate the mechanism by which EVs stimulate wound healing. The four phases of wound healing, which are hemostasis, inflammation, proliferation, and remodeling, have been studied from various perspectives by researchers using various EVs derived from MSCs, and they have made good success.(28)

Main Process in Wound Healing

Homeostasis, inflammation, proliferation, and remodeling are the four overlapping steps that make up wound healing process.(29) The extracellular matrix (ECM) and cells interact intricately during wound healing can manifest as tissue regeneration or scarring because the skin typically loses its capacity to regenerate the normal epidermis and dermis in adulthood. As described in Figure 1, Wound healing process initiated as blood arteries contract to stop bleeding as soon as an injury occurs; platelets then gather and activate in the wound to form the platelet plug, called primary haemostasis.(30)

The next phase is the recruitment of inflammatory cells.(31) Proinflammatory macrophages (M1 types) generate ROS and proinflammatory cytokines (such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-1) to have bactericidal effects during early inflammation. M1 macrophages then execute efferocytosis to promptly eliminate neutrophils. Ultimately, M1 macrophages polarize to the M2 type in response to cytokinesis or other signals, and the wound progressively enters the proliferative phase. Macrophages' polarization is essential for the following healing of wounds. The proliferative phase's arrival is marked by the migration of fibroblasts, granulation, angiogenesis, and re-epithelialization. The thrombus will be replaced with granulation tissue, which will serve as a scaffold for new cells (like macrophages) or other elements (like new blood arteries).(32)

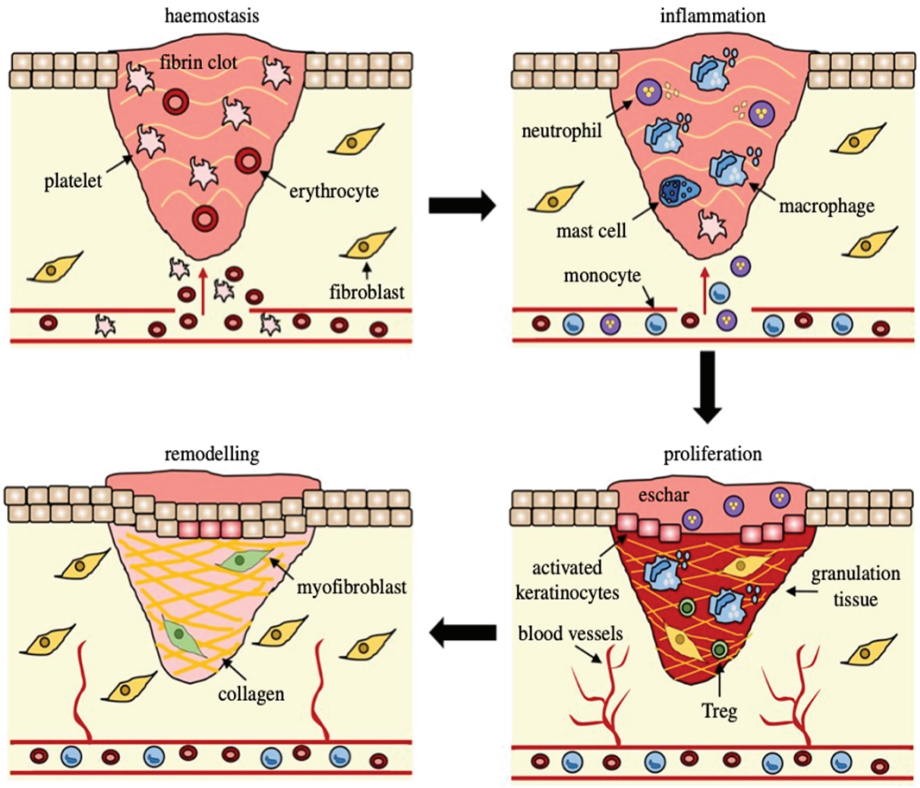

Figure 1. The stages of wound repair and their major cellular components.(30) (Adapted with permission from Royal Society).

The last stage is angiogenesis. Endothelial cells are stimulated to initiate angiogenesis by mild hypoxia, cytokines, and protein hydrolases. Enzymes specifically cause endothelial cells to escape from damaged capillaries, multiply, and move in the direction of proangiogenic signals to create new vascular networks. M2 macrophages promote angiogenesis by expressing cytokines including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), smooth muscle cells, as well as pericytes to maintain the neovascularization.(33)

The main goals of the remodelling stage, which is the last step of wound healing, are to increase tensile strength and restore tissue structure. Lastly, apoptosis, differentiation, or other unidentified processes cause most of the macrophages, myofibroblasts, and endothelial cells engaged in wound healing to vanish. The length of remodeling might range from weeks to years, depending on the severity of the injury. Cell-cell, cell-factor, cell-tissue, cell-vascular systems, etc., all interactions during wound healing, and each healing phase will give the signal for the subsequent step.(34)

Diabetes Foot Ulcers: A Chronic Wound

DFU affects between 19% to 34% of diabetics. DFU treatment is quite difficult since the recurrence rate within a year after complete healing is about 40%.(35) It is widely acknowledged that the primary causes of DFU are diabetic neuropathy and vasculopathy brought on by prolonged hyperglycemia, and that bacterial infections make treating DFU more challenging.(36)

The healing period for DFU, typically lasted longer than four weeks in a clinic, thus regards as a chronic wound with persistent inflammation. Despite having a similar healing mechanism to a typical acute wound, DFU wounds eventually stop healing.(15,37) The extracellular space where the cells and signals (such growth factors) involved in the genesis or healing of DFUs are found is known as the DFU microenvironment. It is made up of elements from the external and internal microenvironments.(38)

One of the microenvironmental features of DFU is hyperglycemia as described in Figure 2, which is linked to insulin disruption (deficiency or resistance).(39) The polyol pathway hardly ever metabolizes intracellular glucose under normal circumstances. Nevertheless, when hyperglycemia is stimulated, the amount of glucose digested by the polyol pathway rises noticeably (by over 30%), and the hexosamine and protein kinase C pathways are activated as well.(40) In addition to causing AGEs and oxidative stress, such aberrant glucose metabolism suppresses the production of antioxidants, which exacerbates inflammation.(41) Furthermore, hyperglycemia prevents inflammation

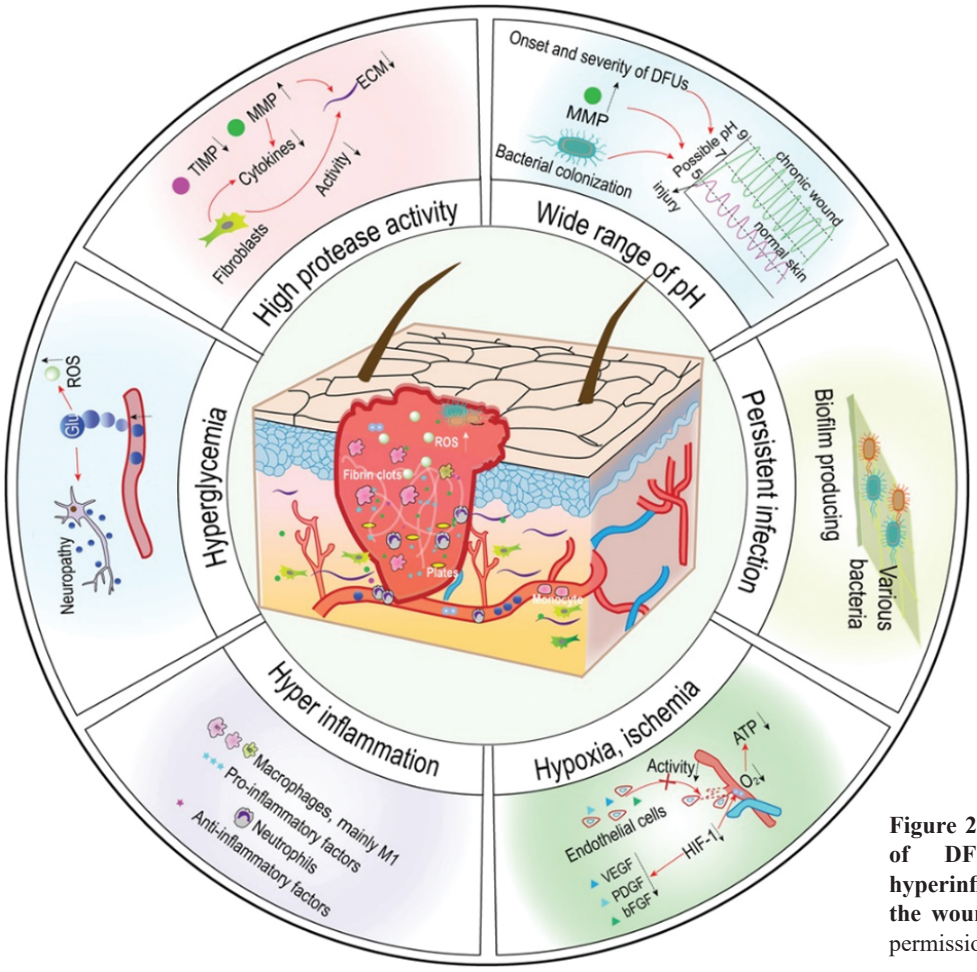

Figure 2. The complex microenvironment of DFU, including hyperglycemia, hyperinflammation, hypoxia, *etc*., renders the wound nonhealing.(39) (Adapted with permission from Wiley-VCH GmbH).

from reducing and raises the risk of bacterial infection. (42) Simultaneously, local hyperglycemia can impede the process of proliferation and remodelling by causing endothelial cells to undergo apoptosis, inhibiting fibroblasts' and keratinocytes' ability to migrate, and substantial matrix metalloproteinase (MMP)-1 production.(43,44)

As was previously mentioned, hyperglycemia-induced redox homeostasis imbalance is linked to hyperinflammation in DFU. In particular, hyperglycemia inhibits the expression of chemokines during the inflammatory phase, which delays the arrival of monocytes and macrophages at the wound site and hinders the prompt removal of neutrophils, increasing oxidative stress in the wound. Furthermore, epigenetic changes brought on by hyperglycemia, such as histone methylation and acetylation, increase the expression of pro-inflammatory factors (such as TNF-α, IL-1, and IL-6) and promote the polarization of M1 macrophages. M1 macrophages contribute to the persistence of inflammation by upregulating proinflammatory markers, which in turn creates a vicious cycle of M1 macrophage infiltration and oxidative stress.(45)

Moreover, chronic oxidative stress promotes the senescence of fibroblasts, endothelial cells, keratinocytes, and MSC, all of which are highly harmful to the development of granulation tissue, blood vessels, and epithelium during the healing process.(45,46) Furthermore, collagen damage and MMP upregulation occur in tandem with protracted inflammation, which inhibits remodeling.(41)

Vasculopathy reduces the ability of immune cells to circulate to the diseased location and results in hypoxia in tissues. Impairment of both intrinsic and adaptive immunity is referred to as immunopathy. Wounds are more vulnerable to infection in a hyperglycemic setting.(47) Additionally, hyperglycemia alters the structure and function of immune cells, impairing their capacity for phagocytosis, chemotaxis, and microbial killing.(48) As a result, under the influence of several conditions, microbial flora (primarily bacteria) from the organism itself and the surrounding environment invade the wound.(49) Even worse, the adhesion and accumulation of several microbes promotes the development of biofilms, which are more challenging to treat than individual germs. (49,50)

The imbalance between the limited oxygen supply (impaired vascular system) and the high oxygen demand (needed for cellular repair of wounds) in healing tissues causes DFU to end up in a chronic hypoxic environment with oxygen tension typically below 20 mmHg, as opposed to the transient hypoxia in acute wounds.(51,52) A key modulator of oxygen homeostasis, hypoxia-inducible factor 1 (HIF-1) influences the expression of hundreds of genes. HIF-1 is active in normal hypoxic settings, which triggers downstream gene expression to support erythropoiesis and generate growth factors (VEGF, PDGF, and basic fibroblast growth factor (bFGF)), which are critical for hypoxia regulation.(51,53) Notably, one of the strongest proangiogenesis factors is VEGF, and a major contributing element to DFU vascular injury may be compromised HIF-1/VEGF axis function.(54) Sadly, HIF-1/VEGF expression is downregulated in chronic wounds, including DFU, due to oxidative stress and hyperglycemia, which renders angiogenesis inadequate and results in ischemia.(39)

Proteases, particularly MMP, are abundant at the wound site and function as decomposers in the wound, assisting in all phases of healing. Wound healing is actively influenced by MMP secretion, activation, and homeostasis. (55) Many cells in the wound, including neutrophils, fibroblasts, and keratinocytes, release inactive MMP zymogens in response to cues such reactive oxygen species (ROS), proinflammatory cytokines, and extracellular matrix. Kallikrein and serine proteases then transform MMP zymogens into active MMP. Tissue inhibitors of metalloproteinases (TIMPs) are primarily responsible for the regulation of MMP homeostasis.(56) Nonetheless, in DFU, MMP expression is increased whereas TIMP expression is downregulated.(55) Microorganisms inhabiting the wound periphery express foreign proteases in addition to endogenous proteases in order to promote their growth. In general, excessive amounts of endogenous and exogenous proteases break down vital proteins, which worsens tissue necrosis in the wound and produces a lot of protein exudate, which prevents the wound from healing.(55)

The undamaged skin surface functions as a natural barrier and is naturally acidic due to the release of organic acids by epithelial cells that have a pH between 4 and 6. The skin's pH rises from the surface to a depth of around 7.4. Damage to the skin exposes the organism's neutral tissues to the outside world. As a result, as acute wounds heal, the pH returns to its acidic state. Nevertheless, depending on the pathophysiology, chronic wounds typically have a more alkaline microenvironment than acute wounds.(56) The pH of chronic wounds with large bacterial burdens is typically higher than 7.3. In a similar vein, it was also found that the pH range of chronic wounds was 7.15 to 8.9. However, clinical records have also documented pH values as low as 6.41 and as high as 9.0. As a result, the pH environment of DFU is typically quite variable and dynamic, and it is linked to a number of variables, including the time of beginning, severity, and stage of the wound, as well as the colonization of various microorganisms.(57) Therefore, the DFU microenvironment restrain the wound to heal.

Diabetic Wound Healing and Macrophage Polarization

The establishment of the chronic inflammatory phenotype of diabetic ulcers may be caused by the inadequate early-stage inflammatory responses that are seen in diabetes chronic wounds as opposed to acute wounds (Figure 3).(58) Compared to non-diabetic settings, injury exhibits significantly decreased levels of IL-6, IL-8, their receptors, the precursor of neuropeptide Y (NPY), prepro-NPY, and the C-C chemokine receptor type 2 (CCR2) for macrophage chemoattractant protein (MCP)-1. Systemic natural killer (NK) and T cells in diabetes patients also demonstrate suppression of IL-6, IL-8, and cluster of differentiation (CD)28 signaling pathways, according to single-cell transcriptome and pathways study.(59) The pro-inflammatory reactions of keratinocytes, fibroblasts, and endothelial cells are stimulated by the persistence high level of neuropeptide substance P.(60)

Granulocytic (Gr)-1$^+$, CD11b$^+$, and CD14$^+$ macrophages are the most common types of hyperpolarized macrophages. (61) Their population and related secreted pro-inflammatory chemicals accumulate as a result of their decreased clearance linked to dysregulation of the cell membrane protein selectin P ligand (SELPLG).(62) In hyperglycemic circumstances, α-defensins are also increased, which enhances the recruitment of neutrophils, basophils, and T cells and promotes the release of IL-8. By inducing pro-inflammatory gene expression in epithelial keratinocytes, excretions from *Staphylococcus aureus* biofilms, the predominant bacterial species in diabetic chronic wounds, were also demonstrated to directly contribute to the chronic inflammatory phenotype.(58) Numerous studies have demonstrated that pro-inflammatory (M1) macrophages' poor transition to anti-inflammatory phenotypes (M2) in wounds is directly linked to chronic wounds. M1 and M2 type macrophages can be characterization based on their surface markers as seen in Figure 4. Due to their plasticity

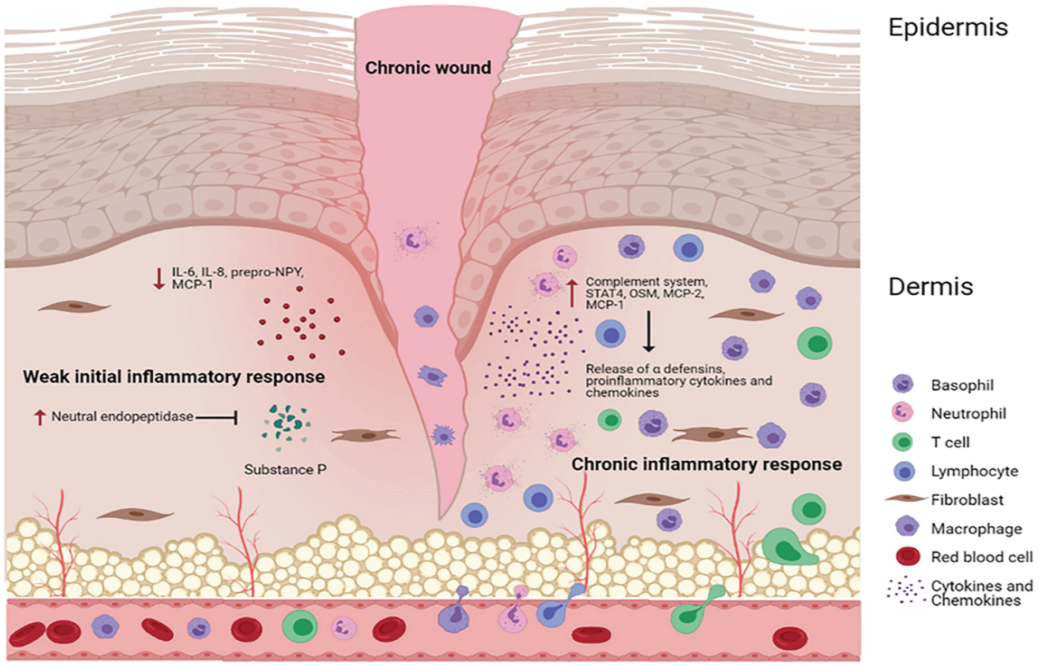

Figure 3. Pathophysiology of diabetic chronic wounds.(58) (Adapted with permission from Medicalhelplines.com Inc (3M) and John Wiley & Sons).

and heterogeneity, macrophages exhibit a wide range of functional characteristics in skin wound models, including scavenging, phagocytosis, and antigen presentation during the inflammatory phase, stem cell recruitment and revascularization during the proliferative phase, and extracellular signaling transduction during the remodeling phase.(61,62)

Additionally, macrophages were identified as background actors that contribute to immunomodulation in a variety of illnesses. This group of immune cells was thought to be a promising target for treatment and may be able to be altered in a tissue-specific way.(63)

The ratio of M1/M2 macrophages are dynamically changing and become challenging to exactly measure even though the characteristics of both phenotypes have been elucidated. The dysregulated of this ratio will disrupt the wound healing. The smooth progression of the repair process is facilitated by the strict modulation of the macrophage phenotype transitioning from an M1-proinflammatory to an M2-anti-inflammatory (pro-healing) phenotype as wounds heal.(64) However, M1 macrophages in wounds were inhibited with an incomplete conversion to M2 phenotype under pathological conditions such diabetes, obesity, infection, and aging, which caused the healing process to stop at the inflammatory phase.(65) Histone acetyltransferase males absent on the first (MOF) is stimulated by TNF-α in macrophages, and it is elevated in a diabetic environment. MOF hinders wound healing processes by increasing TNF-α signaling activation and NFκB-mediated gene transcription via H4K16 acetylation in wound macrophages. (66) Neutralizing monoclonal antibodies that block TNF-α signaling in an obese mouse model inactivated macrophage, decreased circulating monocyte populations, and decreased levels of inflammatory cytokines. This caused the wound to close, indicating that TNF-α signalling is yet another important component that contributes to the development of diabetic ulcers.(67) Impaired macrophage efferocytosis raises the number of apoptotic cells at the wound site, which in turn stimulates pro-inflammatory cytokine responses while attenuating anti-inflammatory ones.(58,68)

There is proof that MSCs have strong immune system regulating effects, especially when it comes to macrophage immunoregulatory activity.(69) The recruitment of macrophages is one way that MSCs influence them. For instance, the conditioned media of bone marrow MSCs dramatically speeds up macrophage movement *in vitro.* (70) In comparison to the control group, the subcutaneous injection and topical administration of bone marrow MSCs conditioned media in wounds enhanced the proportions of macrophages and endothelial progenitor cells, hence improving wound healing.(70) High quantities of the chemoattractant C-C motif ligand (CCL)3/macrophage inflammatory proteins (MIP)-1α, MIP-2, and CCL12/MCP-5 released by MSCs may be responsible for the recruitment

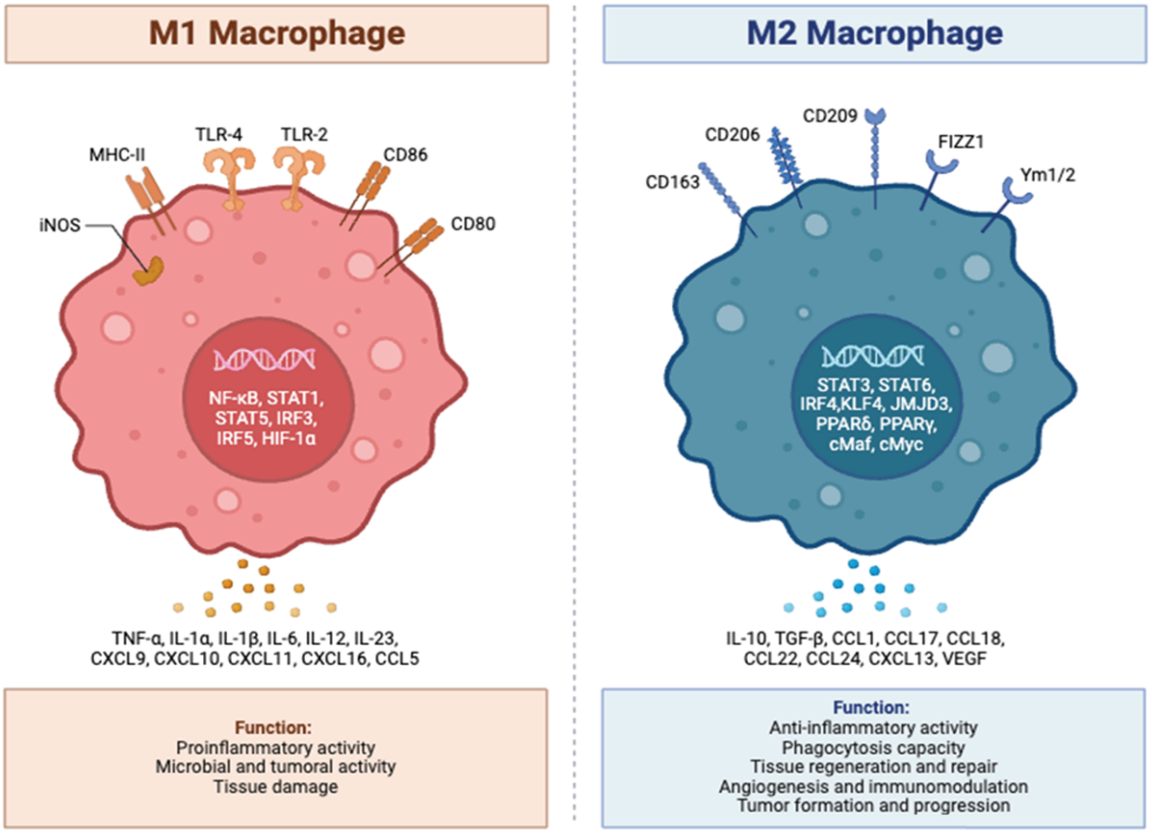

Figure 4. Characteristics of M1 and M2 macrophages based on their surface markers, cytokines, and signals. (Created using Biorender).

of macrophages.(65) An additional way that MSCs affect macrophages is by increasing their capacity to take up apoptotic neutrophils. This is explained by either increased release of soluble extracellular superoxide dismutase (SOD3) from MSCs or upregulation of the intercellular adhesion molecule-1 (ICAM-1) on macrophages.(71) MSCs also influence macrophages by raising the number of M2 macrophages in wounds.(72) The M2 phenotype's secretory traits expressed more IL-6 and IL-10 and less TNF-α and IL-12.(73) In diabetic mice, MSCs or MSC-conditioned medium treatment of wounds resulted in an increase of M2 macrophages and high levels of VEGF and IL-10 but low levels of TNF-α and IL-6.(74) Some study showed that topical MSC administration inhibited TNF-α, which in turn had an immunosuppressive effect *in vivo*.(75) The immunomodulatory processes of a panel of mediators released by MSCs. For instance, the M1-M2 polarization of macrophages was directly impacted by prostaglandin E-2 (PGE-2), a mediator released by MSCs.(76) This mechanism comprises two pathways, including CREB and PI3K signaling, and is primarily controlled by PGE2 binding to the EP4 receptor on M1 macrophages.(76)

Autophagy and Chronic Wound Healing

Although more research indicates that autophagy is specifically necessary for wound healing and repair, we still don't fully grasp the systematic relationship between autophagy and wound healing. Autophagy is a cellular self-defence mechanism under normal physiological settings. A modest degree of autophagy helps cells cope with unfavourable situations and maintain a stable intracellular environment. On the other hand, excessive autophagy in abnormal circumstances can cause excessive cellular content breakdown and initiate autophagic cell death, a type II programmed cell death.(48) Through the lysosome-dependent, self-renewing process of autophagy, unnecessary or potentially harmful cytoplasmic cargos (such as damaged mitochondria or invasive pathogens) are delivered to the lysosome, where they are broken down. The breakdown products are then returned to the cytoplasm and recycled for various cellular uses. Macro autophagy, micro autophagy, and chaperone-mediated autophagy are the three morphological subtypes of autophagy.(49)

Both macro- and micro-autophagy can be selective or non-selective. While selective autophagy targets invading microorganisms and damaged or unnecessary organelles like mitochondria and peroxisomes, nonselective autophagy is employed to turnover bulk cytoplasm under famine conditions.(50)

Both the proliferative and remodelling stages of wound healing are significantly influenced by fibroblasts. The transcription factor EB (TFEB) is crucial for controlling autophagy, mediating it to preserve fibroblast viability and function.(77) Remifentanil is another popular short-acting synthetic opioid analgesic medication that prevents skin fibroblasts from dying due to oxidative stress by triggering autophagy.(78) Furthermore, several investigations have demonstrated a tight relationship between fibroblast differentiation and autophagy activation and inhibition.(79) For instance, a rat model of wound healing showed significant increases in the frequency of LC3-positive dots during the late proliferative phase when spatiotemporal alterations in LC3-positive dots in fibroblasts and myofibroblasts were seen. Interestingly, there were more of them near the edge of the wound than in the middle, indicating that the fibroblasts there were in the differentiation phase.(80) Remarkably, a prior study discovered that the autophagic mechanism is not involved in gingival wound healing, which leads to less myofibroblast differentiation and less scarring. On the other hand, inflammation triggers autophagy, which consistently stimulates myofibroblasts and results in oral mucosal cicatrix repair.(81) Therefore, depending on the tissue or cell in which it occurs, autophagy can have a dual influence on the control of wound healing and decide distinct clinical outcomes.

Several studies indicate that diabetic refractory wounds may be caused by deregulation of autophagy. Furthermore, a hyperglycemic environment significantly reduces keratinocytes' ability to migrate, most likely by suppressing p38 MAPK signalling activation and downregulating the autophagy-related proteins Atg5 and LC3-II, which prevent autophagy and slow cell migration.(82) Under chronic hyperglycemic circumstances, AGEs are the end products of non-enzymatic reactions between the aldehyde groups of sugars and the amino groups of proteins, nucleotides, and nucleic acids.(83) Autophagy plays a significant role in AGE-induced refractory wounds, and numerous investigations have proven that AGEs produce refractory wounds by altering the activities of various cell types. By encouraging macrophage polarization towards the M1 phenotype through autophagy activation, AGEs induce refractory wounds, according to a prior study.(84) According to a different study, melatonin treatment increases autophagic flow in EPCs, which encourages migration and adhesion, while simultaneously decreasing AGE-induced death of these cells.(85) Furthermore, p62 (an autophagy receptor)-dependent autophagy can be used to remove excessively accumulated AGEs in order to lessen their cytotoxicity.(86,87)

According to earlier research, controlling autophagy may be a useful tactic to increase MSC survival and enhance the results of wound healing.(88) According to a prior study, palmitate causes intracellular ROS buildup, which promotes MSC apoptosis, while autophagy activation through the ROS – c-Jun N-terminal kinase (JNK) – p38 mitogen-activated protein kinases (MAPK) signalling pathway shields MSCs from apoptosis.(89) Overexpression of HIF-1α increases MSC survival in hypoxic environments by inhibiting phosphatidylinositol-3 kinase (PI3K)/AKT/mTOR signalling, which in turn triggers autophagy.(90) Furthermore, to shield adipose-derived stem cells (ADSC) from hyperglycemia-induced apoptosis, the serine/threonine kinase aurora kinase A targets Forkhead box class O 3a (FOXO3a) to trigger autophagy. According to related research, blocking microRNA (miR)-34a enhances the therapeutic use of MSCs to treat diabetic wounds by triggering autophagy mediated by the sirtuin-1/FOXO3a pathway.(91) Furthermore, injecting MSCs pretreated with autophagy inducers subcutaneously stimulates VEGF secretion by triggering MSC-specific paracrine signaling via the ERK1/2 pathway, which in turn improves wound healing.(92)

Autophagy has an anti-infection effect during the inflammatory phase and inhibits the inflammatory response, preventing tissue damage from excessive inflammation. Local hypoxia in the wound can trigger autophagy during the proliferative phase, which aids in cell survival by preventing oxidative stress and apoptosis. Vascular endothelial cells' autophagy stimulates wound angiogenesis, while keratinocytes' autophagy stimulates their migration, differentiation, and proliferation, all of which aid in the completion of wound re-epithelialization. Fibroblast autophagy influences the development of hypertrophic scars during the remodelling phase. Furthermore, MSC autophagy regulation may enhance its application to wound healing, and elevated autophagy levels may be linked to resistant diabetic wounds. Therefore, knowing how autophagy and skin wound healing are related, as well as investigating the molecular mechanism of autophagy control, may lead to the development of new therapeutic approaches for wound healing.(93)

Balancing Wound Healing and Scarless Skin Repair

The last stage of mammalian tissue regeneration is skin scarring. Millions of people suffer burns, trauma, or surgical skin ailments each year. Scar formation impacts both normal skin function and appearance, even though it is a typical byproduct of wound healing.(94) Rapid inflammatory responses can help the body heal wounds by preventing infection and other factors from causing harm, but they can also cause scarring.(95) Furthermore, during wound healing, overexpression of TGF-β1, α-SMA, and connective tissue growth factor (CTGF) can encourage fibroblast activation and excessive ECM accumulation, which ultimately results in cell fibrosis and the formation of scar tissue.(95) Scar tissue formation is a very complicated process that is influenced by a variety of elements, including the mechanical force of the wound site, the quantity and rate of cell reproduction, the expression level of cytokines, and the interaction between cells and other factors. Furthermore, the depth of the wound, that is, the distance from the outer layer to the interior, affects the creation of scars.

Scar tissue, which seems thicker and tougher than normal skin but is less functional than normal skin, arises when severely injured skin tissue is repaired. Regeneration is the best healing process for skin wounds. Because the fundamental component of a scar is an aberrant tissue that lacks the physiological function and normal skin tissue structure of normal skin and has lost its vitality.(96) Scar development is caused by cell fibrosis and the excessive deposition of disorganized ECM that is dominated by collagen.(97) Scar formation is not uncontrollable, despite the fact that a large scar or coating on the joint surface can cause significant injury. In the latter stages of pregnancy, the fetal skin begins to produce scars, suggesting that human skin may regenerate to some degree.(98)

Regeneration, as opposed to repair, is the optimal healing process for skin wounds. The process of blood clotting and healing right after an injury is referred to as skin damage repair. Collagen precipitation and local cell repositioning take place during this procedure to repair the injured tissue. However, rather than restoring the original tissue structure, scarring typically occurs.(99) Skin injury regeneration is the process by which tissue that has only been damaged by the superficial dermis or epidermis can regenerate and create some epidermal appendages in addition to the original structure. Without leaving scars, the new skin will be identical to the original. But if the damage is too severe, the skin will unavoidably develop a lot of fibrous tissues to cover it during the healing process, which will lead to scarring and the loss of the skin's integrity and beauty.(100)

Excessive and aberrant ECM deposition results in some degree of fibrosis in all scars.(101) Hypertrophic scars and keloids are the two forms of pathological scars. The scarring from keloids can spread past the wound's edge and infiltrate healthy tissue without regressing, and they are unpleasant and irritating.(102) Strong inflammatory responses and elevated pro-inflammatory factor levels in keloids cause chronic inflammation that pushes the keloid outside the wound's boundaries. Furthermore, myofibroblasts that can secrete alpha smooth muscle actin (α-SMA) survive, cell proliferation is fast, apoptosis is sluggish, and fibroblasts are very sensitive to transforming growth factor (TGF)-β1. Thus, under the influence of cytokines, fibroblasts and myofibroblasts generate a significant amount of collagen and other ECM components, hastening fibrosis and creating pathological scar tissue.(103) Although keloids do not spread to other tissues and organs and can be effectively treated to produce a curative effect, they are classified as benign tumors because of their persistent growth and invasion of surrounding tissues, which are characteristics of tumors, as well as their abnormal fibroblast activity and excessive collagen secretion, which are characteristics of tumors. (104) Numerous factors, including genetic, endocrine, stress, hyperactive inflammation, and immune state, have been linked to the formation of keloid, according to clinical investigations.(105)

The optimal treatment approach should hasten wound healing, reduce the formation of scars, be safe, dependable, sustainable, and economical, and provide individualized care. The development of 3D bioprinting technology has advanced extremely quickly since its inception. It can print biological materials with structure and qualities like the natural extracellular matrix, connect different elements (biocompatible materials, cells and factors, etc.) in the therapy of wound healing, and encourage wound healing toward regeneration.(106)

The Secretion Profile of MSCs and Potential Applications in Treating Human Diseases

According to recent research, the paracrine substances that MSCs release are primarily responsible for the therapeutic activity seen in these cells. Through paracrine signaling

pathways, these biomolecules also initiate antiapoptotic activities to stop the sick organ from further degenerating. The peripheral system of diseased organs has a greater paracrine gradient than normal physiological settings, which facilitates the migration of tissue-specific MSCs towards the site of infection or injury to promote recovery. To expedite the healing process, conditioned media made from mesenchymal stem cell cultures (MSC-CM) may help maintain the elevated paracrine factor gradient between the diseased organ and the stem cell niche. Based on the paracrine signalling mechanism, MSC-CM, also known as the secretome of MSCs, is a rich source of paracrine factors and is being thoroughly researched for a variety of regenerative therapies, including wound healing, bone regeneration, myocardial infarction, stroke, and hair growth.(107)

Some studies showed that stem cells do not work alone, supported by the improvement in organ function shown less than 72 hours after stem cell transplantation.(108) Nonetheless, the quick healing process might be explained by the paracrine substances' antiapoptotic actions.(109) Autocrine and paracrine signalling pathways work together to maintain homeostatic processes in the human body.(110) The paracrine factor gradient seen in the peripheral system will help stem cells migrate to specific tissues when an organ is injured.(111) The process of regeneration at the injured site is made possible by tissue-specific differentiation and engraftment, which come next. These chemicals reach the circulatory system, including the peripheral blood system, after being secreted by activated cells that live in their physiological niches.

The condition media thought to be a rich source of paracrine substances.(112) VEGF, stem cell factor (SCF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1, IGF-2, and stromal cell-derived factor (SDF)-1 are among the common paracrine factors produced during *in vitro* stem cell cultivation.(110) These elements have several physiological functions. Consequently, depending on the biological functions associated with the factors, delivering individual factors or a combination of factors to the site of an injured organ would increase metabolic activity, oxygen supply, and extracellular matrix remodelling, thereby preventing further depletion of the injured organ.(110)

The strict tissue structure surrounding the wounded location, where allografted cells either die or are absorbed by resident cells, typically limits the engraftment of MSCs. Much research has been done on how MSCs cause plerosis and immunoregulatory effects. In addition to improving the function of keratinocytes and endothelial cells in the skin incison, the supernatant of cultivated MSCs also drew macrophages into the wound-healing process.(113) There is a renewed interest in the elements and molecular basis of MSC secretion involved in the interaction between allogeneic cells and the tissue milieu because of the significant role that MSCs' paracrine function plays in tissue repair.

The paracrine effects of MSCs in the treatment of illnesses have been demonstrated by numerous investigations. In clinical translation, signaling molecules produced by MSCs can be separated and refined as cell-free products. In addition to their crucial function in regulating the intrinsic tissue repair process through the release of bioactive fractions, MSCs also express a diverse range of chemokines and receptors, which together create a subtle chemotactic network *in vivo* that can direct circulating cells to injury sites or mobilize immune cells in inflammatory tissues.(114) Crucially, the MSC-mediated tissue remodelling process is not due to a single effector but rather to the coordinated control of several variables to preserve homeostasis. Developing safer and more efficient treatment plans requires a better understanding of how MSCs secrete in both healthy and pathological settings.

The paracrine gradient may be raised by adding conditioned media made from MSC-CM to an injured organ, which would speed up the process of tissue regeneration. An alternative to the cell-based therapeutic strategy could be MSC-CM, which is based on the paracrine signaling principle. The current restrictions on using stem cells as a therapeutic source for tissue regeneration would be removed with the introduction of MSC-CM.(110) The use of MSC-CM to treat conditions like wound healing, skin rejuvenation, increased hair growth, myocardial infarction, myocardial injury, myocardial fibrosis, Friedreich's ataxia, tissue ischaemia, stroke, hindlimb ischaemia, bone marrow regeneration, distraction osteogenesis, and liver regeneration is becoming more and more supported by evidence.

Effective use of MSC-CM for a particular problem requires a thorough understanding of the paracrine processes that reverse the sick situation. Because the homing signals of a diseased organ may vary depending on the clinical circumstance, it is important to keep in mind that the proper dose of the condition media cocktail provided for various diseases may also vary.(115) Additionally, the condition media's distribution method must be tailored to the specific illness state.(116) The right stem cell source that secretes the paracrine factors needed for the particular diseases should be found in addition to the previously listed variables. When grown in *ex vivo* settings, it is also vital to take into account

how each individual's paracrine factor secretion is modulated in relation to the concentration of paracrine factors secreted to the media. Data gathered from *in vitro* research on optimal treatment dosages can be utilized to assess each person's unique reaction to the supplemented MSC-CM and will be a crucial prerequisite for developing a therapy method. A particular treatment's effectiveness can be raised by appropriately altering the paracrine factors' secretion levels. The dosage and frequency of condition media must be carefully determined because to the short half-lives (117) and the consumption of paracrine components within the body following administration (118).

Because of their paracrine functions, MSCs are potent bioactive agents that can be used to treat a wide range of illnesses, particularly resistant immunological disorders, tissue injury, and tissue degeneration. A deeper comprehension of MSCs' immunoregulatory and healing capacities should be possible through studies of the mediators they produce under different circumstances.(119)

Small Extracellular Vesicles: Discovery, Functions, Detection and Application

Development and the equilibrium of the adult body depend on intercellular communication. Recognized mediators of intercellular interaction include hormones, neurotransmitters, and cytokines and chemokines. (120) A recent way of cellular communication through extracellularly produced nanovesicles, or EVs, was discovered in reticulocyte cells in the early 1980s.(121) EVs were once thought to as cellular waste, but numerous research over the past ten years have indicated that they are crucial for intercellular communication and signalling results in the recipient cells.(122)

EV can be divided into three categories which is microvesicles, exosomes, and apoptotic bodies. Apoptotic bodies (50–5000 nm) are discharged from dying cells into the extracellular space, microvesicles (100 nm–1 μm) are created by direct outward pinching of the plasma membrane, and exosomes (30–150 nm) are of endosome origin.(123) Recently, exomere, a non-membranous vesicle smaller than 50 nm, were included in the EV category.(124) According to their biophysical and biochemical characteristics, EV subtypes are classified according to the density: high density (HD) and low density (LD); membrane tetraspanin enrichment: CD63, CD9, and CD81; or size: small EVs (sEVs) and large EVs (lEVs).(125)

EVs can be regard as cargo produced by cells and serve as delivery mechanisms for proteins, lipids, or nucleic acids. Despite being thought of merely a waste disposal mechanism at first, sEVs are currently being studied for a variety of biological and pathological purposes. Immunoregulation, central nervous system (CNS) development, nervous system physiology, development of the mammary glands, induction of immunosuppression during pregnancy, developmental signaling pathways, tissue regeneration, inflammation, angiogenesis, coagulation, apoptosis, stem cell differentiation, and ECM turnover are all significantly impacted by these EVs.(126)

EV characteristics including density, size, and surface composition are the basis for common isolation techniques that divide EVs from the remainder of the cellular compartment. To guarantee the maximum EV yield and purity, the International Society for Extracellular Vesicles advises combining various isolation techniques. For exosome separation, a number of recognized methods have been used, including size exclusion chromatography, density gradients, precipitation, filtration, and differential ultracentrifugation.(127)

The methods most frequently employed to describe the microstructure of MSC-EVs are scanning electron microscopy (SEM) and transmission electron microscopy (TEM), respectively. While TEM is more frequently employed to provide a 2D image of the EV with interior structural information, SEM scans the topography of the EV surface. The ability to integrate immunogold labeling with SEM and TEM-based imaging is a benefit.(128)

Long ago, it was discovered that EVs released by platelets and tumour cells, are present in bodily fluids and tissues with size 20–50 nm, acted as clotting suppressors in human.(129) Later, concurrent studies conducted in the 1980s by two different groups, showed that during reticulocyte maturation, the sheep reticulocyte's transferrin receptors are released in an active vesicular form measuring 50 nm. Experiments conducted in the 1980s demonstrated that these EVs were endosomal in origin, despite the earlier belief that they only came from the plasma membrane. It was also suggested that the exfoliated membrane vesicles with a physiological purpose be called sEVs. The genesis and active role of EVs were then supported by several tests. For example, it was first discovered in 1996 that B-lymphocytes secrete major histocompatibility complex (MHC) class II antigen-presenting EVs, which can cause T cells to respond in a way that is particular to the antigen. (130) The first reports of EVs acting as genetic material transporters between cells were published in 2007.(131)

A lipid bilayer membrane, primarily derived from the plasma membrane, encloses the luminal cargos, such as proteins, lipids, and genetic materials, found in a normal sEV.(132) Instead of displaying a random collection of molecules, its luminal cargos exhibit distinct subcellular components. The sEV content depends on several variables, such as the donor cell's type, physiological state, and pathological state, in addition to the luminal cargo's selective sorting.(133) Nonetheless, some databases that offer a summary of the sEV material have been created, such as Exocarta (*http://www.exocarta.org*) (134), Vesiclepedia (*http://microvesicles.org*) (135), and EVpedia (*http://evpedia.info*) (136). Since the sEV membrane topology is determined by the inner and outer domains of proteins as well as their orientation with respect to the sEV membrane, which influences the functional capability of sEVs, several proteins are extremely prevalent in all types of sEVs, including cytosolic proteins, fusion and transferring proteins, heat shock proteins (HSPs), tetraspanins, signal transduction proteins, the proteins involved in multivesicular bodies (MVB) formation, and cytoplasmic enzymes. On the other hand, certain proteins, such as immunoglobulins, cell surface peptidases, tumor antigens, antigen presentation proteins, cell-specific transmembrane proteins, and cell-specific signaling proteins, determine the origin and target of sEVs.

Serial invagination of endosomal compartments results in the formation of sEVs. First, clathrin- or caveolin-dependent or -independent processes are used to produce early endosomes, which obtain their membrane and content from the endocytic vesicles.(138) The early endosomes then incorporate several tiny intraluminal vesicles (ILVs) to develop into the late endosomes or MVBs. The specialized transmembrane proteins known as tetraspanins-enriched microdomains undergo rearrangement to generate the ILVs.(139)

Hematopoietic cells (like red blood cells, platelets, lymphocytes, dendritic cells, mast cells, and reticulocytes) and non-hematopoietic cells (like adipocytes, fibroblasts, neurons, intestinal epithelial cells, and tumor cells) are among the majority of cells from which sEVs are derived (140), and circulated through body fluids including blood, saliva, synovial fluid, urine, breast milk, cerebrospinal fluid, semen, amniotic fluid, and fluids from malignant ascites (141). Numerous studies have shown that sEVs can transport a variety of bioactive cargos from the origin cells to nearby or distant recipient cells, including DNA, lipids, nucleic acids like mRNA, lncRNA, and miRNA, and functional proteins like transcription factors, heat shock proteins, and surface receptors. Therefore, sEVs play essential roles in a variety of physiologic and pathological processes as a result of these widespread cell-to-cell contacts.(142)

Based on their function in intercellular signaling and communication, sEVs regulate immune and inflammatory responses, angiogenesis, tissue homeostasis, nerve regeneration, and osteocyte metabolism in addition to a variety of other biological processes like cell proliferation, migration, and apoptosis. The recent emphasis on their use in therapeutic, diagnostic, and therapeutic fields is based on their many activities as well as some special qualities, which appears to be highly promising given their special qualities. These applications are still in the research stage, nevertheless, and have not yet been used in clinical settings. (143) Reducing the release of sEVs by aberrant cells can be a therapeutic strategy in many disorders because of the role that sEVs play in cellular communications. Therefore, the treatment goals in such cases will be to either stop the creation and release of such sEVs or stop their absorption. (144)

SEVs have had a significant impact on many areas of biology since they are the body's natural storehouse and carrier of a wide variety of substances, such as proteins, lipids, nucleic acids, and chemicals. They can be used to treat a variety of illnesses when certain unique molecules or compounds are needed to restore the body's equilibrium. Because of their unique physicochemical properties, sEVs has potentials to be used as medication and vaccine delivery systems.(145) Since sEVs are endosomal-derived vesicles encased in lipidic membranes, they are readily absorbed by cells by fusing with their membrane.(146) sEV membranes are actually derived from cell membranes; they can readily reattach to it and transfer their contents into the cell. They make excellent candidates for delivery applications because of this fact. Promising information on all facets of sEVs as potential medications, targets, and biomarkers is provided by the quick conversion of fundamental research into preclinical models. Future therapeutic uses are probably coming, but we must proceed cautiously because there is still a lot to learn.(126)

MSC Extracellular Vesicles Application in Diabetic Wound Management

MSC treatments are one strategy to treat DFU.(147-153) Studies have demonstrated that the use of MSCs, both autologous and allogenous, improves the healing of chronic wounds. However, several studies have shown that when it

comes to treating chronic wounds, the conditioned media made from MSCs are just as effective and have a greater ability for regeneration than MSCs alone. Because of this, scientists are now more interested in the paracrine activity of MSC cells' regenerative potential than in their therapeutic potential.(154,155) Specifically, soluble factors, metabolites, and genetic elements are carried by EVs, which are tiny particles released by MSCs. Because of these cargoes' significant contributions to wound healing, EVs are a unique, adaptable, and powerful healing stimulator. (156,157)

Both *in vitro* and *in vivo* studies have assessed the effects of EVs derived from various MSC sources on wound healing. ADSC, umbilical cord-derived MSCs (158), bone marrow-derived MSCs, induced pluripotent stem cell-derived MSCs (iPSC-MSCs), Wharton's jelly-derived MSCs, placenta-derived MSCs, menstrual blood-derived MSCs, corneal MSCs, fetal dermal MSCs, urine-derived stem cells, gingival-derived MSCs, and UCB-derived MSCs are some of the sources of MSCs used to produce EVs.(159)

MSCs and EVs both could modulate all types of immune cells, such as neutrophils, T lymphocytes, B lymphocytes, and macrophages.(160) At the areas of injury, EVs typically have an anti-inflammatory impact. EVs could limit B cell maturation, suppress T cell proliferation, stimulate macrophages to adopt the anti-inflammatory M2 phenotype (161,162), and secretly activate T cells into T regulatory cells (163). Exosomes could lower the levels of inflammatory cytokines, specifically TNF-α, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), IL1-β, and MCP-1, through these processes. EVs can therefore aid in preventing subsequent tissue damage caused by an overabundance of cytokines. According to certain research, exosomes can increase the synthesis of IL-10 at inflammatory areas; IL-10 is a crucial anti-inflammatory cytokine that controls the creation of scars and the healing of wounds.(159)

It is known that the immunomodulation of exosomes is comparable to that of the MSCs that create them. Nonetheless, there are some distinctions between the ways that exosomes and MSCs affect target cells. Exosomes primarily influence immune cells based on their microRNA (miRNA) content, whereas MSCs influence immune cells by producing cytokines. Exosomes produced from MSCs might promote cutaneous wound healing and induce M2 polarization. The M2 macrophage phenotype decreased when exosomes were depleted, suggested that exosome-derived miR-223 controls macrophage polarization.(164)

By improving the biological characteristics of the primary cell types participating in the crucial stages of wound healing, MSC-derived EVs may hasten this process. Therefore, the impact of EVs on macrophage activation, angiogenesis, migration and proliferation of keratinocytes and dermal fibroblasts, and myofibroblasts' ability to control extracellular matrix turnover was assessed. Numerous investigations have demonstrated that EVs have the ability to introduce miRNAs into immune cells, altering the actions of immune cells.(165,166) By inhibiting the TLR4/NF-kB and STAT3/AKT pathways, EVs could deliver their miRNA-let7b to macrophages and induce macrophage polarization, thereby reducing the inflammatory response. (166) Other miRNAs that affect immunomodulation in EVs include miR-181, miR-145, miR-221, miR-145, miR-126, miR-233, miR-124-3p, and miR-135b-5p.

The findings demonstrated that EVs promoted angiogenesis even if they lacked anti-inflammatory qualities. The transfer of certain miRNAs from EVs to target cells is linked to the effects of EVs on angiogenesis.(167) mRNAs linked to the endothelial NOS signalling and PI3K/AKT signalling pathways may be transferred to endothelial cells via MVs derived from endothelial progenitor cells.(168) Several of the miRNAs, such as miRNA-125a, miRNA-31 (in EVs produced from ADSCs), miR-126, miR-130a (in $CD34^+$ stem cells), and miR-31, were discovered to be associated with angiogenesis.(169)

By encouraging resident cell proliferation and differentiation and fostering angiogenesis at injury sites, stem cell-derived EVs can have a direct impact on the proliferation phase of wound healing. The re-epithelialization of the wound was considerably improved by both umbilical cord-derived MSCs and EVs from both umbilical cord-derived MSCs. Additionally, they discovered that by lowering the amount of the pro-apoptotic protein Bax, these EVs prevented keratinocytes and fibroblasts from undergoing apoptosis.(170) When keratinocytes and dermal (myo)fibroblasts were exposed to EVs, their ability to proliferate and migrate was increased. When fibroblasts isolated from diabetic wounds were exposed to EVs derived from MSCs, their proliferation was markedly enhanced. Additionally, the migration of exosome-treated fibroblasts improved.(152) Furthermore, EVs enhanced MMP-13 expression during the fibroblast–myofibroblast transition and inhibited the elevation of gene expression for type I and III collagen, α-SMA, and MMP-2 and MMP-14. A wound healing skin organotypic model was used to validate the regenerative qualities of EVs, and after being exposed to EVs, the skin showed complete re-epithelialization. All

things considered, these findings suggest that EVs improve the biological characteristics of fibroblasts, endothelial cells, and keratinocytes, making them a dependable therapeutic agent for skin regeneration.(171)

According to reports, engineered sEVs have modulated the function of skin repair cells, such as immune cells, vascular endothelial cells, keratinocytes, fibroblasts, myofibroblasts, and skin appendage-associated cells involved in the inflammatory phase, proliferative phase, and remodeling phase, in the healing processes of diabetic wounds.(172) More significantly, sEVs are easily customizable to increase their targeting and therapeutic efficacy.(126,173)

The final phase of wound healing is matrix remodeling. Tissue remodeling and ECM production are related to this stage. Recent research has noted how EVs affect matrix remodeling. A study regarding the the wound treatmtn with EVs from ADSCs, suggested that this method might encourage the production of collagen and elastin, which would lessen the formation of scars.(174) Furthermore, by raising the ratio of collagen III to collagen I by up to 50% EVs could lessen excessive scarring. In a different study, it was found that EVs from UC-MSCs suppress myofibroblast accumulation, which reduces scar formation in mice treated with them.(175) miR-21, miR-23a, miR-125, and miR-145 in UC-derived EVs can inhibit myofibroblast development and over-aggregation. Additionally, by downregulating TGF-β1, EVs derived from epidermal stem cells can aid in wound healing (Figure 5).(176,177)

Some studies have employed hydrogel-formed EVs to improve the impact of exosomes in wound healing. EVs are transported via the hydrogel, which also aids in maintaining the EVs at the wound sites. This approach has been employed in certain instances to increase the effectiveness of EVs as a therapy for wound healing.(178-180) A study observed how EV-loaded chitosan hydrogel affected mouse models' ability to heal wounds. In contrast to employing sterile gauze, which only reduced wound size by 51.5%, the use of chitosan hydrogel and EV complexes facilitated an 83.6% reduction in wound size (with a high degree of re-epithelialization), which increased wound closure.(179) Animal models' skin sores were treated using EVs loaded in alginate hydrogel. Additionally, these compounds significantly improved collagen production, vascular development, and wound healing in the affected area.(180) Additionally, EVs infused in pluronic F127 hydrogel effectively facilitated full skin regeneration and the healing of chronic diabetic wounds. (178) In order to cure ulcers in diabetic rats, EVs made from human UC-derived MSCs was employed. These EVs were subsequently injected into pluronic F-127 hydrogel. In comparison to the usage of EVs alone, hydrogel, or a placebo, the scientists demonstrated that the complexes of EVs with hydrogel greatly accelerated the rate of wound closure. The administration of EV and hydrogel complexes improved

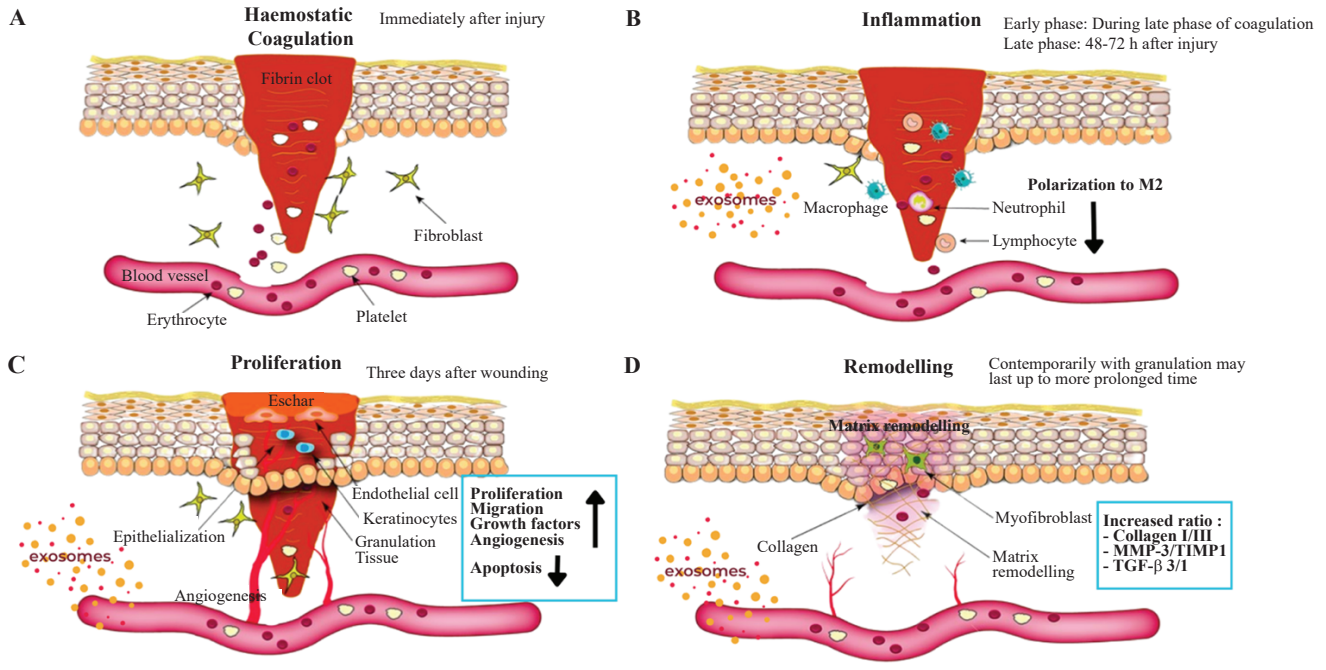

Figure 5. Bioeffects of stem cells derived exosomes on wound healing at different consditions. A: hemostasis; B inflammation; C: proliferation; D: remodelling stage.(177) (Adapted with permission from Frontiers).

granulation tissue regeneration and raised VEGF and TGF β-1 expression. In a different work, skin abnormalities in diabetic mice was treated by using gingival stem cell EVs to mix with chitosan/silk hydrogel.(181) By encouraging collagen remodeling and boosting angiogenesis, these therapies helped diabetic rats' skin wounds heal.(23,181) Table 1 summarizes some examples of MSC-derived EV application for wound healing based on various study.

EVs Controls Inflammation and Acelerate Cutaneous Wound Healing

Despite their therapeutic potential for wound healing, natural EVs' low concentration, short half-life, quick disintegration in the wound site, and inefficient payload restrict their potential for additional clinical uses. In order to get over the

Table 1. Application of EVs deriving from various stem cell source for the use of wound healing treatment.

Source of MSC-derived EV	Markers	Therapy Effects	References
Bone marrow-derived MSC	Akt, ERK, STAT3, HGF, IGF-1, NGF, SDF1	- Activation of Akt, ERK, and STAT3 signaling pathway - Increase the expressions of HGF, IGF-1, NGF, SDF1 - Enhancement of proliferation and migration of fibroblasts	(152)
Bone marrow-derived MSC	Wound closure rate	- Wound closure after 27 hours (examined by scratch assay)	(155)
Umbilical cord-derived MSC	miR-181c, TLR4, TNF-α, IL-1β	- Overexpression of miR-181c - Suppression TLR4 signaling - Decrease ecpressions of TNF-α and IL-1β - Reduction of burn-induced inflammation	(158)
Bone marrow-derived MSC	CD206, IL-10, TNF-α	- Increase of CD206 (M2 macrophare marker) - Higher expressions of IL-10 and TNF-α - Promotion of M2 polarization	(164)
Umbilical vein endothelial cell-derived MSC	Cell migration, tube formation, β-Catenin	- Promotion of endothelial cells migration - Improvement of tube-formation ability of endothelial cells - Activation of Wnt/β-catenin signaling - Promotion of angiogenesis	(167)
Human iPSC-MSC	Wound closure, scar width, collagen, CD31	- Wound closure after 16 days (examined macroscopically) - Reduction of scar widths - Increase of collagen maturity - Increase of CD31 (marker for newly formed vessels and mature - Promotion of angiogenesis	(170)
Bone marrow-derived MSC	Cell proliferation, cell migration, *COL1* , *COL3, α-SMA, MMP2, MMP14, MMP13*	- Enhancement of proliferation and migratory capacity of fibroblast - Inhibition of COL1, *COL3, α-SMA, MMP2, MMP14* gene expression - Increae MMP13 gene expression - Promotion of angiogenesis - Enhancement of skin regeneration	(171)
Adipose-derived MSC	N-cadherin, cyclin-1, PCNA, collagen I, collagen III, wound closure	- Increase of N-cadherin, cyclin-1, PCNA - Promotion of collagen expression - Wound closure after 21 days (examined macroscopically) - Improve cell proliferation and cell migration	(174)
Epidermal stem cell	TGF-β1, miR-16, let-7a, miR-425-5p, miR-142-3p	- Inhibition of TGF-β1 activity - Enrichment of miR-16, let-7a, miR-425-5p and miR-142-3p - Inhibition of myofibroblast differentiation	(176)
Umbilical cord-derived MSC	Wound closure rate, CD31, Ki67, VEGF, TGF-β1	- Acceleration of wound closure rate - Increase expressions of CD31 and Ki67 - Upregulation of VEGF expression and TGF-β1	(178)
Adipose-derived MSC	Wound closure rate, collagen fiber	- Wound closure after 14 days (examined macroscopically) - Improvement of collagen synthesis and vessel formation	(180)
Gingival-derived MSC	Wound closure rate, collagen fiber	- Wound closure after 14 days (examined macroscopically) - Deposition and remodeling of collagen - Promotion of angiogenesis	(181)

drawbacks of natural EVs, synthetic EVs have lately been investigated as novel EV-based nanotherapeutics. Several EV engineering techniques, including modifying the EV membrane, adding EV cargo, and combining EVs with other nanomaterials, have been used to treat wounds with encouraging results.(182)

Microvesicles and exosomes undergo distinct EV biogenesis processes. The endosomal system is the source of exosomes, which are produced when the plasma membrane fuses with MVBs that contain ILVs. On the other hand, the cell membrane budding outward is how microvesicles are secreted.(183) Despite coming from distinct parts of the cell, these two EVs share identical biogenesis processes. (184) The synthesis of exosomes mostly occurs in multiple stages. The inward budding of the plasma membrane results in the generation of early endosomes. They then develop into MVBs with ILVs after forming into late endosomes. MVB then takes two distinct routes. Fusion with lysosomes degrades MVB. However, following MVB's transport to the cell membrane and subsequent fusion with the membrane, exosomes are released.(184) In MVBs, exosomes can develop independently or in dependence on endosomal sorting complex required for transport (ESCRT). Following MVB production, exosomes are released from the cell when the secretory MVBs fuse with the plasma membrane.(185)

Several engineering approaches have been explored to enhance the use of EVs in clinical illness treatment.(186) The two primary categories of EV engineering strategies are direct approaches (EV modification) and indirect ways (cell modification). A common technique for modifying EVs for therapeutic purposes is genetic engineering (Figure 6A). When mRNA is injected into a cell, it can be enclosed in EVs. These EVs can then merge with a specific target cell, causing the production of transgenic proteins.(187,188) Another strategy is via the modification of EV membrane proteins and the loading of particular cargos into EVs, gene transfection can create tailored EVs (Figure 6B). For optimal EVs, endogenous biogenesis boosts nucleic acid expression by transferring exogenous nucleic acids (such as DNA, mRNA, siRNA, and miRNA) to parental cells.(188) Furthermore, proteins that express on the EV membranes can be altered with targeting and homing properties by applying transgenic expression.(189) Even if it is possible and simple to create tailored EVs using this technology, the low loading efficiency and specificity still prevent better clinical medicine application. Therefore, future studies should look more closely at improving transfection efficiency and specificity.

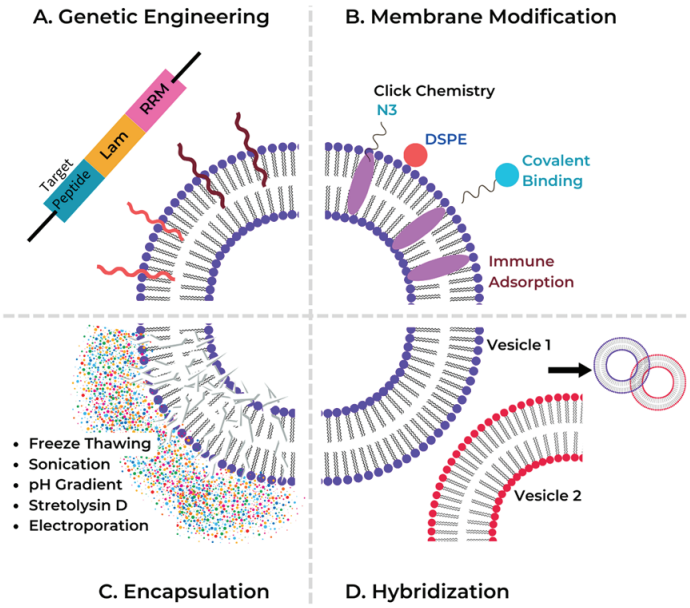

Figure 6. Generic engineering strategies for EVs divided into genetic engineering (A), membrane modification (B), encapsulation (C), and hybridization (D). Lam: Laminin; RRM: RNA-recognition motif; DSPE: Distearoyl phosphoethanolamine; N3: Azide. (Created using Canva).

The endowment of EV membrane alterations can be covalent or non-covalent, with reduced clearance and desired capabilities. Click Chemistry (Figure 6B), which makes use of the copper-catalyzed azide-alkyne cyclo-addition reaction, is the most often used covalent technique. The chemicals are directly covalently bonded to the EV surface using this method, which creates chemical bonds quickly. (190) EVs are coupled to neuropilin-1-targeted peptide and c (RGDyK) peptide to treat gliomas and ischemic brain, respectively.(191,192) One popular technique for altering EV surfaces with polyethylene glycol is PEGylation, which can increase the EVs' circulation half-life.(193)

It is expected that engineered EVs would overcome these problems and serve as a tool for focused therapies. By blocking the action of miR-21 in leukemia cells, for instance, alteration of the AS1411 aptamer can enhance the leukemia cell targeting effect of EVs, resulting in evident cell death. (194) Biological, chemical, and physical engineering are the three groups into which EV engineering techniques can be divided.(195)

Drug loading or encapsulating into EVs (or any other lipid vesicles) can be delivered by passive or active loading. Passive loading was conducted by incubating EV with drug solution, then the drug will diffuse into EVs. While active loading includes freeze thawing, sonification, pH gradient, electroporation, or treatment with streptolisyn D

(Figure 6C). EVs can also be combined with another EVs or synthetic nanoparticles, known as hybridization (Figure 6D). (196)

According to recent research, native EVs are employed as therapeutic agents to support tissue repair by releasing a variety of trophic and growth factors, including as VEGF, epidermal growth factor (EGF), TNF-α, IL-1β, and TGF-β, to support all physiological wound healing processes. Moreover, the content and surface of EVs can be created, altered, and manipulated to increase their specificity, safety, and effectiveness for wound healing. The two categories of EV engineering methodologies are indirect EV modification and parent cell modification. It is still necessary to investigate the therapeutic potential of existing EVs and modified EVs for wound healing on a broad clinical scale using creative methods.(197)

To change EVs and create particles with specialized therapeutic capabilities and higher concentrations, the idea of bioengineering technology was introduced. One intriguing method to concentrate dosage, provide the intended therapeutic efficacy, and sustain a prolonged releasing effect may be to load MSC-EVs with biomaterials. However, the potential of MSC-EVs modified by bioengineering is still unknown, despite the fact that the positive effect of MSC-EVs in wound healing is generally acknowledged.(198)

Future Direction and Challenges

MSC-Evs have shown great potentials as future therapy for chronic wound including DFUs. Currently, the lack of standard methods for EVs isolation, characterization, and protocol for massive production poses a significant challenge to their clinical application.(199) Due to their temperature-sensitive, MSC-EVs stability become another challenge in bench to bedside application. Developing a stable hydrogel patch for MSC-EVs will hold a promising utility of DFU treatments.(200)

Conclusion

DFU are caused by a variety of pathogenic mechanisms, managing them calls for an interdisciplinary and multimodal strategy that should involve prevention, addressing the several mechanisms that lead to their creation, and promoting wound healing. The DFU microenvironment is quite complex due to its etiology and is typified by chronic infection, hyperglycemia, ischemia, hypoxia, and hyperinflammation. MSC-EVs can be a safe, effective and ethical therapy for DFU by increasing M2 macrophages polarization, improving the proliferation, reducing scar, and improving angiogenesis.

Authors Contribution

AM drafted the original manuscript, critically revised the manuscript manuscript, and designed the figures. NMD edited and revised the manuscript. AW proposed and concepted the manuscript topic, and gave critical suggestions to the final draft. All authors have agreed with the final revisions of the manuscript.

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