Nitric oxide (NO) is a critical molecular signal and mediator for normal wound healing. Nitric oxide deficiency has been established as an important mechanism responsible for poor healing in diabetic foot ulcer (DFU) patients. Preliminary clinical wound healing studies using wound fluid nitrate determinations, as measures of wound NO bioactivity, suggest that threshold wound fluid nitrate values may function as an effective diagnostic indicator of successful wound healing for the DFU patient. In this case, wound fluid nitrate measurements could predict the suitability of a wound for specialized wound therapies such as topical growth factors or bioengineered skin substitutes. Wound fluid nitrate determination would also identify DFU patients with wound NO deficiency–related impaired healing that may promote wound complications possibly leading to lower-extremity amputation. With clinical validation, wound fluid nitrate may qualify as a wound healing biomarker and surrogate endpoint for DFU treatment. Additionally, combined diagnostic determinations of wound NO bioactivity, bacterial load, and matrix metalloproteinase production will enhance DFU management by providing quantitative parameters for clinical wound treatment. The impact of wound fluid nitrate determination and the combined platform of wound diagnostic indicators for wound healing prediction and clinical treatment, respectively, is anticipated to provide substantial costs savings and improved outcomes for the DFU patient.

Introduction

There are over 20 million people in the United States with diabetes mellitus and the cost of treatment is approximately $132 billion.1 Within this group, 15% will experience foot-related lower-extremity ulcerations, including the most common of these ulcerations, the plantar neuropathic diabetic foot ulcer (DFU) (Figure 1). It is estimated that 20% of hospitalizations related to diabetes mellitus involve treatment of complications of lower-extremity ulcers. Of more than 100,000 nontraumatic lower-extremity amputations (LEAs) performed annually in the United States, 45% to 83% involve diabetics with lower-extremity ulcer complications.2 It has also been estimated that 5% to 15% of persons living with diabetes mellitus will have an LEA during their lifetime. Further, after an initial amputation, 9% to 17% will experience a second amputation within the same year. The 5-year survival rate after LEA is between 40% and 70%. Unfortunately, up to 10% may succumb before leaving the hospital after amputation.3

For the past 2 decades, research supporting the development of specialized wound therapies for impaired DFU healing has focused on the role of growth factor and cytokine deficiency. Recombinant growth factor therapy for DFU treatment was clinically introduced in the mid-1990s with the synthesis of platelet-derived growth factor (rhPDGF, becaplermin) following early promising results in experimental diabetic wound models and Phase II clinical trials.4 The working hypothesis supporting becaplermin (Regranex Ortho-McNeil, Titusville New Jersey) treatment for impaired DFU healing suggested elevated levels of matrix metalloproteinases (MMPs) and reduced levels of their endogenous tissue inhibitors (tissue inhibitors of MMP [TIMPs]) resulted in excessive proteolysis of tissue, as well as of growth factors and their receptors.5,6 However, subsequent studies of specialized wound therapies that combined pressure offloading with platelet releasates, bioengineered skin substitutes, and recombinant platelet-derived growth factor demonstrated healing rates that varied from 8% to 75%, averaging less than 50% overall.7 These results suggest that additional biological factors responsible for impaired DFU healing were not addressed by the use of growth factor–based treatments and have not been incorporated into widespread practice.
Subsequently, emerging wound-healing research with nitric oxide (NO) provided evidence of a critical dependency of effective cellular wound repair mediation on optimal wound NO bioactivity for the diabetic wound.\(^8\),\(^9\) These findings provided a biological basis for the marginal performance of growth factor therapy for many hard-to-treat DFUs related to deficient wound NO bioactivity.\(^1\)\(^0\)–\(^1\)\(^2\) Our preliminary human wound healing studies suggest that a threshold level of wound NO bioactivity and supernormal wound NO production may be required for successful wound repair for subjects with diabetes and lower extremity ulcers.\(^1\)\(^0\)–\(^1\)\(^2\) Validation of these relationships could establish determination of wound NO bioactivity as a biomarker for wound healing and a surrogate endpoint for DFU management.

**Nitric Oxide and Clinical Wound Healing**

Nitric oxide is formed by the enzymatic combination of molecular oxygen and the amino acid l-arginine. Nitric oxide provides cellular signaling by activation of its target molecule, guanylate cyclase, which elevates intracellular concentrations of cyclic guanosine monophosphate (cGMP), which is frequently used as an indirect measure of nitric oxide production.\(^1\)\(^3\) Signaling occurs without the need for signal transduction; NO crosses cell membranes without mediation of channels or receptors and diffuses isotropically.\(^1\)\(^4\) Because of its high diffusion coefficient, short half-life of about 5 seconds, and prompt decomposition, NO is ideal as a dynamic intercellular signal for wound repair.

Three isoforms of NO synthase (NOS) metabolize l-arginine and molecular oxygen to citrulline and NO. Two of the 3 isoforms are constitutive enzyme systems (cNOS) that are described in neuronal cells (nNOS) and endothelial cells (eNOS). With these enzymes, increased levels of intracellular calcium activate the cNOS via calmodulin. The calcium-dependent cNOS systems produce low (picomolar) quantities of NO. The third system is the inducible isoform (iNOS), which is calcium independent. Expression of iNOS is controlled by tissue-specific stimuli such as inflammatory cytokines, exogenous materials, that is, bacterial lipopolysaccharide, and activation of NF-kB. Once induced, production of NO within tissue can increase as much as 1,000-fold, thereby producing an environment that is toxic to invading microorganisms. Currently, it appears that the cNOS enzymes are involved in maintaining skin homeostasis and providing regulatory function. The iNOS enzymes are mainly associated with inflammatory and immune responses that are implicated in certain skin diseases.\(^1\)\(^5\) Keratinocytes in humans possess both constitutive (eNOS and nNOS) and inducible (iNOS) isoforms.\(^1\)\(^6\) Epithelial migration,\(^1\)\(^7\) wound angiogenesis,\(^1\)\(^8\) and granulation tissue formation\(^1\)\(^9\),\(^2\)\(^0\) are critical processes of tissue formation and repair that are mediated by the inducible expression of NO in keratinocyte and endothelial cells, respectively. Experimental studies also document a biphasic effect of NO on keratinocytes in a wound, with cellular proliferation or cytostasis occurring as a result of low or high NO tissue concentrations, respectively.\(^2\)\(^1\)

The major metabolic pathway for NO is conversion to nitrate [NO\(_{3}^-\)] and nitrite [NO\(_{2}^-\)], collectively termed NOx, which exists as stable metabolites within tissue, plasma, and urine.\(^1\)\(^4\) Tracer studies in humans have demonstrated that approximately half of NOx originates from the NO synthesis substrate, l-arginine, although this percentage varies based on dietary intake of NOx.\(^2\)\(^2\) Fasting plasma and urine samples allow us to use variations in NOx values as a means of evaluating changes in NO production and bioactivity.\(^2\)\(^3\) In experimental and clinical wound healing research, WFNOx has been used extensively as a reliable surrogate marker for wound NO bioactivity.\(^2\)\(^0\),\(^2\)\(^4\)–\(^2\)\(^6\) In all cases, WFNOx determinations have been highly sensitive to conditions or factors that reduce NO production and impair normal wound healing, such as diabetes mellitus,\(^9\) protein-calorie malnutrition,\(^2\)\(^7\) cutaneous irradiation,\(^2\)\(^8\) steroid therapy,\(^2\)\(^9\) and metabolic inhibition of NO synthesis.\(^3\)\(^0\) In theses cases, decreased wound fluid NOx and impaired wound closure were associated with decreased collagen accumulation,\(^2\)\(^4\) wound tensile strength, type I and III collagen gene expression,\(^3\)\(^1\) vascular endothelial growth factor expression, granulation tissue formation, and wound microvascular perfusion.\(^1\)\(^9\)
In a retrospective evaluation of diabetic subjects with DFUs that used becaplermin, we measured endogenous fasting urine and plasma NOx levels upon admission to the study and following 24 hours of hospital bed rest with a specialized, low nitrate diet. This preliminary retrospective study of diabetic wound healing was designed to evaluate the relationship between wound outcomes and endogenous NO bioactivity, as determined by fasting urine and plasma NOx measurement. Three groups with 5 subjects were studied: (1) healthy nondiabetics with no history of foot ulcers (control group), (2) subjects with type I/II diabetes who had neuropathic foot ulcers and wound healing after rhPDGF (Regranex) treatment for 20 weeks (responder group), and (3) subjects with type I/II diabetes and neuropathic ulcers that remained unhealed at 20 weeks after treatment with rhPDGF (nonresponder group). The 3 groups were well matched for age, sex, body mass index, HgbA1c, serum creatinine, creatinine clearance, and foot ulcer size/area. None of the subjects had microalbuminuria or renal insufficiency. Subjects were tested after an overnight fast prior to hospitalization (day 1 results). They were then hospitalized for 24 hours and received a low nitrate, low arginine diet, and bed rest, and fasting urine NOx values were repeated (Day 2 results). On day 1, the nonresponder group had significantly lower fasting urine NOx values than control subjects or responder group subjects ($P < .05$). On day 2, following a 24-hour hospitalization, bed rest, and overnight fast, the same relationships were observed; nonresponders continued to have significantly lower fasting urine NOx than control or responder group subjects. No significant differences in fasting urine NOx values were found when control and responder groups were compared on days 1 and 2. Plasma NOx values for the study groups demonstrated essentially the same relationships on days 1 and 2. Specifically, plasma NOx was significantly higher among subjects in the responder group. Interestingly, plasma NOx in the responder group was also significantly higher than that in the control group on days 1 and 2.

These results suggest that poor wound outcomes with rhPDGF are associated with significantly lower endogenous NO production as determined by fasting urine NOx measurements. Although these metabolites are indirect measures of the true target (biologically active NO), plasma and urine samples of NOx obtained in individuals on a controlled diet of low nitrate and low arginine provide a reasonable index of NO production.

In a prospective wound healing study, wound fluid NOx values were documented for chronic LEU subjects undergoing weekly treatments with the fibroblast-derived, dermal substitute Dermagraft (Smith & Nephew, Largo, Florida) over a period of 8 weeks. For the purposes of measuring wound fluid NOx, 12 subjects were divided into 2 equal-sized groups at the end of a 12-week observation period based on treatment response (responders vs nonresponders). While no significant differences in wound areas at baseline were found, the responder group experienced significantly greater wound area reduction at 2 weeks after treatment ($P < .05$), more robust granulation tissue formation, and a higher rate of wound closure at 12 weeks as compared to the nonresponder group (67% vs 0%). A pattern of early wound fluid NOx elevation was documented in responder group subjects who experienced early wound closure (62% area reduction at 2 weeks) or complete wound healing (Figure 2). In each case, peak wound fluid NOx levels were observed at about 2 weeks after initial treatment.

Responders also had significantly higher baseline wound fluid NOx values than nonresponders ($13 \pm 2 \mu mol/L vs 4 \pm 1 \mu mol/L$, Mean $\pm$ SD). Wound fluid NOx values in responders fell quickly after reaching their peak to subthreshold levels before wound closure or at the end of the 8-week treatment period. Wound fluid NOx values in nonresponders were not significantly elevated above baseline values at any point during the 8-week treatment period. All responders had normal serum homocysteine levels, but 5 out of 6 nonresponders had elevated levels. In one nonresponder subject with type 2 diabetes and bilateral ankle venous ulcers, elevated serum homocysteine was corrected by daily multivitamin treatment (2.5-mg folic acid, 25-mg pyridoxine, and 2-mg cyanocobalamin) leading to a 4-fold increase in wound fluid NOx values (from 1.5 to 6 µmol/L). Following this improvement in wound NO bioactivity, a second series of Dermagraft treatments (4-week treatment) resulted in successful healing of both ulcers. Nonlinear regression analysis of preliminary
data from this study demonstrates correlation between baseline wound fluid NOx values and the percentage of healing (wound area) in Dermagraft-treated subjects (Figure 3).

The results of this preliminary study suggest that elevated serum homocysteine should be considered a risk factor for successful wound healing with Dermagraft therapy and that wound outcomes with this specialized wound therapy might be predicted from baseline wound fluid NOx measurement. These data further suggest the presence of a functional threshold of related wound NO bioactivity may be required for successful wound treatment. Early supernormal production of wound NO in responder subjects may offer evidence of an important relationship between inducible NO mediation of inflammatory wound processes and successful wound closure. In a preliminary study of successful wound healing mediated by hyperbaric oxygen therapy, a similar association between supernormal wound fluid NOx values and wound closure was found.12

Wound Fluid NOx as a Diagnostic Indicator for Wound Healing

At this point, no molecular biomarker of a tissue repair mediator has been validated for clinical use. The absence of a wound healing biomarker has contributed significantly to the marginal efficacy of specialized wound therapies for patients with DFU. The NIH Biomarkers Definitions Working Group defines a biomarker as a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or response to a therapeutic intervention.34 A clinical endpoint (eg, a closed wound) is defined as a characteristic or variable that reflects how a patient feels, functions, or survives. Valid biomarkers may function as a surrogate endpoint, which are intended to substitute for a clinical endpoint. In this case, a surrogate endpoint should predict clinical benefit (or lack of benefit) on the basis of therapeutic or other “scientific” evidence.34 Consideration of wound fluid NOx as a valid biomarker or surrogate endpoint for wound healing will require more extensive research. However, the following statements summarize our current understanding of the importance of NO mediation in wound repair and the value of WFNOx determinations in studies of this biological process.

1. Scientific evidence of the critical role of NO mediation in cutaneous repair has been well established from prior experimental studies of various models of wound epithelialization, angiogenesis, collagen deposition, and wound remodeling.
2. Wound fluid NOx determinations appear to function effectively as measures of wound NO bioactivity and suggest the “state of readiness” of related NO-modulated processes required to initiate successful wound repair.
3. The positive and negative effects of wound interventions or pathologic conditions on wound closure appear to be reliably and appropriately reflected in wound NO bioactivity and in comparative determinations of WFNOx.

Three types of clinical tests that may be used in wound management are (1) nonspecific tests, (2) diagnostic tests or indicators, and (3) theranostic tests.35 Nonspecific tests are used to generate data relevant to the patient and clinical context but require interpretation and information from other sources. An example would be the use of erythrocyte sedimentation rate values as part of the clinical evaluation of a chronic infected wound patient. In this case, additional information is needed to indicate the value and impact of these data on the developing plan of wound treatment. A diagnostic indicator for wound management should provide unambiguous diagnostic or discriminatory information about a specific physiological or biochemical condition or disease state; results of this test may be interpreted without the need for additional parameters. An example is the results of a wound tissue culture and sensitivity. This information is specific to the wound and contributes directly to the decision process for treatment. Theranostic tests are specialized clinical tools that do not necessarily provide or lead to a diagnosis but indicate the need for a particular therapy in the treatment of a specific condition and may indicate the form of treatment as well. An example would be the diagnostic selection of the monoclonal antibody trastuzumab for a breast cancer patient with overexpression of human epidermal growth factor receptor-2. As a diagnostic indicator for wound healing,
wound fluid NOx determination has the potential to provide an assessment of the wound environment specific to related NO-mediated processes for tissue repair and to indicate the type of correction needed for unimpaired healing to occur in an otherwise well-prepared wound bed. This determination of the magnitude and direction of therapy should require little or no interpretation but ideally should influence treatment decisions and wound outcome.

Currently, our nonspecific and diagnostic wound testing has been laboratory based with specimens sent to centralized hospitals or offsite facilities for preparation and testing. The time required for interpretation is often in the order of days following specimen retrieval. In many cases, such time-related factors slow the delivery of effective medical treatment. A new generation of wound diagnostics, including measurement of wound fluid NOx, will be characterized by mobile (hand-held), noninvasive, self-contained testing kits designed for bedside or point-of-care use. Results of this one or more selected wound assays are anticipated to be available in a matter of minutes or hours of wound-specimen retrieval. It is strongly anticipated that such a system will provide accurate and prompt wound assessment that will significantly improve the initial clinical evaluation process and enable more rapid delivery of effective management.

Clinical algorithms for wound fluid NOx will be provided to establish guidelines for diagnostic test administration. This process is anticipated to provide early identification of patients with difficult-to-heal wounds and enhance identification of risk factors during wound evaluation and more aggressive medical or surgical preparation when indicated. Clinicians would also be alerted to the need for wound NO enhancing interventions such as hyperbaric oxygen or the application of NO-releasing dressings. The wound fluid NOx diagnostic algorithms could be integrated with currently used wound bed preparation paradigms, alerting the clinician to the presence of “favorable” or “unfavorable” conditions for specialized wound treatments. Such diagnostic markers may trigger a change to a more effective form of therapy and may also prove useful in demonstrating the futility of expensive treatments for patients with wounds that have little to no prospect of healing. For these reasons, outcomes from the clinical use of diagnostic indicators for wound management will benefit from cost savings provided by an increased incidence of wound closure or the adoption of an alternative treatment demonstrated as an acceptable form of therapy.

**Wound Diagnostics and DFU Management**

While optimal cellular NO-mediated processes are required for successful wound repair, the complexity of the healing process suggests that it is unlikely that a single marker will be identified for all forms of chronic wounds. Rather, it seems more likely that a range of new diagnostic tools will be required to assist with decision making in clinical wound treatment and that these may be applicable at different stages of healing. In addition to deficient NO bioactivity, DFU healing may be impaired by dysfunctional cells in the wound bed and imbalances in key proteases, cytokines, and growth factors. For example, an intensified protease response, in particular MMPs and neutrophil elastase, influences DFU healing. Higher concentrations of MMP-2, -9, and -8 and reduced concentrations of tissue inhibitors of MMPs (TIMPs) have been observed in chronic diabetic wounds when compared with trauma lesions of a control group with normal glucose metabolism. Overexpression of these proteases appears to impair DFU healing. Thus, in addition to the need for adequate wound NO bioactivity, an optimal range of wound MMP production and the absence of significant bacterial colonization are required to promote tissue repair and the clinical recovery of the chronic wound.

Understanding that DFU healing may be adversely affected by deficient wound NO bioactivity, excessive wound MMP production, and a significant bacterial microbial burden and systemic factors promoting endothelial dysfunction or increased NO scavenging.

**Evaluation of NO Bioactivity**

Wound fluid NOx measurement of the DFU will be performed to identify the level of NO-related wound bioactivity needed for unimpaired healing. It will also be used to monitor the effect of therapies designed to enhance NO production or decrease its removal or scavenging. This evaluation will include identification of metabolic risk factors such as elevated serum homocysteine or systemic factors promoting endothelial dysfunction or increased NO scavenging.

**Evaluation of Bacterial Load**

Rapid diagnostic identification and assessment of bacterial colony counts will be used for quantitative or semiquantitative determination of microbial burden in the DFU. This information will be used to initially direct surgical wound debridement and guide antibiotic therapy. Controlling bacterial load will prevent cellular necrosis and excessive MMP production.

**Surgical Wound Debridement**

Surgical wound debridement will be performed to remove biofilm in the wound bed, devascularized tissue, and necrotic material. Surgical debridement also
FIGURE 4. (a) Three key diagnostically determined wound factors (circles) that may simultaneously influence healing outcomes for the DFU patient. Nitric oxide—a primary wound healing factor, bacterial load (microbial burden/biofilms)—an inhibitory factor compromising tissue viability, and excessive wound MMP production—an inhibitory factor responsible for the degradation of granulation tissue, wound proteins, growth factors, and cytokines. (b) Selected clinical therapies that will optimally modulate or enhance key wound factors (a), enable the clinical objectives of diagnostic-based DFU management, and encourage successful wound recovery and DFU healing. DFU indicates diabetic foot ulcer; MMP, matrix metalloproteinase; NO, nitric oxide.
reduces the dysfunctional cell population and may help stimulate a new cycle of cytokines and growth factors needed to restore the normal healing process. Surgical debridement is beneficial for initiating healing in an indolent DFU and maintaining healing in a responsive foot ulcer.

Assessment of Wound MMP Production

This assessment will use a tissue and fluid assay to determine specific MMP (eg, MMP-2, -8, and -9) and TIMP activity. This information will be used to quantitatively document the need for specialized dressings or wound pharmacology for the accelerated removal, inactivation, or inhibition of excessive MMP production. It will also be used to monitor the efficacy of therapy directed at correcting excessive wound MMP and protease production in the DFU.

Implementation of this multifactor, diagnostic process, when combined with currently accepted principles of wound bed preparation is expected to significantly improve the likelihood of successful wound closure. Alternatively, in the absence of a favorable diagnostic assessment for DFU healing, alternative wound treatments employing more aggressive surgical wound debridement, or amputation, could be pursued in a more timely and cost-effective manner.

Summary

Effective mediation of NO-related cellular processes is critical to the normal wound healing. Ongoing research suggests that measurable thresholds and patterns of wound NO bioactivity, using wound fluid NOx measurements, may predict wound outcomes for the DFU patient. Therefore, it requires consideration as a biomarker and, perhaps, as a surrogate endpoint for DFU wound management. If validated by additional research, wound fluid NOx-related diagnostic technology may lead to improved wound healing outcomes for DFU patients, decrease associated complications, and provide substantial cost savings associated with wound-therapy selection. Finally, we suggest that the combination assessment of wound fluid NOx, bacterial load in the wound bed, and MMP production may be more valuable than measurement of wound fluid NOx alone.

KEY POINTS

✔ Deficient wound NO bioactivity is an important mechanism responsible for impaired wound healing in the DFU patient.

✔ Preliminary wound healing studies have demonstrated that wound fluid NOx determinations may predict wound healing in diabetic foot patients.

✔ With further clinical validation, wound NO bioactivity, as determined by wound fluid NOx measurements, may qualify as the first biomarker for clinical wound healing.

✔ Wound diagnostic indicators of healing, including measurement of wound fluid NOx, bacterial load, and wound MMP production may enhance treatment of DFUs resulting in accelerated wound patient evaluations, costs savings, and a decreased frequency of serious complications.

References

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