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Nitric oxide-releasing nanoparticles accelerate wound healing in NOD-SCID mice

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Abstract

Wound healing is a complex process, coordinated by various biological factors. In immunocompromised states wound healing can be interrupted as a result of decreased numbers of immune cells, impairing the production of effector molecules such as nitric oxide (NO). Therefore, topical NO-releasing platforms, such as diethylenetriamine (DETA NONOate), have been investigated to enhance wound healing. Recently, we demonstrated a nanoparticle platform that releases NO (NO-NPs) in a sustained manner, accelerating wound healing in both uninfected and infected murine wound models. Here, NO-NPs were investigated and compared to DETA NONOate in an immunocompromised wound model using non-obese, diabetic, severe combined immunodeficiency mice. NO-NP treatment accelerated wound closure as compared to controls and DETA NONOate treatment. In addition, histological assessment revealed that wounds treated with NO-NPs had less inflammation, more collagen deposition, and more blood vessel formation as compared to other groups, consistent with our previous data in immunocompetent animals. These data suggest that NO-NPs may serve as a novel wound-healing therapy in the setting of immunocompromised states associated with impaired wound healing.

From the Clinical Editor: Wound healing in an immunocompromised host is often incomplete and is a source of major concern in such conditions. This work demonstrates in a murine model that in these settings NO releasing nanoparticles significantly enhance wound healing.

Key words: Wound healing; Nitric oxide; Nanotechnology; Diazeniumdiolate; Immunodeficiency

Wound healing is a complex process divided into three distinct phases: inflammation, proliferation, and remodeling. Progression from one phase to the next is carefully orchestrated by numerous cytokines, growth factors, and cellular elements.[1] One agent that plays a vital role in wound healing is nitric oxide (NO), a highly reactive, lipophilic molecule with a variety of physiological functions. NO is generated endogenously by three distinct isoforms of nitric oxide synthases (NOSs), two of which are predominantly involved in wound healing: endothelial (eNOS) and inducible (iNOS) nitric oxide synthase.[2,3] During the early phases of wound healing, NO mediates vasodilation, inhibits platelet aggregation, and protects against invading pathogens. In the proliferative phase, NO stimulates fibroblasts, keratinocytes,

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and endothelial cells, the last of which promote angiogenesis via production of additional NO and vascular endothelial growth factor (VEGF).[1] During remodeling, NO plays a critical role in fibroblast production and deposition of collagen.[1,4,5]

When any of the steps of wound healing are disrupted, delayed and incomplete healing can result in a chronic wound.[6] Although the etiologies of impaired healing are complex and varied, wound-healing problems can be associated with decreased immune function, such as with diabetes mellitus, radiation therapy, advanced age, and immunosuppressive therapy.[7] Within the myriad alterations of cell responses in these conditions, there is speculation that lymphocytes, particularly $CD4^+$ and $CD8^+$ T cells, play a vital role in wound healing.[7-10]

Impaired wound healing, evidenced by increased closure time, decreased collagen deposition, and lower resistance to wound breaking, can occur in NO-deficient states, specifically in both eNOS[11] and iNOS[12] knock-out mice as well as in animals treated with inhibitors of NOS.[4,13] In fact, NO deficiency may contribute to impaired wound healing in diabetes and other immunocompromised states, which have been shown to be associated with reduced NO generation.[2,14] As a result, several organic and synthetic NO donors have been investigated to deliver exogenous NO topically to enhance wound healing.[15-17] One synthetic donor, diethylenetriamine (DETA NONOate), a type of diazeniumdiolates, spontaneously releases NO in a controlled manner and has been shown to accelerate wound closure in rats.[16] However, triamine-based DETA NONOates are water soluble and can migrate from the wound site before NO release. In addition, soluble diamine and triamine byproducts of DETA NONOate are potentially toxic to tissue.[17]

Recently, we described a nanoparticle platform composed of silane-based sol-gel and sugar-derived glasses that can generate, store, and deliver NO in a controlled and sustained manner.[3,18-20] In addition to demonstrating promising antimicrobial activity, these NO-NPs were shown to accelerate wound healing in both uninfected[21] and infected[22] murine full-thickness wound models. It had previously been shown that in vivo, NO-NP treatment increases tissue concentrations of transforming growth factor-β (TGF-β), a potent cytokine with broad and extensive effects on wound healing ranging from macrophage and fibroblast recruitment to collagen deposition to neoangiogenesis—features that were found to be enhanced histologically in wounds treated with NO-NPs as compared to controls.[21] Based on these findings, we hypothesized that the NO-NPs can accelerate healing in a murine model of impaired wound healing in the setting of immune deficiency. In this study the efficacy of NO-NPs and DETA NONOate are compared in non-obese, diabetic, severe combined immunodeficiency (NOD-SCID) mice. These mice can be used as a model of impaired wound healing due to immune compromise, as they lack T and B cells.

Methods

Synthesis of NO-NPs

The synthesis of NO-NPs and control nanoparticles without NO (NPs) was previously reported.[20,22] Briefly, a hydrogel-glass composite was synthesized using a mixture of tetramethylorthosilicate, polyethylene glycol, glucose, chitosan, and sodium nitrite in a 0.5 M sodium phosphate buffer (pH 7). The nitrite was reduced to NO within the matrix because of redox reactions initiated by thermally generated electrons from glucose and affected by the glass properties of the composite. After the redox reactions, the ingredients were combined and dried using a lyophilizer, resulting in a fine powder composed of NO-containing NPs. Control NPs were similarly generated, although without the addition of sodium nitrite. All NPs were stored in dry-powder form at room temperature (20°–22°C) before use. Single lots of NO-NPs and NPs were used for all experiments.

Amperometric detection of NO release

Once the NO-NPs are exposed to an aqueous environment, the hydrogel properties of the composite allow for the opening of water channels inside the particles, facilitating the release of the trapped NO over extended periods of time. Amperometric detection of this released NO was achieved using the Apollo 4000 Nitric Oxide Detector (World Precision Instruments, Sarasota, Florida), as previously reported.[20,22]

Wounding procedure

All procedures for animal experimentation were approved by the Institutional Animal Care and Use Committee at the Albert Einstein College of Medicine (Bronx, New York). Fifteen female NOD.SCID/NCr mice (6–8 weeks; National Cancer Institute, Frederick, Maryland) were divided into three treatment groups (NPs, NO-NPs, and DETA NONOate) comprising four animals each, and three mice were used as wounded untreated controls. NOD mice typically develop diabetes after 40 weeks of age[23]; therefore, at the time of wounding, glucosuria and serum glucose levels were not monitored. On day 0, the hair on the back of each mouse was shaved and the skin disinfected with ethanol. A rubber washer (1/2-inch Flat Washers; Danco, Dallas, Texas) was then secured to the back of each mouse by cyanoacrylate (The Gorilla Glue Company, Cincinnati, Ohio). In contrast to wound healing in humans, the primary healing response in mice involves wound contracture; therefore, rubber washers were used to minimize contraction and simulate human skin healing.[24] Single punch biopsies (5 mm Punch Biopsy; Miltex, York, Pennsylvania) were performed within the center of the washer on day 0, resulting in 5-mm diameter, full-thickness excision wounds. Diet was not restricted during wounding or subsequent treatment.

NO Treatment

NO is a known inhibitor of platelet aggregation[1,25]; therefore, treatment was begun on post-wounding day 1 to provide adequate time for hemostasis and fibrin clot formation. Five milligrams of NPs, 5 mg NO-NPs, or 7 mg of DETA NONOate powder were topically applied to wounds on post-wounding days 1, 3, 5, 7, 9, and 11. These treatments were chosen because 5 mg NO-NPs and 7 mg DETA NONOate release approximately similar concentrations of NO. It was determined that there is

~5.9 mg of sodium nitrite per 5 mg of NO-NPs (the dose used). Knowing that the structure of the particles allows for long-range thermally induced protonation, it is believed that all nitrite is reduced to NO via the following equation:

$$2NO_2^- + 4H^+ \rightarrow 2NO + 2H_20$$

Based on this conversion, 0.0008557 mol NO should be generated. DETA-NONOate liberates 2 mol of NO per 1 mol of parent compound (package insert; Cayman Chemical, Ann Arbor, Michigan). The molecular weight of DETA NONOate is 163.6 g/mol, and therefore 7 mg = 0.00004279 mol of parent compound = 0.0008557 mol NO released.

After application, powders were moistened with 10 μL phosphate-buffered saline solution. Control mice were not treated. Photographs of the wounds were taken on treatment days to follow gross visual wound healing as assessed by the area of wound uncovered by the migrating epithelium. Wounds were also measured using a dial caliper by two treatment-blinded (NPs vs. NO-NPs vs. DETA NONOate) investigators. On day 13 after wounding, skin lesion tissue was excised and histological sections were obtained for analysis.

Histological processing of wound area

Tissue from skin lesions was fixed in 10% formalin for 24 hours, processed, and embedded in paraffin. Full wound areas were excised from two animals in each experimental arm. Multiple levels (three recuts per tissue block) of the tissue block were examined. Four-micron vertical sections were fixed to glass slides and stained with hematoxylin and eosin (H&E), Masson's Trichrome, and CD34 to observe morphology, collagen deposition, and angiogenesis (microvessels), respectively. The slides were examined using light microscopy, and images were digitally captured without further processing. Slides were numbered without indication of cohort to blind the histological interpretation. Collagen deposition was measured by intensity using ImageJ software (National Institutes of Health, Bethesda, Maryland). Twenty high-power fields (HPFs; 40×) were evaluated per section.

Statistical analysis

All data were subjected to statistical analysis using GraphPad Prism 5.0 (GraphPad Software, La Jolla, California). *P* values were calculated by analysis of variance and were adjusted by use of the Bonferroni correction. *P* values of <0.05 were considered statistically significant.

Results

Detection of NO release using amperometric analysis

Amperometric analysis revealed immediate NO release after the NO-NPs were added to solution. A 1 mg/mL solution of the formulated particles resulted in a steadily increasing rate of NO release (10 minutes = ~70 nM/min; 100 minutes = ~180 nM/min; 200 minutes = ~215 nM/min). A steady-state level of ~245 nM/min was achieved after 5 hours, and was maintained for ~24 hours (data not shown).

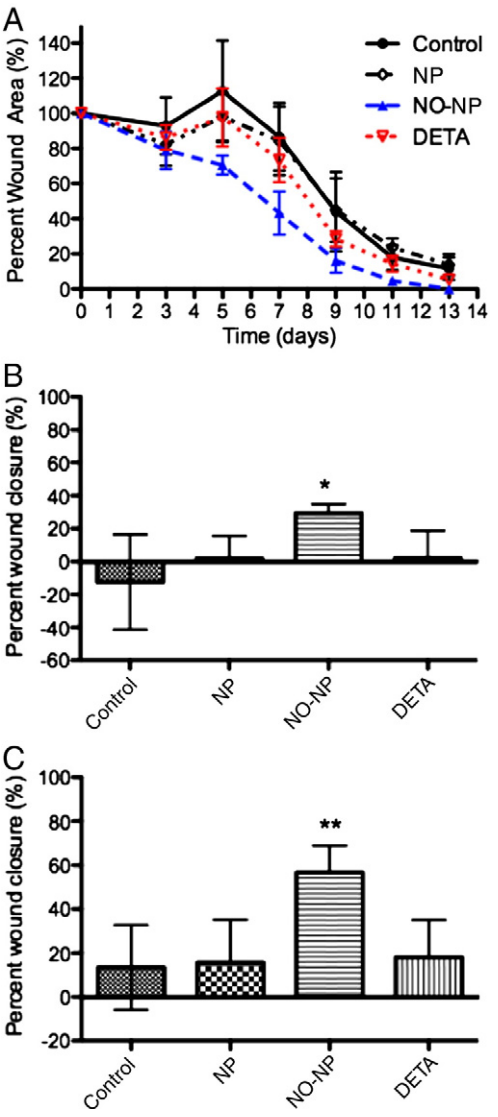

Figure 1. NO-NPs accelerate wound closure. Clinical evaluation of wound in control animals and in animals treated with NPs, NO-NPs, and DETA NONOate. **(A)** Percentage of initial wound area on 3, 5, 7, 9, 11, and 13 days after wounding (mean ± SD). **(B)** Percentage wound closure on day 5 after wounding (mean ± SD). **(C)** Percentage wound closure on day 7 after wounding (mean ± SD). Asterisks denote *P* value significance comparing NO-NPs to control (*$P < 0.01$; **$P < 0.05$).

NO increased wound closure

The effect of NO-NPs, DETA NONOate, control NPs, and no treatment on wound healing in NOD-SCID mice was evaluated. Topical application of NO-NPs onto wound beds decreased the size of eschar considerably as compared to the other treatment groups (Figures 1 and 2). At day 5, the wound area of mice treated with NO-NPs decreased by 29.4% relative to day 0, compared to 1.9% in mice treated with NPs and 2.3% in DETA NONOate–treated mice (Figure 1, *A*; Figure 2). In contrast,

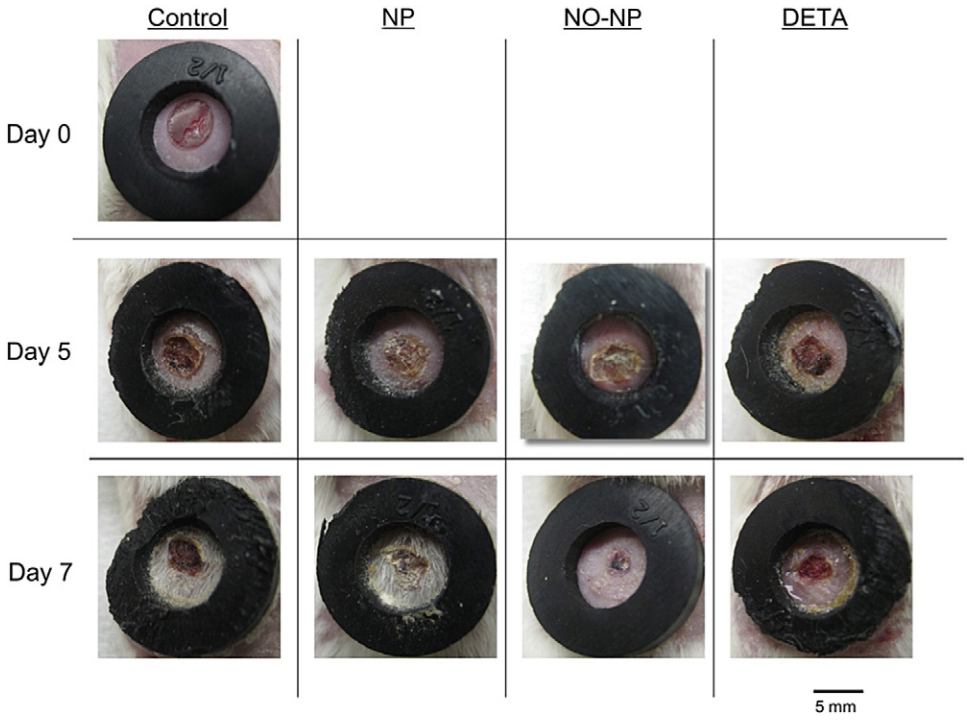

Figure 2. NO-NPs treatment macroscopically accelerates wound closure. Photographs of wounds in control, NP, NO-NP, and DETA NONOate animals were taken on days 0, 1, 3, 5, 7, 9, 11, and 13. Days 0, 5, and 7 are presented. Scale bar, 5 mm.

wound area at day 5 increased by 12.5% in control mice ($P < 0.01$; as compared with groups treated with NO-NPs). At day 7, mice treated with NO-NPs had achieved 56.7% wound closure relative to day 0, whereas there was ~15% closure in control and NP-treated ($P < 0.05$), and 27% in DETA NONOate–treated mice (Figure 1, *B*; Figure 2). At day 9, NO-NPs treatment resulted in 84% wound closure relative to day 0, while closures of 71.6% in DETA NONOate and ~55.5% in NP and control groups were noted. On day 13, total wound closure was only appreciated in mice treated with NO-NPs. No clinically significant weight loss or evidence of infection in any mouse was appreciated throughout the study period.

Histology

Granulation tissue, characterized by neoangiogenesis and proliferating fibroblasts, was present in all groups on day 13 following wounding. Figure 3 shows micrographs of granulation tissue on day 13 in the no-treatment control group, and in the groups treated with NPs, NO-NPs, and DETA NONOate. Reductions in the number of inflammatory cells and an increase in the number of fibroblast-like cells in both superficial and deep areas of granulation tissue in the NO-NPs group were visualized, and these alterations were significantly different from the control and other treatment groups. Fibroblast cells in the NO-NPs group were mainly fusiform and orientated parallel to the surface. The dermis was difficult to appreciate in the DETA NONOate–treated wounds, as there were extensive inflammatory infiltrates and fibrinous debris present. Minimal granulation tissue was appreciated in the wound bed of the DETA NONOate–treated mice, along with hyalinized necrosis noted in the subcutis. In light of these findings, collagen intensity and CD34 staining were not evaluated in the DETA NONOate mice, as the disorganized granulation tissue in this treatment arm would not be an adequate comparison to the other groups.

Figure 4 shows the histological features of the collagen fibers in the groups. Masson's Trichrome staining demonstrated a significant increase in the arbitrary collagen staining intensity in the mice treated with NO-NPs as compared to other groups (∗*P* value <0.0004 as compared to control and NP groups). In the group treated with NO-NPs, immature collagen fibers (thin, dark-blue collagen fibers) were arranged mainly parallel to the epidermis, and more organized mature fibers were prevalent in deep areas. In contrast, thin green-blue collagen fibers, orientated in some regions perpendicularly to the surface, were observed in both superficial and deep areas of the granulation tissue in the control group and NP groups.

New-vessel formation, a hallmark of the proliferative phase of wound healing, was evaluated using CD34 staining (Figure 5). Multiple small vessels were visualized in the group treated with NO-NPs (Figure 5, *C*). There was significantly more neovascularization in the mice treated with NO-NPs as compared to controls (*P* value <0.0001) and the NP groups ($P = 0.0031$), as determined by number of stained microvessels per HPF (40×; 20 fields) (Figure 5, *D*).

Discussion

Chronic wounds, caused by diabetes and other immunocompromised states, impose extraordinary economic and societal burdens on health-care systems worldwide. In the United States

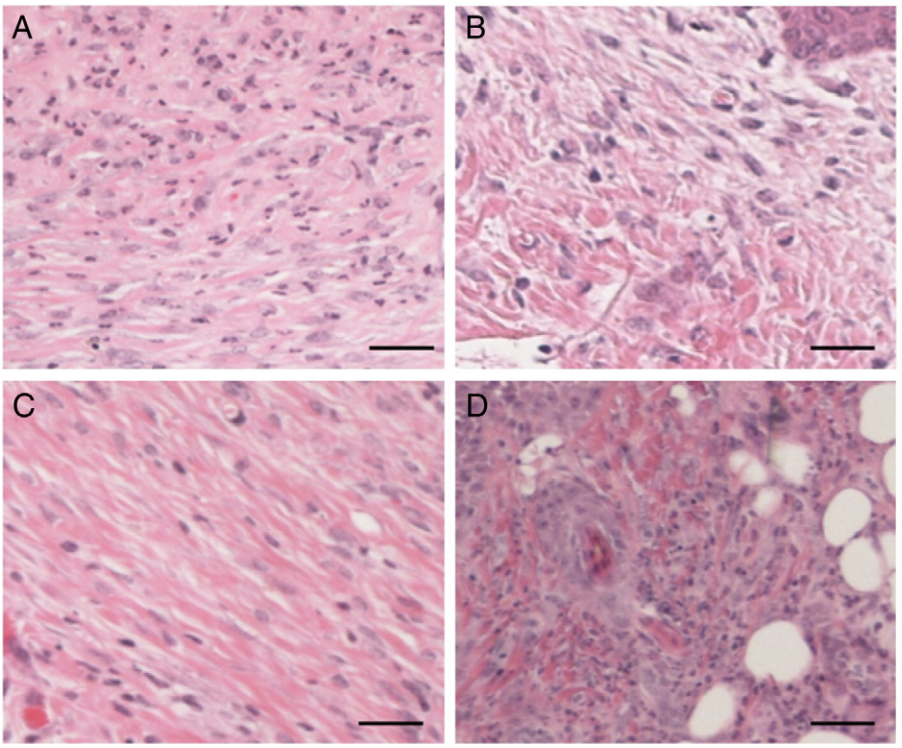

Figure 3. NO-NPs enhance fibroplasia and formation of granulation tissue. Granulation tissue in **(A)** control-, **(B)** NPs-, **(C)** NO-NPs-, and **(D)** DETA NONOate–treated groups 13 days after wounding. Control, NPs, and DETA NONOate groups present a high amount of inflammatory cells in granulation tissue **(A**, **B**, **D)**; in the group treated with NO-NPs, fibroblastic cells predominate **(C)**. H&E, magnification 20×; scale bar, 50 μm.

alone, 4 to 6 million people are affected by chronic wounds each year, consuming over $25 billion dollars of health-care spending.[26,27] These startling statistics and the health-care risks inherent in the setting of this chronic disease serve as the driving force behind the development of novel wound therapies. NO-releasing vehicles are promising therapeutics in this arena, as NO has been shown to play a vital role in wound healing. In this study we investigated the role of NO-NPs and DETA NONOate in the wound healing of NOD-SCID mice.

The NOD-SCID mice used in this pilot study have various impairments resulting in severe immune dysfunction. Most notably, they lack $CD4^+$ and $CD8^+$ T lymphocytes, which play a critical role in normal wound healing. Lymphocytes predictably migrate into wounds with a peak at day 7 after wounding,[9] and have been shown to regulate fibroblast replication and collagen synthesis in vitro[7,9,28,29] via stimulatory (TGF-β and lymphotoxin) and inhibitory (interferon-γ) signals. Their role in vivo is less well clarified, as wounds have been shown to heal in the absence of T lymphocytes.[7,30] Recently, it has been suggested that alterations in T-lymphocyte subpopulations dictate the healing process.[7,10] For instance, whereas animals depleted of $CD4^+$ lymphocytes have decreased strength, resilience, and toughness of wounds, those depleted of $CD8^+$ lymphocytes showed significant increases in these parameters. Depletion of both $CD4^+$ and $CD8^+$ lymphocytes resulted in wounds with intermediate properties, and is expected in SCID mice, as they lack both subpopulations of lymphocytes.[7] Therefore, wound healing, and specifically collagen synthesis, are believed to be regulated by a delicate balance of both stimulatory and inhibitory factors released by $CD4^+$ and $CD8^+$ cells.

In addition to T-lymphocyte involvement, B lymphocytes have recently been demonstrated to play a critical role in wound healing, as evidenced by impaired wound healing in CD19-deficient mice, a critical positive-response regulator of B cells.[31] Specifically, CD19-deficient mice displayed decreased infiltration of neutrophil and macrophages, as well as diminished cytokine expression of fibroblast growth factor, interleukin-6, platelet-derived growth factor, and TGF-β, which positively impact wound healing. Cytokine inhibition is thought to result from inhibition of Toll-like receptor 4–mediated cytokine production from B cells. In contrast, enhanced wound healing and cytokine expression was demonstrated in mice with CD19 overexpression.[31] NOD-SCID mice have deficiencies in both T and B lymphocytes. As such, wound impairments in these mice are multifactorial.

Beyond regulating cytokine expression, lymphocytes also activate important protective mechanisms to combat oxidative stress within the wound bed. In particular, they upregulate oxidoreductase thioredoxin, which maintains a reducing intracellular redox state thereby protecting migrating cells and newly formed structural components from oxidative stress. NO also has the capacity to counter oxidative stress and protect cells from damaging reactive oxygen species by scavenging superoxide,[32] even in the absence of lymphocytes. However, immunocompromised states have been associated with decreased NO generation systemically and locally within wound

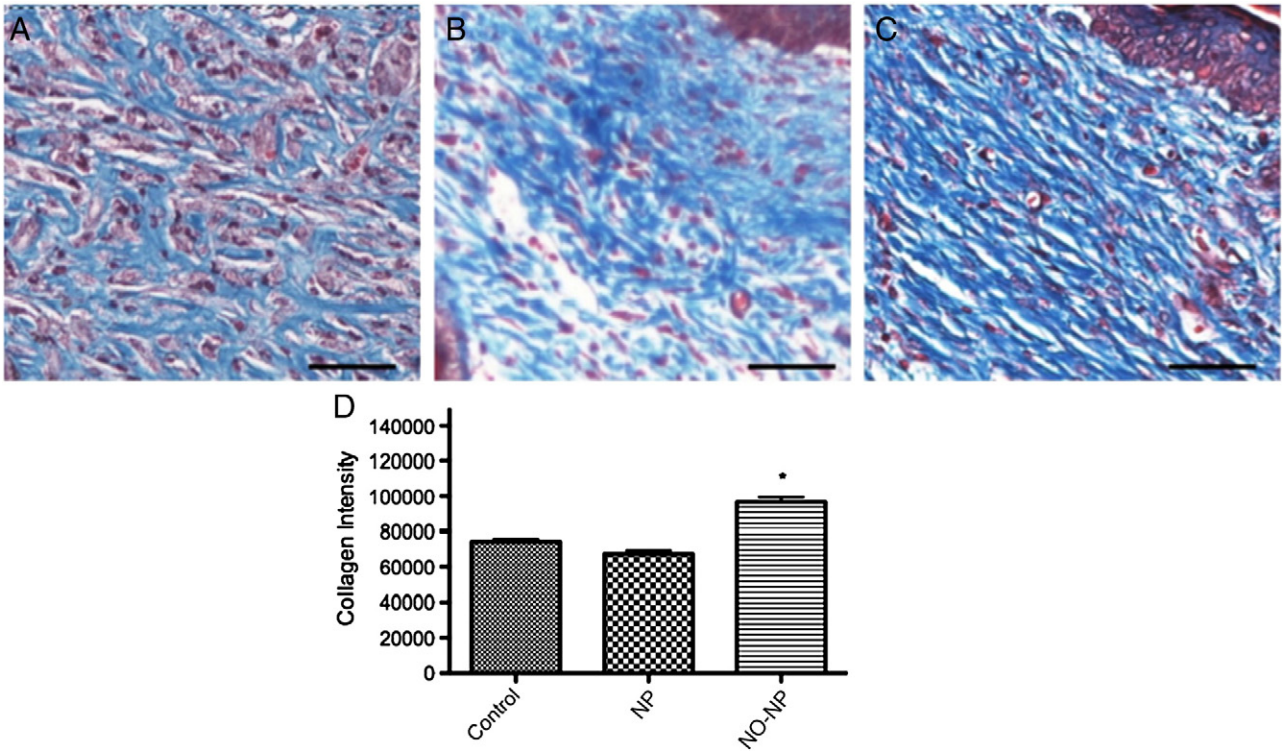

Figure 4. NO-NPs enhance collagen production. Histological tissue sections of **(A)** control, **(B)** NPs-treated, **(C)** and NO-NPs-treated wounds, and **(D)** quantitative measurement of collagen intensity in 20 representative fields of the same size for control, NPs, and NO-NPs. Bars are the averages of the results, and error bars denote standard deviations. Asterisks denote *P* value significance (*$P < 0.0004$ as compared to control and NPs). Masson's Trichrome stain; magnification 20×; scale bar, 50 μm.

tissue.[14] This is especially evident in diabetes, as diabetic animals have reduced collagen deposition, reflecting NO's role in fibroblast stimulation.[4,14] In light of these various impediments, topical application of NO-NPs to NOD-SCID mice should be expected to significantly improve wound closure. Although results were not statistically significant between NO-NPs and DETA NONOate, mice treated with NO-NPs clinically demonstrated improved healing as compared to DETA NONOate–treated mice, specifically on post-wounding days 5 and 7 (~30% difference in wound closure; Figure 1), perhaps reflecting enhanced delivery to and increased concentrations of NO in the wound bed as seen in our previous studies.[21] These results here are also supported histologically by increased intensity of collagen production on Masson's Trichrome staining, representing organized collagen deposition, and minimal inflammation in mice treated with NO-NPs as compared to the other cohorts. In addition, the collagen visualized in the DETA NONOate–treated group appeared disorganized, degenerated, and fragmented by H&E staining.

Among its many roles, NO regulates angiogenesis during the proliferative phase of wound healing. Blood vessels compose up to 60% of repair tissue in wounds, and without this newly established blood supply, metabolic requirements for wound repair cannot be met.[11] This is substantiated by reduced endothelial cell sprouting appreciated in eNOS-deficient mice, probably contributing to delayed wound healing and reduced wound tensile strength.[11] Although NOD-SCID mice have no reported impairments in eNOS expression, decreased concentrations of NO-associated immunodeficiency may also hinder angiogenesis. Consistent with previous reports on the effect of NO in promoting angiogenesis,[33] we found greater vascularization histologically in mice treated with NO-NPs than in other cohorts. NO can contribute to enhanced wound healing by recruiting angiogenesis factors such as TGF-β and VEGF to ensure adequate blood supply toward a healing wound .as suggested by increased blood vessel formation in mice treated with NO-NPs compared to the other treatment groups.

Both NO-NPs and DETA NONOate release NO, suggesting that both therapies should have positive effects on wound healing. However, there was no significant difference in wound closure among DETA NONOate–treated mice and controls, which differs from previous studies that report enhanced wound healing with topical DETA NONOate.[16,17] This may result from the small number of animals in each group reducing the power of this study; however, the histology from DETA NONOate–treated mice suggests otherwise, as there was increased inflammation, decreased and dysfunctional collagen deposition, and impaired angiogenesis compared to both NO-NPs and control groups. Clinically, DETA NONOate–treated wounds appeared more crusted and violaceous than NO-NP, NP, and control wounds, suggesting irritation from the product itself or from toxic diamine and triamine byproducts of DETA NONOate. The clinical utility of DETA NONOate has been limited in the past by the potential to form carcinogenic

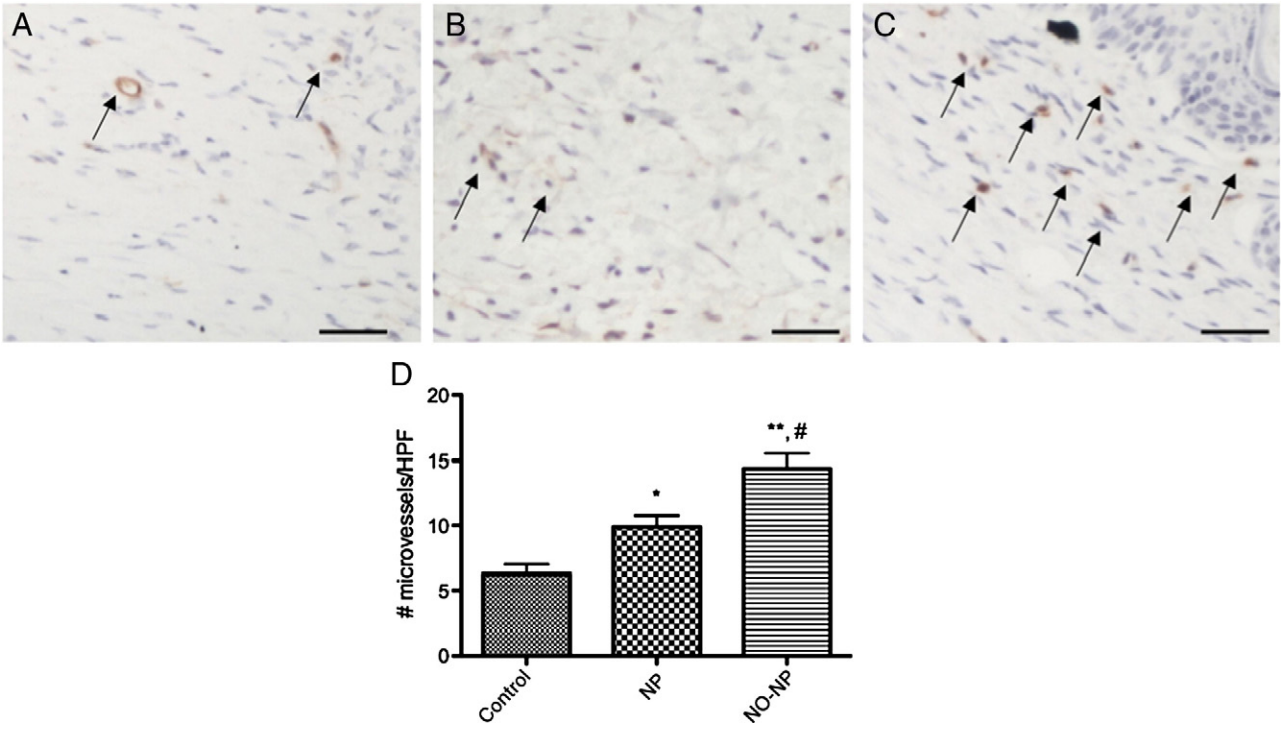

Figure 5. NO-NPs increase neoangiogenesis. Small new-vessel formation on day 13 following wounding was evaluated using CD34 immunohistochemistry. **(A)** Control group tissue demonstrated a small amount of small vessels, with more vessels appreciated in NP controls **(B)**. **(C)** Numerous small vessels were visualized in the wounds treated with NO-NPs (magnification 20×; scale bar, 50 μm). **(D)** Quantitative measurement of microvessels in 20 representative HPFs of the same size (magnification 40×). Bars are the averages of the results, and error bars denote standard deviations. Asterisks denote *P* value significance (**P* = 0.0023 in comparing NPs to controls; ***P* < 0.0001 in comparing NO-NPs to controls, $^{\#}P$ = 0.0031 in comparing NO-NPs to NPs); calculated by analysis of variance and adjusted by use of the Bonferroni correction.

nitrosamines.[3] For example, O-alkenyl/O-alkyl derivatives of DETA NONOate (V-PYRRO/NO) developed to target NO release in liver cells can be converted to *N*-nitrosopyrrolidine, a potent hepatocarcinogen.[34] To date, we have not encountered toxicity with topical or systemic application of NO-NPs.[20,35,36] In the current study, this is reflected both clinically and histologically, as wounds treated with NO-NPs demonstrated well-organized and vessel-rich granulation tissue and less inflammation compared to the other groups.

There are several limitations to this pilot study. Most notably, the small number of subjects in each treatment group may have influenced our results. However, these results still reflect the important role of NO in wound healing, as both NO-NPs and DETA NONOate release NO into the wound bed and resulted in improved wound closure. Nonetheless, repeating this study using a larger number of animals will allow us to substantiate our results, and we anticipate the superiority of NO-NPs vs. DETA NONOate to be further highlighted, reflecting the nontoxic and predictable release of NO over a sustained period of time. Another limitation was the use of rubber washers to prevent healing by wound contraction. Although washers were checked daily, on occasion they were found to have fallen off the dorsal surface of the mice, potentially allowing wound closure by contraction. In the future, washers would need to be more securely applied to mice, perhaps with sutures, or with Tegaderm (3M, St Paul, Minnesota), as recently reported.[37]

In this study we demonstrated that NO-NPs have profound wound-healing properties in NOD-SCID mice. This is evidenced clinically by accelerated wound closure and histologically by reduced inflammatory cell infiltration and increased fibroblast cells, collagen deposition, and neovascularization in mice treated with NO-NPs. These results suggest that this NO-releasing platform has the potential to serve as a novel topical wound-healing therapy in chronic wounds, including in settings of impaired wound healing caused by immunocompromised states.

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