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Nitric oxide-based treatments improve wound healing associated with diabetes mellitus

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Abstract

Non-healing wounds are long-term complications of diabetes mellitus (DM) that increase mortality risk and amputation-related disability and decrease the quality of life. Nitric oxide (NO·)-based treatments (i.e., use of both systemic and topical NO· donors, NO· precursors, and NO· inducers) have received more attention as complementary approaches in treatments of DM wounds. Here, we aimed to highlight the potential benefits of NO·-based treatments on DM wounds through a literature review of experimental and clinical evidence. Various topical NO·-based treatments have been used. In rodents, topical NO·-based therapy facilitates wound healing, manifested as an increased healing rate and a decreased half-closure time. The wound healing effect of NO·-based treatments is attributed to increasing local blood flow, angiogenesis induction, collagen synthesis and deposition, re-epithelization, anti-inflammatory and anti-oxidative properties, and potent broad-spectrum antibacterial effects. The existing literature lacks human clinical evidence on the safety and efficacy of NO·-based treatments for DM wounds. Translating experimental favors of NO·-based treatments of DM wounds into human clinical practice needs conducting clinical trials with well-predefined effect sizes, i.e., wound reduction area, rate of wound healing, and hospital length of stay.

Key Words: angiogenesis; diabetes mellitus; diabetic foot ulcer; inflammation; *L*-arginine; nitric oxide; nitrite; non-healing wounds; re-epithelization; wound healing

Introduction

Diabetes mellitus (DM), a leading cause of death and disability worldwide, affected 10.5% (536.6 million) of the adult population (20–79 years) in 2021, projected to reach 12.2% (783.2 million) in 2045.[1] DM wounds, especially diabetic foot ulcers (DFUs), are life-threatening complications with a prevalence of 4–10% and an annual population-based incidence rate of 1–4.1%.[2] The lifetime risk of developing DFU in DM patients is estimated as high as 25%.[2] Around 60–80% of these wounds are capable of healing, 10–15% may remain active, and 5–24% lead to amputation of the limb after the first evaluation within 6–18 months.[3,4] 40–70% of all non-traumatic amputations of the lower limbs occur in patients with DM.[4] DFU precedes ~85% of all amputations and 20% of hospital admissions amongst patients with DM.[4,5] DFU is mainly caused by lack of foot sensation and high plantar pressure secondary to peripheral neuropathy, ischemia secondary to peripheral artery disease (PAD), and impaired wound healing in patients with DM.[6] The updated guidelines on the prevention of DFU considered loss of protective sensation and PAD as the most potent predictors of DM wounds.[7]

Comprehensive management of DM wounds necessitates a multifaceted approach, encompassing meticulous wound care, optimized glycemic control, pressure offloading, rigorous infection control, debridement of devitalized tissue, and techniques promoting wound closure. Various complementary therapeutic strategies (e.g., growth factors, synthetic drugs, stem cells, and natural products) targeting critical molecules involved in the healing process have been developed to manage DM wounds.[8] Nitric oxide (NO·, a multifunctional gasotransmitter) is a critical component of a normal wound healing process.[9] Research evidence supports NO·-based therapeutics for DM-wound healing.[10-15]

Here, we focus on the potential benefits of NO·-based treatments on DM wound healing through a literature review of current evidence. Two primary databases, including PubMed and Scopus, were searched for published papers using the search strategy incorporated search terms for core concepts, including “nitric oxide” and “wound healing” alongside terms specific to diabetes (“diabetes mellitus,” “diabetic foot ulcer,” and “diabetic wound”) and mechanisms relevant to wound healing (“angiogenesis,” “inflammation,” and “re-epithelialization”). Additionally, terms like “*L*-arginine” and “nitrite” were included to capture studies investigating the NO· synthesis pathway, its precursors, and metabolites. The search strings were designed using Boolean operators (AND,

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OR, NOT) to refine the results and ensure they aligned with the specific focus of the manuscript. Following the outlined search strategy, studies were selected based on the following criteria to ensure their relevance to the review aim. For inclusion, experimental studies had to investigate the effects of NO· or NO·-releasing agents on wound healing, wound closure, and re-epithelialization in DM wound models (*in vivo*). Clinical studies were included if they assessed the efficacy and safety of NO·-based therapies for promoting wound healing in patients with DM (type 1 or type 2). The primary outcomes of interest were objective measures of wound healing progress (closure rate and closure time) and relevant biological markers (angiogenesis, re-epithelization, inflammation, and infection). As a narrative review, a pre-defined timeframe was not considered when searching databases. However, studies published within the last years were prioritized to capture recent advancements in the field.

Definition of Wound

Traditionally, wounds were defined as a break in tissue continuity caused by external violence (wound) and a lesion with inflammation, a gradual occurrence, and/or a chronic nature caused by an internal factor (ulcer).[16] Regardless of origin or internal/external cause, most skin lesions that impair the structural and functional integrity of the skin in the affected site are now called wounds and are primarily classified into acute and chronic.[16] Acute wounds heal quickly[17] in 5–10 days[18] or within 30 days from injury,[18,19] whereas chronic wounds are not repaired after 12 weeks of initial insult.[17,19]

Considering the diverse etiology/pathophysiology, morbidity and mortality, and required therapeutic approaches, reinstating the traditional nomenclature for different skin lesions seems to be essential.[16] The most typical wounds can be classified as DM wounds, including DFUs, surgical wounds, venous ulcers, and pressure ulcers.[20]

Etiology and Classifications of Diabetic Wounds

DM wounds are complex lesions caused by multiple factors.[21,22] Both internal (e.g., neuropathy, ischemia, prior deformities, and edema) and external (e.g., mechanical, thermal, and chemical) factors may be associated with the onset of DM wounds.[21,23]

As indicated in **Figure 1**, chronic hyperglycemia in DM culminates in the development of wounds through its effects on the peripheral nervous system, vasculature, immune system, and normal healing process. The interplay between hyperglycemia-induced neuropathy, angiopathy, and immunopathy increases sensitivity to the external forces, enhances the onset of lesions, and hinders the normal healing process, altogether leading to developing non-healing DM wounds, including DFUs.[23] Neuropathy, encompassing sensory, motor, and autonomic dysfunction causes loss of protective sensation, bone deformity and increased plantar pressures, callus formation, and dry and fissured skin (a favorable environment for fungal infections) rendering the foot highly vulnerable to ulceration, where even minor trauma can trigger a cascade of events leading to DFUs.[24] Furthermore, angiopathy manifests as a spectrum of vascular complications, including both microangiopathy and macroangiopathy, leading to a state of reduced oxygen (O_2) and nutrient supply known as ischemia. In addition, microvascular complications associated with infections can cause edema.[23] Hyperglycemia-induced immunopathy, which makes the environments prone to infections, also contributes to developing DM wounds.[23]

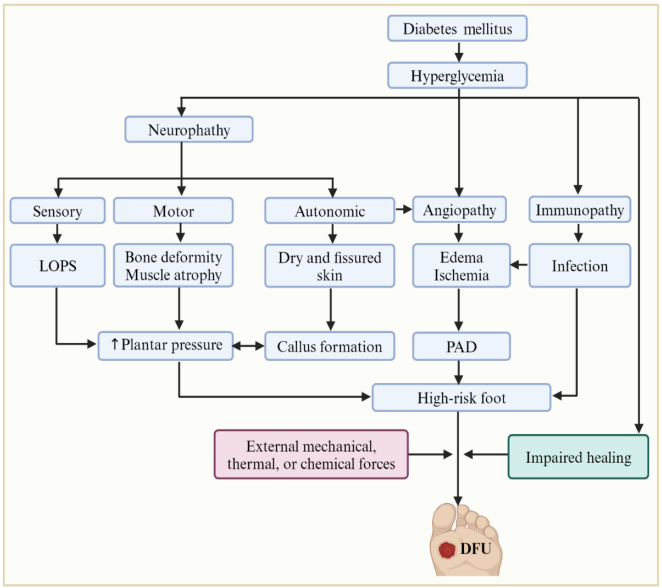

Figure 1 | Pathophysiology of diabetic foot ulcer (DFU). Hyperglycemia, a hallmark of diabetes mellitus (DM), initiates a cascade of pathologic conditions, including neuropathy, angiopathy, and immunopathy, leading to DFU. The hyperglycemia-induced neuropathy encompasses sensory, motor, and autonomic dysfunction. Sensory neuropathy results in loss of protective sensation (LOPS), leading to unnoticed injuries that can progress. Motor dysfunction can contribute to bone deformities and increased plantar pressures, promoting callus formation. Autonomic neuropathy manifests as dry and fissured skin, creating a favorable environment for fungal infections, further increasing vulnerability to ulceration. The hyperglycemia-induced angiopathy manifests as microvascular and macrovascular complications. Microvascular dysfunction disrupts blood flow, leading to ischemia and compromised oxygen and nutrient delivery necessary for healing. Additionally, microvascular complications associated with infections can cause edema. Macrovascular complications, such as peripheral artery disease (PAD), further exacerbate ischemia. On the other hand, hyperglycemia may impair the immune system, potentially increasing susceptibility to infections that further complicate wound healing. Consequently, the interplay between hyperglycemia-induced neuropathy, angiopathy, and immunopathy increases sensitivity to external forces, enhances the onset of lesions, and hinders the normal healing process, leading to the development of non-healing DM wounds, including DFUs. Created with BioRender.com.

The etiology, complexity, and severity of DM wounds exhibit significant heterogeneity. This variation encompasses the extent and depth of tissue destruction, the specific anatomical areas affected, and the presence of co-morbidities such as ischemia, infection, edema, and neuropathy.[25,26] DFUs, the most prevalent form of DM wounds, can be classified based on the underlying pathophysiology into three main categories, i.e., neuropathic, ischemic, and neuroischemic.[27,28] Neuropathic ulcers develop in patients with peripheral

neuropathy, whereas ischemic ulcers are associated with PAD in the absence of neuropathy; neuroischemic ulcers represent a combined etiology occurring in patients with both peripheral neuropathy and PAD.[26-28] Neuropathy can further contribute to developing various lesions beyond ulcers, including arterial, venous, or mixed ulcers.[27] This highlights the complex interplay between neuropathy and vascular insufficiency in the pathogenesis of DFUs. While established classifications for DFUs typically focus on underlying pathologies like neuropathy and ischemia, external forces, e.g., accidents, surgical procedures, burns, radiation therapy, thermal injuries, and mechanical trauma, can develop another class of wounds, i.e., "traumatic DM wounds."[23,26] Notably, trauma, as the primary cause of DM wounds,[23,29] can occur in the context of any DFU classification; so, considering "traumatic DM wounds" alongside traditional classifications for a more comprehensive understanding of DFU etiology seems to be crucial.[23] The primary external causes of "traumatic DM wounds" are likely to be puncture wounds, ill-fitting footwear, and self-care practices.[23]

Traumatic DM wounds exhibit distinct characteristics and prognoses compared to other classifications.[23,28] An ischemic DFU displays features of PAD, i.e., an ankle-brachial index (ABI) < 0.9.[28] Ischemic DM wounds present with a pale, yellow, and cool appearance, often accompanied by weak or absent pulses, whereas neuropathic DM wounds are preceded by callus formations and contain fibrotic and hyperkeratotic tissues.[23,28] Plantar callus, tinea pedis, onychomycosis, and foot deformity are prevalent in neuropathic and neuroischemic DM wounds.[28] Arterial DM wounds manifest with intense pain, a punched-out appearance, a shiny surface, reduced hair growth, pallor upon leg elevation, weak or absent pulses, and delayed capillary refill.[23] The average healing time of different types of DM wounds, including neuropathic, neuroischemic, and ischemic ulcers, is reported to be 70, 113, and 233 days, respectively.[28] Although traumatic DM wounds often exhibit a poorer prognosis, studies suggest potentially faster healing for those with a normal ABI ≥ 0.8 and in the absence of co-morbidities such as neuropathy, PAD, or infection.[26] Several established classification systems have been developed and validated, i.e., Meggitt-Wagner, University of Texas, IDSA (i.e., Infectious Disease Society of America), SINBAD (i.e., acronym for site, ischemia, neuropathy, bacterial infection, area, and depth), WIfI (i.e., acronym for wound, ischemia and foot infection), and PEDIS (i.e., acronym for perfusion, extent, depth, ischemia, sensation).[27,30,31] The PEDIS system, developed by the IWGDF group, is a research-based classification system categorizing DFUs based on the five key independent factors.[32]

Wound Healing in Normal Condition and Diabetes Mellitus

Wound healing is one of the most complex processes in the human body, involving the spatial and temporal synchronization of various cell types with distinct roles in the four phases.[33-35] The normal wound healing process involves the hemostasis phase (i.e., vasoconstriction, formation of a platelet plug, coagulation, and reinforcement of the platelet plug), the inflammation phase (i.e., immune cell infiltration, cytokine secretion), the proliferation phase [i.e., extracellular matrix (ECM) generation, angiogenesis, and epithelialization], and the remodeling phase (i.e., collagen crosslinking and reorganization).[34]

Wound formation triggers an immediate response to stop bleeding, known as hemostasis. Damaged arteries constrict rapidly, within minutes, to restrict blood flow and achieve initial hemostasis.[17] However, this decrease in blood flow can lead to tissue hypoxia and acidosis within a few minutes,[17] triggering the production of vasodilators like NO· and adenosine.[18] Simultaneously, clot formation occurs at the injury site as a primary mechanism to prevent further bleeding.[17,19] Trapped platelets within the clot are activated, resulting in the degranulation of α-granules and dense granules and releasing cytokines and growth factors, including platelet-derived growth factor, transforming growth factor-beta (TGF-β), epidermal growth factor, and insulin-like growth factors.[17,18]

The inflammatory phase of wound healing (days 1–5 after wounding) is a critical defense mechanism, preventing infection and initiating tissue repair.[17,18] Neutrophils infiltrate the wound by chemotaxis immediately upon wounding and migrate in sustained levels for the first 48 hours, with a peak at 24 hours; neutrophils release reactive oxygen species (ROS) to destroy bacteria and dead host tissue.[17] Monocytes are recruited within 48–96 hours post-injury and transform into tissue-activated macrophages at the wound site.[36] Macrophages, peaking at 48–72 hours, regulate inflammatory responses, stimulate new blood vessel formation, and promote granulation tissue (GT) growth.[15,16] The pro-inflammatory M1-like macrophages, induced by necrotic cells and/or infection, produce pro-inflammatory cytokines, proteases, and ROS to support host defense.[37] Finally, lymphocytes arrive later and contribute to the ECM and collagen remodeling, which are essential for successful wound healing. A prolonged inflammatory phase, however, can hinder the healing process.[17,18] Later, at 72–120 hours post-injury, lymphocytes initiate wound repair by generating components of the ECM and promoting collagen remodeling, a vital process for restoring tissue integrity.[17]

The proliferation phase (also called the growth phase) includes angiogenesis, granulation, collagen deposition, re-epithelialization, and wound retraction.[17] Following the inflammatory phase, wound healing progresses with angiogenesis, i.e., involved endothelial cell proliferation, migration, and branching, ultimately forming new blood vessels.[34] Alongside endothelial cell proliferation, pericytes within the basal lamina become activated, providing scaffolding and structural support for the newly formed blood vessels.[38] Additionally, circulating progenitor cells from the bone marrow are also recruited to contribute to new blood vessel formation during wound healing.[39] Dominated by activated fibroblasts, GT plays a vital role in wound healing. Fibroblasts provide structural support and contribute to wound contraction by synthesizing new ECM. Additionally, GT serves as a temporary platform for other essential components, including newly formed blood vessels,

inflammatory cells, and further ECM deposition.34 Ultimately, during wound remodeling, normal connective tissue gradually replaces this specialized tissue.[33,34]

Building upon the formation of GT, wound contraction is another crucial aspect of the proliferation phase. This process minimizes the surface area requiring re-epithelialization; collagen fibers realign perpendicularly to the wound edges for increased strength.[34] This change in stiffness triggers the transformation of specific fibroblast subpopulations into contractile myofibroblasts, a transient cell type that synthesizes collagen types I and III and exhibits characteristics of contractile smooth muscle.[40,41] M2-like macrophages, exhibiting pro-fibrotic capacities, are key players that contribute to both GT formation and wound contraction; they actively produce growth factors, specifically TGF-β1 and platelet-derived growth factor, and influence the persistence of ECM components within the wound environment, lasting up to 10 days after the initial injury.[37]

Following successful re-epithelialization, the wound proceeds into the remodeling stage with the emergence of a new fibrinolytic profile of resident macrophages within the wound.[35] These reprogrammed macrophages, called M2c or M_{reg}-like macrophages, release proteases and phagocytize unnecessary cells and excess ECM no longer needed for wound closure.[37] This activity ensures proper remodeling and prevents the accumulation of ECM and cells, which can lead to scar formation if dysregulated.[37]

DM wounds exhibit a dysregulated healing cascade due to a confluence of pathological factors. Restricted microvascular perfusion limits O_2 and nutrient delivery to the wound bed, compromising tissue regeneration.[42] Additionally, deficiencies in key growth factors like insulin-like growth factor-1 and TGF-β impair cellular proliferation and differentiation, essential for timely vasculoneogenesis and wound closure.[43] Furthermore, matrix metalloproteinase dysregulation can occur, potentially driven by oxidative stress and advanced glycation end products, leading to excessive degradation of the ECM, which provides structural support and facilitates cell migration during wound healing.[44-46] Finally, delayed recruitment of inflammatory cells and increased pro- to anti-inflammatory cytokine ratio,[47] and excessive ROS production,[48-50] results in further inhibition of cell proliferation, vasculoneogenesis, M1-to-M2 macrophage polarization, and inflammation-to-proliferation transition, i.e., a critical step during wound healing.[51]

Nitric Oxide and Wound Healing in Normal State and Diabetes Mellitus

Historically, the involvement of NO· in normal wound healing was first documented indirectly using its precursor *L*-arginine (*L*-Arg) in 1978,[52] and then directly in 1996–2000 through a series of investigations, including time-course assessment of NO· synthase (NOS) expression and synthesis of NO· metabolites [i.e., nitrate (NO_3), nitrite (NO_2)] and citrulline during wound healing,[53] and pharmacologic-[54] and genetic-manipulations[55] of NOS enzymes. As reviewed elsewhere,[13,19,56] normal wound healing requires NO· (1) in the inflammation phase (for cytokine modulation), (2) in the proliferative phase (for re-epithelialization, angiogenesis, and neo-vascularization), and (3) in the remodeling phase (for collagen deposition).

Building on the established role of NO in wound healing, research conducted in 1997 provided the first evidence linking impaired NO synthesis to the pathophysiology of DM wounds.[57] This impairment was in line with lower concentrations (~20–68%) of NO metabolites observed in DM wounds compared to non-DM ones.[56] Several contributing factors may explain this deficiency, including downregulated NOS enzymes, including endothelial NOS and inducible NOS (iNOS),[58-60] defective migration of NO·-producing cells (e.g., macrophages, keratinocytes, and fibroblasts) into the wound, and diminished capacity of these cells to produce NO·.[57] Insufficient NO· in DM wounds disrupts natural healing, leading to an impaired inflammatory response,[59,61] decreased collagen synthesis,[57,61-63] inadequate re-epithelization and angiogenesis,[60,63] and diminished wound breaking strength (i.e., a good index of functional recovery of healing wounds, measured as the minimum force required to break a wound).[60,64] An impaired inflammatory response refers to either a deficient initial inflammatory response (manifests as insufficient initial cytokine production and recruitment of immune cells, leading to delayed debris clearance and impaired wound healing) or excessive/prolonged inflammation (i.e., uncontrolled production of pro-inflammatory cytokines and infiltration of immune cells, preventing the deposition of matrix components, remodeling, and wound closure).[65,66]

Considering the essential roles of NO· in wound healing, NO·-based treatments have emerged as new approaches for wound healing.[10-13] Several NO·-based therapeutic strategies have been proposed, ranging from systemic administration of NO· donors [e.g., *L*-Arg, molsidomine (SIN-10), dinitrosyl iron complexes (DNIC)] to topical applications of engineered NO·-based biomaterials.[13] Topical NO·-based therapies broadly include gaseous NO·, acidified NO_2 creams, NO·-probiotic patches, nanoparticle platforms, and NO·-releasing hydrogels.[13] Commonly used NO· donors/NO· releasing substances for topical treatments of DM wounds are inorganic NO_2, *L*-Arg, nitroglycerine (also known as trinitroglycerin, TNG), metal-NO· complexes, *N*-diazeniumdiolates, and *S*-nitrosothiols (e.g., *S*-nitrosoglutathione and *S*-nitroso-*N*-acetyl-*DL*-penicillamine).[67-70] TNG, a common clinically used NO·-releasing drug for management of hypertension and angina pain, produces NO· upon activation by mitochondrial aldehyde dehydrogenase, cytochrome p450 enzymes, and xanthine oxidoreductase.[71,72] *N*-diazeniumdiolates are the most widely studied NO· donors hydrolyzed under physiologic conditions or upon thermal, photochemical, or enzymatic stimuli and release 2-mole equivalents of NO·.[73] *S*-nitrosothiols are naturally occurring NO·-releasing substances (e.g., *S*-nitrosohemoglobin, *S*-nitrosoglutathione, *S*-nitrosocysteine, and *S*-nitrosoalbumin) biologically produced through thiol nitrosation reaction.[74]

Gaseous NO· (200–500 ppm) is a straightforward way of NO· delivery to wounds; however, its application has some

limitations (e.g., requiring industrial NO· gas cylinders and hospital settings) and concerns (e.g., NO·'s high-reactivity, especially with O_2 and producing harmful byproducts like nitrogen dioxide).[19,75] NO·-generating acidified-NO_2 cream is another simple mode of NO· delivery to the wound tissue. Hydrogels, i.e., highly-hydrated crosslinked polymers (made up of collagen, alginate, hyaluronic acid, gelatin, cellulose, and chitosan), are popular materials for wound dressings because of their flexibility, adhesion, stability, and mimicking native ECM,[76] and a platform for NO· storage and delivery that provide a controlled NO· release for sustained exposure to wounds.[12]

Effects of Nitric Oxide-Based Treatments in Animal Models of Diabetic Wound

An overview of animal models of diabetic wound

Ethical and practical concerns limit the direct investigation of therapeutic interventions, like NO·-based treatments, for DM wounds in humans. Thus, various models in different animal species (e.g., mouse, rat, rabbit, dog, guinea pig, pig, and zebrafish) have been developed to mimic the healing process of DM wounds.[77,78] However, these models often only capture a single aspect of the multifaceted nature of human DM wounds. The ongoing challenge is developing a model that resembles the human DM wound environment with acceptable reproducibility, quantifiable interpretation, therapeutic significance, and effective translation into clinical applications.[77,78]

Rodents, particularly mice and rats, are commonly used animals for studying DM wounds because of their genetic and biological similarities to humans, cost-effectiveness, and ease of handling.[77-79] Mice have been preferred over rats because of the much larger genetic toolbox available for mice.[80] However, having higher body weight, demonstrating lower stress response to human interaction, and increasing availability of genetic tools in rats paved the way for the rising use of rats in wound research.[80] The induction of DM wounds in animals includes a two-stage process: (1) Induction of DM, including type 1 (T1DM), i.e., spontaneously developed autoimmune models or chemically-disrupted pancreatic β-cells models [using streptozotocin (STZ), or alloxan[81]] and type 2 (T2DM), i.e., genetically-manipulated models (e.g., *db*/*db* mice, KK-Ay mice, *ob*/*ob* mice, Goto-Kakizaki rats) or dietary models (e.g., monosodium glutamate, high-fat diet[82,83]); (2) Induction of wound.[77,78] A network meta-analysis of 267 studies indicates that among all models, only *db*/*db*, *ob*/*ob*, STZ, and STZ + HFT models display significantly delayed wound healing.[84] Upon establishing DM, the wound will be created by cutting, radiation, burning, or other methods, resulting in heterogenic lesions in size and depth. The commonly used wound models are[77,85]: (1) excision; (2) incision; (3) burn; (4) ischemic; (5) dead space; (6) tape stripping; (7) pressure ulcer; (8) para-biosis (by the surgical joining of two animals at their flank skin, used for study of circulating factors that play a vital role in a different phase of tissue repair and regeneration); (9) denervated (i.e., performed by hemisection of the spinal cord, followed by a 15 mm diameter skin wound, resembling neuropathic DM wound); (10) skinfold chamber (performed using two complementary plates sandwiching a laterally positioned fold of dorsal skin of animal, i.e., a good model for microvascular research); (11) xenograft (performed using putting a xenograft from human skin on the full-thickness wound created earlier in animal, i.e., used for resembles wound healing by re-epithelization); (12) infected wound model.

In experimental studies, the excision wound model is the most commonly used and clinically relevant model for DM ulcers.[77,78,85] The model involves a surgical excision (a circular full-thickness wound ~2 cm²) on the dorsal thoracic region of rodents, which is appropriate for the pharmacological evaluation of new entities and formulations.[77,78] The second most common model is the incision wound model (controlled cut), i.e., induced using two para-vertebral long cuts ~4-6 cm length made through the skin and cutaneous muscles that are separated ~1.5 cm from the midline on each side of the depilated back of the rodents. This model is appropriate for investigating the quality of healed tissue and scar formation.[77,78] The pressure wound models, which are more superficial, resulting in partial thickness loss of the dermis, are less likely appropriate as a DM wound model.[84]

Systemic nitric oxide-based treatments

Table 1 summarizes the effects of systemic NO·-based treatments, including *L*-Arg and NO· donors, i.e., SIN-10, DNICs on DM wounds in animal studies.[62,86-89]

L-Arg supplementation restores the impaired NO· synthesis in the DM wound environment and promotes healing factors.[62,86,88] The healing effect of systemic supplying *L*-Arg was associated with increased collagen synthesis and deposition, induction of vasculoneogenesis, and decreased inflammation.[62,87] *L*-Arg supplementation restored NO· metabolites but not ornithine concentration in wound fluid toward normal, an observation indicates that systemic supplying *L*-Arg is preferentially utilized by NOS enzyme(s), not arginase[86]; this speculation was supported by evidence obtained in iNOS-knockout mice without DM that failed to improve wound healing in response to *L*-Arg supplementation.[55] *L*-Arg is catalyzed by M1 macrophages-iNOS to produce NO· at the early phase of wound healing (which promotes vascular repair), and then by M2 macrophages-arginase to produce ornithine at the late phase of wound healing (which promotes tissue repair).[68]

Supplementation with other NO· donors, e.g., SIN-10 and DNIC-1, also promotes wound healing in animal DM models.[61,88,89] Administration of SIN-10 (1 mg/kg twice daily) in STZ-induced DM male rats, compared to saline-treated ones, increased wound-breaking strength, hydroxyproline concentration, matrix metalloproteinase-2 and arginase activity in DM wounds.[86] Systemic supplementation with SIN-10 (4 mg/kg/d) improved wound repair in rats with T1DM. However, the healing rate was better in the normoglycemic (insulin-treated) than in hyperglycemic rats. This finding highlights the importance of concurrent glycemic control and systemic NO· boosting to facilitate wound healing among patients with DM.[88]

Table 1 | Effects of systemic NO·-based treatment on wound healing in animals with DM

Study	Year	Species	DM model	Body weight (g)	Wound	Donor	Dose	Route	Endpoint	Findings
Witte et al.[86]	2002	Male rats	STZ	225–250	Incision (7 cm)	*L*-Arg	2× 1 g/kg/d	G	Wound healing	↑ Wound fluid NO· metabolite concentrations ↑ Wound breaking strength ↑ Hydroxyproline concentration ↔ Wound fluid arginase activity ↔ Wound fluid ornithine concentration
Shi et al.[62]	2003	Male rats	STZ	225–266	Incision (7 cm)	*L*-Arg	1 g/kg/d	IP	Wound healing	↑ Wound fluid NO· concentration ↑ Wound fluid hydroxyproline ↑ Wound breaking strength ↑ Expression of procollagen I, and III
Jerônimo et al.[87]	2016	Male mice	STZ	20–25	Incision (3 cm)	*L*-Arg	2 g/kg/d	G	Wound healing	↑ Collagen synthesis and deposition ↑ Vasculoneogenesis ↑ TGF-β expression ↓ Expression of IL-8 ↓ Polymorphonuclear cell infiltration ↓ Fibrosis
Witte et al.[86]	2002	Male rats	STZ	225–250	Incision (7 cm)	SIN-10	2× 1 mg/kg/d	G	Wound healing	↑ Wound breaking strength ↑ Hydroxyproline concentration ↑ MMP-2 ↑ Wound fluid arginase activity
Schäffer et al.[88]	2007	Male rats	BBDP	220–250	Incision (7 cm)	SIN-10	4 mg/kg/d	G	Wound healing	Restoring NO· metabolite concentrations, wound-breaking strength, and collagen deposition in normoglycemic rats ↑ NO· metabolite concentrations, wound-breaking strength, and collagen deposition in hyperglycemic rats
Chen et al.[89]	2019	Female mice	*db/db*	NR	Ischemic-excision (6 mm)	DNIC-1	0.18 mg/kg every other day	IV	Angiogenesis, wound healing	↑ Healing rate ↓ Wound closure time ↑ Expression of eNOS, MMP-11, CD31, CD34, and VEGF ↑ Angiogenesis ↓ Inflammation

Control groups received water or normal saline. BBDP: Biobreeding (i.e., an inbred laboratory rat strain that spontaneously develops autoimmune type 1 diabetes mellitus); CD31: endothelial marker; CD34: endothelial marker; DM: diabetes mellitus; DNIC-1: dinitrosyl iron complexe-1; eNOS: endothelial nitric oxide synthase; G: gavage; GSNO: *S*-nitrosoglutathione; HIF-1: hypoxia-inducible factor-1; IL: interleukin; IP: intraperitoneal; IV: intravenous; *L*-Arg: *L*-arginine; MMP: matrix metalloproteinase; NO·: nitric oxide; NR: not reported; SIN-10: molsidomine; STZ: streptozotocin; TGF-β: transforming growth factor-β; VEGF: vascular endothelial growth factor.

Intravenous injection of DNIC-1 (0.18 mg/kg) increased expression of endothelial NOS, matrix metalloproteinase-11, CD31, CD34, vascular endothelial growth factor (VEGF), restored the impaired angiogenesis, and accelerated the recovery rate of wound closure in mice with T2DM.[89]

The safety profile of systemic NO·-based therapies for DM wound healing is yet to be fully characterized due to limited *in vivo* data on potential toxicity. However, it is worth noting that *L*-Arg supplementation has been well-tolerated, as indicated by normal serum levels of urea, creatinine, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase.[87]

To sum up, studies provide preliminary evidence for the efficacy of systemic *L*-Arg and other NO·-based therapies in promoting DM wound healing in animal models. However, generalizability to humans remains to be established due to potential species-specific physiological differences. Whereas short-term safety is indicated by normal serum markers of liver function with *L*-Arg supplementation, a comprehensive evaluation of long-term safety profiles for all NO· donors is essential. Furthermore, the optimal dosing and specific mechanisms of action for each NO· donor require further elucidation.

Topical nitric oxide-based treatments

Acidified NO_2 (3.0% sodium nitrite ($NaNO_2$) + 4.5% citric acid containing cream) was documented to accelerate wound healing [i.e., indicated as decreased half closure time (CT50%) from 8 to 5 days] in rats with T2DM (low dose of STZ + high-fat diet).[59] Acidified-NO_2 increased the numerical density of basal cells (1070 *vs*. 936.6 mm^3) and epidermal thickness (58.5 *vs*. 44.3 μm).[90] Daily topical administration of a similar formulation with various doses of sodium nitrate ($NaNO_3$) and citric acid on wound healing in male mice with T2DM indicated a wound closure rate of 98.1%, 100%, and 97.4% (in 0.5%/0.75%, 3.0%/4.5% and 9.0%/13.5%, $NaNO_3$/citric acid, respectively), at day 12 after wounding.[91] The healing rate, i.e., indicated by both higher wound closure rate (91.8 *vs*. 60% at day 18) and lower CT50% (12.9 *vs*. 17.9 days), was significantly greater in the NO·-treated (3.0%/4.5% $NaNO_3$/citric acid) compared to the untreated group when administered on day

2 after wounding (i.e., after the coagulation phase).[91] This dose of acidified-NO_2 was effective when applied before day 4 after wounding (days 1, 2, and 4), parallel to the migratory/proliferative phase of healing.[91]

Although topical TNG was proposed as a promising potent healing agent for clinical management of DFUs,[92] this idea remains to be investigated. Only one experimental study in rats assessed the healing effect of TNG ointment and gel with and without aloe vera on DM wound reported a significantly lower wound area at day 8 (12.2 *vs*. 18.7 mm^2), higher wound closure (54.9% *vs*. 31.2%), and higher hydroxyproline content and NO· concentration in the wound tissue (1.45 *vs*. 0.8 ng/mg protein) in the TNG ointment group compared to the non-treated group.[93]

Topical application of a newly-developed dual acting NO··-based agent (TOP-N53, NO· donor S-nitroso-N-acetyl-DL-penicillamine + phosphodiesterase 5 inhibitor sildenafil) on full-thickness DM wound in *db/db* mice, induced angiogenesis (indicated by higher expression of vascular marker CD31) enhanced keratinocyte migration and proliferation (indicated by the length and the thickness of the wound epidermis, respectively) and increased area of GT.[94] The favorable effect of TOP-N53 on DM wound healing was confirmed by semiquantitative wound scoring, demonstrating a significant increase in re-epithelialization and a mild acceleration of GT maturation.[94]

Table 2 summarizes the investigations that used newly developed NO··-releasing hydrogels for promoting wound healing in DM.[67-70,95-103] The effects of NO··-releasing hydrogels on DM wound healing are mainly documented in mice. Treatment of DM wounds with NO··-releasing hydrogels in animal models resulted in an increased expression of angiogenesis factors (e.g., hypoxia-inducible factor-1, VEGF, TGF-β), promotion of angiogenesis and neovascularization, inhibition of inflammation by concomitant upregulating of pro-inflammatory cytokines and downregulating anti-inflammatory cytokines, polarization of M1 to M2 macrophages, promoted fibroblast migration and differentiation, and collagen synthesis and deposition.[67-70,96,98,100] NO··-releasing hydrogels also suppressed bacterial growth (i.e., *Staphylococcus aureus* and *Escherichia coli*) and facilitated ROS clearance.[67,97,102] These effects resulted in accelerated wound healing.[67-70,96,98]

Current evidence suggests a gap in knowledge regarding the *in vivo* safety of NO··-based therapies for DM wounds. Experimental studies have evaluated the biocompatibility of hydrogel-containing NO· donors using cell viability (cytocompatibility) and hemolytic activity (hemocompatibility) assays. These studies demonstrated good biocompatibility and no apparent cytotoxicity on various cell lines, including L929 fibroblasts, HaCaT keratinocytes, and human umbilical vein ECs.[73,91-93,95-97] In addition, no apparent cytotoxicity was shown in wound tissue at the investigated doses96 (refer to **Table 2** for details).

Future studies should prioritize translating the promising *in vitro* biocompatibility of NO··-releasing hydrogels to a clinically relevant *in vivo* setting. Dose-response studies are essential to establish the optimal therapeutic window for NO· delivery, balancing efficacy and potential cytotoxicity. Long-term *in vivo* studies are warranted to assess the sustained safety and efficacy of NO··-based treatments, as well as potential delayed adverse effects. Furthermore, exploring synergistic wound healing strategies by combining NO··-based treatments with other established or emerging therapeutic modalities (e.g., growth factors, stem cells) holds promise for accelerating wound closure and improving overall healing outcomes in patients with DM. Through systematic investigation of these key areas, a robust experimental foundation can be established to inform the development of NO··-based treatments for clinical translation in DM wound healing.

Clinical Evidence of Nitric Oxide-Based Treatments on Diabetic Wound

Human clinical trials did not support experimentally investigated beneficial effects of systemic *L*-Arg supplementation on DM wounds. For instance, a multicenter design randomized double-blind clinical trial in patients with T1DM and T2DM (27.9% were women, mean duration of DM was 13 years, mean glycated hemoglobin was 8 ± 1.5%) reported no additional healing properties (wound closure and time to wound healing) for a 16-week supplementation with *L*-Arg, glutamine, and β-hydroxy-β-methylbutyrate as an adjunct to standard therapy.[104] Only in patients with low albumin (≤ 40 g/L) or decreased limb perfusion (ABI < 1.0) was there evidence of a higher healing rate at week 16 (odds ratio = 1.70, 95% confidence interval = 1.04–2.79, and odds ratio = 1.66, 95% confidence interval = 1.15–2.38, respectively).[104]

Most recently, a portable on-demand NO··-generating device (NO· jet healing device, NJHD, producing NO· through a simple reaction of $NaNO_2$ with citric acid) has been developed for DM wound healing that overcomes the limitations and concerns of using gaseous NO·.105 Clinical application of NJHD was assessed in patients with DM (22–85 years, glycated hemoglobin < 12%, at least one full-thickness wound below the ankle with 1–16 cm^2 area, and ABI > 0.7). Use of NJHD (4 sessions per week, each session 12 minutes with 500 ppm of NO·) significantly reduced wound closure time (19 *vs*. 25 days, compared to standard therapy).[105] Upon 12 minutes of use of NJHD, the relative index of transcutaneous O_2 pressure significantly increased, indicating an enhanced blood supply around the wounds.[105] A multicenter randomized controlled trial, including 135 participants with T1DM and T2DM (13% were women, fasting serum glucose 178 ± 102 mg/dL) with a chronic (at least 6 weeks), full-thickness DFU, assessed the safety and efficacy of EDX110 (novel dressing system comprising two layers generating NO· *in situ* with adequate moisturizing that facilitates debridement) in the treatment of DFU compared against an optimal standard of care.[106] Both groups received standard care (debridement, offloading, and antimicrobial treatment); however, the NO-treated group also received EDX110 dressing. The result indicated improved healing, with an 88.6% reduction in ulcer area at 12 weeks compared with 46.9% in those receiving standard dressings.[106] Wound size reduction of the EDX110-treated patients at 4 weeks was similar to that achieved by the standard dressing at 12 weeks.[106]

Table 2 | Animal studies investigated effect of NO·-releasing hydrogels on DM wounds

Study	Year	Species	DM model	BW (g)	Wound model	NO· donor	Concentration in hydrogel	Endpoints	Findings	
Liu et al.[95]	2024	Male mice	STZ	NR	Excision (8 mm)-MRSA- infected	BNN6	0.2 mg/mL	Antibacterial, antioxidant, and angiogenic properties	↑ Re-epithelialization ↑ Collagen deposition ↓ Expression of IL-6 ↑ Expression of IL-10	↑ VEGF and CD31 ↓ ROS generation ↑ Wound healing rate Eradication of S.A (97.3%)
Huang et al.[96]	2024	Male mice	STZ	20–22	Excision (8 mm) MRSA- infected	GSNO	50 μmol/mL	Antibacterial, angiogenic, anti-inflammatory, and wound-healing properties	↑ M1 to M2 polarization ↓ Inflammatory cells ↓ Expression of TNF-α ↑ Fibroblasts ↑ Collagen concentration and deposition	↑ Expression of VEGF ↑ Re-epithelialization and dermal regeneration ↓ Remaining scar Eradication of S.A and E.C (100%)
Yang et al.[68]	2024	Male mice	STZ + HFD	20±5	Excision (6 mm)	*L*-Arg	200 μg/mL	Antioxidant, angiogenic, anti-inflammatory, and wound-healing properties	↑ NO· production ↓ ROS generation ↑ O2 in wound tissues ↓ Expression of IL-1β and IL-6	↑ Polarization of M1 to M2 macrophages ↑ Expression of HIF-1α, VEGF, and PCNA
Sivaraj et al.[69]	2023	Female mice	*db/db*	NR	Excision (6 mm)	$NaNO_2$	2 g/100 mL, 10 nM NO·/h	Wound healing	↑ Tissue density ↑ Collagen networks ↑ Expression of fibronectin, and TGF-β1	↑ Wound healing rate ↓ Wound closure time
He et al.[97]	2023	Male mice	STZ	NR	Excision	BNN6	2 mg/mL	Antioxidant, angiogenic, anti-inflammatory, and wound-healing properties	↑ Wound contraction ↑ Collagen content ↑ Thickness of newly formed epidermis ↑ Angiogenesis	↓ ROS generation ↑ Expression of VEGF ↓ Expression of TNF-α, Eradication of S.A and E.C (100%)
Zhang et al.[98]	2023	Male mice	STZ	20–25	Excision (10 mm)	*L*-Arg	1 g	Epithelization, angiogenic, anti-inflammatory, and wound-healing properties	↑ Epithelial formation ↑ Collagen deposition ↓ Inflammation	↑ Expression of HIF-1α ↑ Angiogenesis
He et al.[99]	2024	Male rats	STZ	200–250	Excision (15 mm) S.A-infected	BNN6	2 mg/mL	Antibacterial, angiogenic, anti-inflammatory, and wound healing properties	↓ ROS generation ↓ Expression of IL-6, TNF-α, CD86 ↑ Expression of IL-10	↑ Expression of CD31 and CD206 ↑ Wound healing rate
Zhao et al.[100]	2022	Male rats	STZ	180–220	Ischemic-excision (15 mm)	NO· microbubble	6.0×10^8 NO· microbubbles	Angiogenic, anti-inflammatory, and wound healing properties	↓ Expression of IL-1β, IL-6, and TNF-α ↑ Expression of IL-10, IL-22, and IL-13	↑ Angiogenesis ↑ Blood perfusion at the wounds ↑ Wound healing rate
Liu et al.[101]	2022	Male mice	*db/db*	NR	Excision (10 mm)	NONOate	100 μmol	Angiogenic and wound healing properties	↑ Granulation tissue ratio ↑ Vascularization	↑ Expression of CD31 and α-SMA
Yang et al.[102]	2021	Mice†	STZ	NR	Excision S.A-infected	SNP	200 μg/mL	Antibacterial, angiogenic, anti-inflammatory, and wound healing properties	↑ HIF-1α protein stability ↑ VEGF secretion from endothelial cells ↑ Wound closure rate	↓ S.A and E.C (86% and 94%) ↓ Inflammation score
Zheng et al.[67]	2024	Male rats	STZ	180–220	Excision (10 mm)	L-Arg + GSNO	0.5 mg + 3.66 mg/mL	Antibacterial, angiogenic, anti-inflammatory, antioxidant, and wound healing properties	↑ Expression of HIF-1α and VEGF ↑ Cell migration rate at the 36-h ↑ Blood vessel regeneration	↑ Collagen deposition ↓ S.A and E.C ↑ ROS clearance ↑ Wound healing rate
Xie et al.[103]	2024	Male rats	STZ	NR	Excision (10 mm) S.A-infected	GSNO	15 g, 25 μmol NO· over 6 h	Antibacterial, angiogenic, and wound healing properties	↑ Angiogenesis ↑ Wound healing rate ↑ Epidermal thickness	↓ Scar widths Eradication of S.A and E.C
Ahmed et al.[70]	2021	Rabbits	ALX	1000±200	Excision (20 mm)	SNAP	5%	Angiogenic and wound healing properties	↑ Wound healing rate ↑ Blood vessels density ↑ Expression of VEGF, PCNA, SDF-1α, Bcl-2, and TGF-β1	↑ Hydroxyproline and collagen deposition ↑ Epithelial layer formation ↓ Healing time

Phosphate-buffered saline-releasing hydrogel was used as control group. † Not reported sex. ALX: Alloxan; Bcl-2: B-cell lymphoma 2; BMSCs: bone marrow stem cells; BNN6: N,N′-di-sec-butyl-N,N′-dinitroso-1,4-phenylenediamine; BW: body weight; CMCS: carboxymethyl chitosan; DM: diabetes mellitus; E.C: *Escherichia coli*; GSNO: *S*-nitrosoglutathione; HFD: high-fat diet; HIF-1: hypoxia-inducible factor-1; IL: interleukin; *L*-Arg: *L*-arginine; MRSA: methicillin-resistant *Staphylococcus aureus*; $NaNO_2$: sodium nitrite; NO·: nitric oxide; NONOate: diazeniumdiolate; NR: not reported; PCNA: proliferating cell nuclear antigen; ROS: reactive oxygen species; S.A: *Staphylococcus aureus*; SDF-1α: stromal cell-derived factor-1α; SNAP: *S*-nitroso-*N*-acetyl-penicillamine; SNP: sodium nitroprusside; STZ: streptozotocin; TGF-β: transforming growth factor-β; TNF-α: tumor necrosis factor-α; VEGF: vascular endothelial growth factor; α-SMA: α-smooth muscle actin.

Mechanisms Underlying Wound Healing Effects of Nitric Oxide-Based Treatments

The effectiveness of NO·-based treatments on DM wound healing has been attributed to the anti-microbial,[67,95-97,102,103] anti-inflammatory,[68,95,96,98-100] and anti-oxidative properties[67,68,95,97,99] of NO· and its positive impacts on promoting healing factors (e.g., growth factors and angiogenesis factors[67-70,95-97,99,100,102]) facilitating angiogenesis and re-epithelization in the wound.

As illustrated in **Figure 2**, NO·-based therapies are involved in various phases of the wound healing process. The role of NO· in the early wound healing phase is unclear. On the first day of injury (known as hemostasis or coagulation phase), activation of platelets, coagulation, and clot formation are dominant. Theoretically, NO· may be essential for the vasodilation of intact vessels to support enough blood supply around the wound tissue and attenuation of platelet aggregation in the hemostasis phase. However, considering the antiplatelet and anti-thrombotic properties of NO·/NO· donors,[107] NO· may prevent platelet activation, attachment, and aggregation, counteracting the hemostasis phase. For instance, treatment of incisional wounds with acidified-NO_2 impaired wound healing when applied immediately after wounding (a decreased wound closure rate of 58% *vs*. 80% and longer CT50% of 9.2 *vs*. 8.4 days in the treated group compared to control).[91] A *db*/*db* mouse model of DM that used NO·-releasing hydrogel dressing reported no effect on wound closure when applied in the coagulation phase.[108]

At the inflammatory phase, NO· acts as a pro-inflammatory mediator, facilitating the migration and activation of neutrophils and macrophages, i.e., essential for clearing debris and initiating the healing process. NO· also modulates the production of cytokines, both pro-inflammatory [e.g., interleukin (IL)-1β, tumor necrosis factor-α] and anti-inflammatory (e.g., IL-10); this balanced response is crucial for a controlled inflammatory environment.[109] Beyond its initial pro-inflammatory role, NO· appears to play a critical role in downregulating the inflammatory phase through inhibiting RANTES (regulated upon activation, normal T cell expressed and secreted), i.e., a monocyte chemoattractant; NO·-mediated suppression of RANTES might initiate the transition from inflammation to the regenerative phase.[110] Additionally, NO· may decrease the expression of monocyte chemoattractant protein-1 by hyperproliferative keratinocytes at the wound edge.[111] Finally, NO· may contribute to reduced chemoattraction by activating TGF-β1, which could subsequently suppress the expression of iNOS.[109] In contrast to its initial pro-inflammatory role, NO·, in later stages, promotes a more anti-inflammatory environment by inducing apoptosis of neutrophils and macrophages to prevent excessive and prolonged inflammation hindering normal healing.[109,112] Overall, this evidence highlights the complex interplay between NO· and various signaling molecules regulating the inflammatory response during wound healing.[109]

NO·-releasing treatments displayed potent antimicrobial activity in wounds.[96,97,113] The antimicrobial properties of NO· donors, e.g., S-nitrosoglutathione, are attributed to their interaction with various proteins, DNA, and bacteria enzymes by forming reactive nitrogen species and induction of nitrosative stress.[114] *Staphylococcus aureus*, i.e., the most frequently isolated bacterium from DFU causing severe necrotic infections and leading to amputations, is eradicated by NO_3.[113] Under hypoxic conditions (like that observed in DM wounds), *Staphylococcus aureus* survives by switching metabolic flux between fermentative growth and anaerobic NO_3 respiration, depending on the availability of NO_2 and NO_3 (that serve as terminal electron acceptors); lack of NO_2/NO_3 impairs anaerobic respiration leading to fermentative growth of *Staphylococcus aureus* that increases biofilm formation and expression of *Staphylococcal* toxins.[113] NO_3 significantly promoted anaerobic respiration and inhibited the expression of *Staphylococcus aureus* virulence factors and overgrowth.[113] Likewise, *L*-Arg increases NO· synthesis, increasing NO_2/NO_3 concentration in the wound and promoting NO_3 respiration of *Staphylococcus aureus* and its virulence in hypoxic DM wounds.[113]

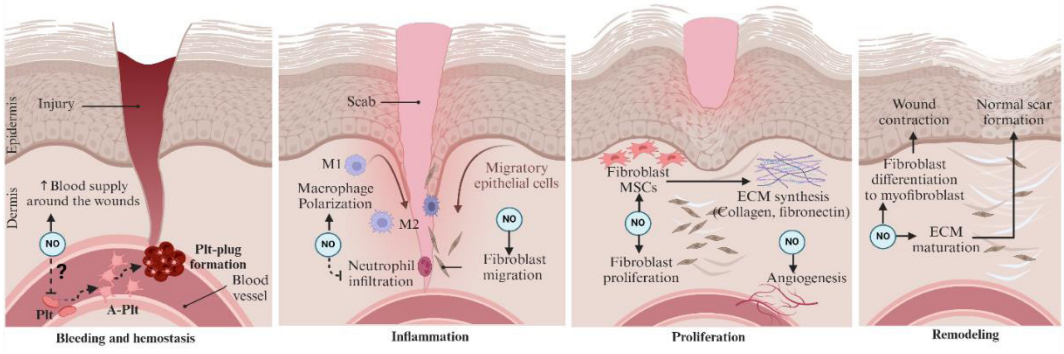

Figure 2 | Effects of NO·-based treatments at various phases of healing in DM wound.
NO·-based therapies may offer a multifaceted approach to improve DM wound healing. (1) The role of NO· in the hemostatic phase is not clear. (2) At the inflammatory phase, NO· therapy can reduce excessive inflammation while promoting the recruitment of beneficial immune cells like macrophages, which are crucial for debris clearance and healing. (3) NO· may positively influence the proliferative phase by stimulating the growth and migration of endothelial cells, necessary for angiogenesis. This enhanced blood flow can deliver vital oxygen and nutrients to the wound site, promoting epithelialization, i.e., the regeneration of the skin's surface layer. (4) NO· can regulate collagen deposition during remodeling, potentially influencing scar formation. However, it is important to note that the specific effects of NO·-based therapies on each stage of wound healing require further investigation. Optimizing their dosage and delivery methods will be crucial to stablish their potential for promoting DM wound healing. Created with BioRender.com. A-Plt: Activated platelet; ECM: extracellular matrix; MSCs: mesenchymal stem cells; NO·: nitric oxide; Plt: platelet.

NO·-based treatments can enhance wound healing by inducing fibroblast migration, decreasing neutrophil infiltration, and polarizing M1-to-M2 macrophages at the inflammatory phase.[68,96,115] M1-macrophages act as a major source of pro-inflammatory cytokines (i.e., tumor necrosis factor-α, IL-1β, IL-6, IL-12, IL-23) involving in pathogen phagocytosis and removing damaged cells, whereas M2-phenotype plays an anti-inflammatory role by upregulating IL-10, TGF-β1, and IL-12 and has repair and regeneration functions in wound healing process.[116,117] Through macrophage polarization, NO· can facilitate the transition of wounds from the inflammatory phase to the proliferative phase, which in turn enhances collagen deposition.[67,96]

NO· also promotes wound healing in the proliferative phase, i.e., characterized by angiogenesis, GT formation, re-epithelialization, and collagen deposition.[118,119] NO· increases expression of stromal cell-derived factor-1α (SDF-1α), a key factor of DM wound healing[120] that involves the migration, recruitment, and retention of endothelial progenitor cells and promotion of angiogenesis through activation of heme oxygenase 1.[121] SDF-1α enhances epidermal stem cell migration and proliferation, accelerating wound healing.[122] SDF-1α acts as a potent chemokine for bone marrow-derived stromal stem cells expressing C-X-C chemokine receptor type 4 (a SDF-1α receptor), which in turn facilitates recruiting bone marrow-derived stromal stem cells to wound tissues, promotes secretion of growth factors by bone marrow-derived stromal stem cells and neovascularization.[123] NO· upregulates VEGF,[67,69,70] an important pro-angiogenic growth factor that induces vasculogenesis and angiogenesis and acts as a chemoattractant for angioblasts during the wound-healing process.[124] VEGF-induced angiogenesis and subsequent perfusion might enhance the nutrient supply of wound tissues.[70] Along with VEGF, NO· treatment induces another important angiogenic factor, CD31 (also known as platelet/endothelial cell adhesion molecule-1[69,125]); CD31 promotes EC-cell adhesion, cellular transmigration, and diapedesis, angiogenesis, and vascular integrity maintenance,[126] resulting in wound healing at early stages.[125] NO· upregulates B-cell lymphoma 2 (Bcl-2, a mitochondrial protein preventing apoptosis) expression in the wound tissue[70]; upregulated expression of Bcl-2 has been shown to improve cell survival and differentiation of neuroepithelial stem cells.[127] The expression of proliferating cell nuclear antigen (a cell-proliferating gene) in the wound tissue increases upon NO· treatment.[70] Upregulated proliferating cell nuclear antigen may facilitate cell viability and cell proliferation in mesenchymal stem cells,[70,128] the self-renewing multipotent stem cells that coordinate the healing process by recruiting other host cells and secreting growth factors and matrix proteins (e.g., ECM proteins).[129]

NO·-based treatments promote wound healing in the remodeling phase, i.e., characterized by developing new epithelium and normal scar formation through establishing a balance between synthesis and degradation of ECM (i.e., collagen, fibronectin, and other ECM components),[17,130] and wound contraction via myofibroblasts.[119] NO· upregulates expression of α-smooth muscle actin, a factor facilitating differentiation of fibroblasts into myofibroblasts (i.e., shared phenotypes of both fibroblasts and smooth muscle cells act essentially in collagen deposition and wound healing[101]); α-smooth muscle actin-expressing myofibroblasts promotes contraction and accelerates wound closure and upregulates both ECM components and matrix-degrading proteases.[131,132] NO· treatment upregulates expression of fibronectin and TGF-β1 throughout the healing process.[69] Fibronectin is a large glycoprotein that crosslinks ECM and integrins and acts as a building block facilitating the maturation of ECM, GT, and re-epithelization.[133]

Conclusion and Perspective

Existing experimental evidence suggests a promising role for NO·-based therapies in DM wounds. Translating findings from animal studies to clinical practice is challenging. Although animal models are valuable tools, their limitations in simulating the multifaceted nature of DM wounds in humans must be considered when interpreting results. Furthermore, humans and rodents have distinct skin morphophysiology (e.g., skin thickness, epidermis layers, adherence to underlying tissues), immunology, and genetics; their skin differentially expresses several immunologically related genes and has specific stem cell niches.[134] The wound healing process also differs between rodents and humans; for instance, wound healing in mice is much faster than in humans and heavily relies upon wound contraction (i.e., mediated by a unique muscle layer, panniculus carnosus) than re-epithelialization.[135] Panniculus carnosus is considered vestigial in humans and found only in specific anatomical regions, including the palm, neck, and heel.[136] The relevance of animal models to the human pathophysiology of DM wounds remains a critical issue because a specific model cannot capture all underlying causes of healing defects in patients with DM.[77] However, animal models help investigate specific pathways underlying DM wounds (e.g., the relationship between hyperglycemia, microvascular dysfunction, neuropathy, and impaired healing).[77,78,84] Heterogeneous protocol of DM and wound inductions, use of different NO· donors and diverse NO·-releasing biomaterials and platforms, confounding variables (e.g., age and sex of animals, duration of DM, use of a splint to inhibit early wound contraction) making between-studies comparisons implausible, is another challenge of bench-to-bedside application of NO·. In the splinted wound model, silicone splints are used around the wound to prevent wound contracture by the panniculus carnosus muscle and to promote tissue granulation formation to mimic human the wound healing process.[69,137]

A critical limitation of the current research field is the paucity of robust clinical trials investigating NO·-based therapies in DM wounds. The lack of human data necessitates well-designed randomized clinical trials to definitively assess the safety and efficacy of NO·-based therapies. These trials should consider well-defined primary endpoints, i.e., wound reduction area, rate of wound healing, and hospital length of stay. Developing a simple, cost-effective, and controlled mode of NO· delivery to wounds with a stable formulation is a promising approach in DM wound treatment.

Some concerns, including the short half-life of NO· and its highly-reactive properties generating excessive amounts of toxic molecules (e.g., peroxynitrite, nitrogen dioxides), uncontrolled release of NO· in the wound tissue, and potential systemic effects (e.g., acute hypotensive effects) remain to be addressed. Some open questions might call for further attention, e.g., the optimum intervention time with NO·-releasing materials and duration.[91,138] For instance, NO· improves the healing process (indicated as wound contraction and re-epithelialization) when applied every day at the inflammatory and proliferative phases (corresponding to the day of lesion until the sixth day after wounding) rather than at the inflammatory or the proliferative phase *per se*.[138]

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