Effects of nitric oxide releasing poly(vinyl alcohol) hydrogel dressings on dermal wound healing in diabetic mice

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Healing of chronic wounds such as diabetic foot ulcers is a significant clinical problem. Methods of accelerating healing in these difficult lower extremity sites include use of growth factor–loaded gels, hyperbaric oxygen, grafts, and artificial skin replacements. Nitric oxide (NO) has been proposed as a possible active agent for enhancing wound healing. This study examines the in vitro and in vivo responses to a novel hydrogel that produces therapeutic levels of NO. A hydrogel wound dressing was fabricated using ultraviolet light-initiated polymerization from poly(vinyl alcohol) with a NO donor covalently coupled to the polymer backbone. NO release from the NO-modified hydrogel was shown to occur over a time period of up to 48 hours, and there was no associated decrease in fibroblast growth or viability in vitro associated with NO hydrogels. Fibroblasts in culture with NO hydrogels had an increased production of extracellular matrix compared with cells cultured without the NO hydrogels. Preliminary animal studies in a diabetic mouse, impaired wound healing model were conducted comparing low (0.5 mM) and high (5 mM) doses of NO. Time to complete closure was similar in control wounds and NO-treated wounds; however, at 8 days control wounds were significantly smaller than NO-treated wounds. By days 10 to 13 this delay was no longer apparent. Granulation tissue thickness within the wounds at days 8 and 15 and scar tissue thickness after wound closure were increased in animals exposed to higher dose NO hydrogels. The results of this study suggest that exogenous NO released from a hydrogel wound dressing has potential to modulate wound healing. (WOUND REP REG 2002;10:286–294)

Chronic wounds such as pressure ulcers, diabetic foot ulcers, and venous ulcers make up approximately 70% of all skin wounds. Chronic wounds like these incur a huge cost and impair quality of life for millions of people. Diabetic foot ulcers alone account for 800,000 chronic wounds in the United States each year, with annual treatment costs of more than $1 billion. Additionally, approximately one in every five diabetic foot ulcers ultimately leads to amputation. A wound is considered chronic or nonhealing if it does not heal in an orderly or timely sequence, or if the healing
process does not result in structural integrity. Wounds that heal improperly may not possess the necessary mechanical integrity to remain healed.

Wound healing is a complex process that involves interaction between a number of cell types, extracellular matrix (ECM) molecules, and growth factors. Recent evidence suggests that nitric oxide (NO) may play a critical role in wound healing, and several studies have suggested an association between decreased NO production and delayed wound healing. The impaired wound healing common in people with diabetes has also been associated with reduced wound NO synthesis. The importance of NO in wound healing has been clearly shown using inducible nitric oxide synthase (iNOS) knockout mice. Studies using iNOS knockout mice showed that delayed cutaneous wound closure occurred following skin injury, and that this effect was reversed by the delivery of an adenoviral vector expressing the iNOS gene at the wound site. Examination of the influence of arginine dietary supplements has also suggested that their beneficial effects on wound healing are lost in mice without the iNOS gene.

Expression of NO by macrophages and other cell types has been shown to be important in regulation of their angiogenic activity, specifically by enhancing the expression of vascular endothelial growth factor (VEGF). Additionally, the importance of NO in wound angiogenesis has been shown by examining the impaired wound healing in endothelial nitric oxide synthase (eNOS)–deficient mice. The impaired wound healing process does not result in structural integrity. Wounds that heal improperly may not possess the necessary mechanical integrity to remain healed.

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The well-known antimicrobial and vasodilatory actions of NO may also be important in the process of wound healing, particularly because vasodilation increases blood flow in the microvasculature, thus facilitating the delivery of both nutrients and cells to the site of injury. During the early postinjury stages, NO may act to prevent infection, thereby also preventing excess inflammation.

Numerous compounds that spontaneously produce NO under physiologic conditions (NO donors) have been identified. These exogenously applied NO donors mimic the normal biological functions of endogenous NO. Several classes of NO donor molecules exist, including S-nitrosothiols and NO-nucleophile complexes (NONOates or diazeniumdiolates). Additionally, direct generation of NO via chemical reaction between sodium nitrite and ascorbic acid can stimulate vasodilation in healthy skin when applied through a gas permeable membrane. However the duration of NO release and the dose control was limited in this system.

Materials that are suitable for prolonged, localized NO release at the site of a dermal wound have not yet been developed. A linear polyethyleneimine (PEI)-nitric oxide (-NO) donor that was applied to full-thickness wounds in aged rats did not favorably affect the wound healing process. Wounds to which PEI or PEI-NO were applied experienced impaired wound healing when compared to untreated wounds, indicating that PEI itself may contribute to dermal toxicity and failure to enhance wound healing. Sustained delivery of an elevated and prolonged dose of NO well beyond physiological levels also resulted in increased inflammation in the PEI-NO treated wounds.

Poly(vinyl alcohol) (PVA) is a polymer containing many pendant hydroxyl groups to which a nitric oxide donor may be attached. It can be crosslinked by a variety of mechanisms to form hydrogels that are easily applied to wounds. Hydrogels are known from previous studies to enhance moist wound healing. The goal of this study was to develop and test the efficacy of a NO-releasing hydrogel wound dressing. Specifically, this study aimed to synthesize NO donors from PVA derivatives that release physiologically relevant levels of NO and to test the effect of the PVA-NO hydrogels on wound healing in a genetically diabetic mouse (db/db) model that exhibits impaired wound healing.

**MATERIALS AND METHODS**

PVA (Mowiol 4-88) (Hoechst AG, Frankfurt, Germany) was modified with amine groups and crosslinkers via reaction with an amine acetal (aminobutyraldehyde diethyl acetal: 0.067 g/g Mowiol 4–88) and a crosslinkable acetal (methacrylamidoacetaldehyde dimethyl acetal: 0.13 g/g Mowiol 4–88) in the presence of glacial acetic acid and 37% hydrochloric acid for 20 hours at room temperature (modified from). The solution was then adjusted to pH 3.6 using triethylamine and precipitated in acetonitrile in a ratio of 1 : 10. Excess salts were removed through dialysis. The percent substitution of pendant groups by amine groups was from 1% to 3% and by acrylamide crosslinkers from 2% to 12%.

Amine groups were then modified to form diazeniumdiolates by dissolving the precipitated material in water and reacting with NO gas (1% in nitrogen) for approximately 24 hours. The pH of the PVA solution during this process was approximately 7.4. NO donor modification of aminated PVA ranged from 55% to 80% of...
the available amine groups (as measured by the ninhydrin assay described below). Different concentrations of NO were achieved by blending PVA-NO with aminated PVA that had not been reacted with NO. Blends using ratios of PVA-NO to PVA that had not been reacted with NO varied according to the conversion of amines to NO donors, but were approximately 1:20 to produce 0.5 mM NO hydrogels and 1:2 to produce 5 mM NO hydrogels, where molarity refers to the theoretical maximum yield of NO from a unit volume of polymer.

Uncrosslinked polymer was sterilized by filtration though a 0.45-μm filter and photocrosslinked in sterile molds (polypropylene 15 mm diameter × 0.2 mm thick) before packaging. Photocrosslinked hydrogels were formed from 20% (w/v) PVA-NO by adding 0.1% Irgacure initiator (CIBA, Basel, Switzerland) and then exposing to ultraviolet light (peaks at 310 and 365 nm, 2 mW/cm²) for 20 seconds. Hydrogels were stored frozen at −20 °C and used within 6 weeks from the time of preparation.

Analysis of NO donor incorporation
To determine the amount of NO donor incorporation that was achieved, the PVA-NO–modified polymers were tested for percent of amine groups reacted to form NO-nucleophile complexes using the ninhydrin assay. Unknown samples were mixed with sodium citrate buffer and ninhydrin reagent (Sigma-Aldrich, St. Louis, MO) was added. Tubes were boiled for 15 minutes and allowed to cool to room temperature before reading the absorbance at 570 nm. Calculation of the amine concentration before and after reaction of aminated polymer with NO gas was conducted based on a standard curve constructed using appropriate l-leucine standards. The ninhydrin assay detects the concentration of amines either on a surface or in a solution, and can thus be used to indicate how many moles of NO are present per gram of polymer. The ninhydrin assay was performed on aminated PVA both before and after reaction with NO and a decrease in the amine concentration after NO reaction was assumed to correspond with successful modification of the amine groups to form NO donors. NO donor concentration was calculated as the difference in amine concentration before and after the NO reaction. This method has been validated previously in the authors’ laboratories for various polymer systems and has been found to reflect the degree of amine modification.

Characterization of release kinetics
Hydrogels (following photocrosslinking) were incubated with stirring at 37 °C in pH 7.4 HEPES buffered saline solution. HEPES buffer volume was greater than 20-fold that of the hydrogel “volume.” At designated time points up to 48 hours, the buffer was removed and replaced with fresh buffer. Buffer removed was analyzed for nitrite content using the Griess reaction and the pH tested. Briefly, solution samples were mixed with freshly prepared Griess reagent and allowed to react for 15 minutes. The absorbance was read at 540 nm and calculation of nitrite in the unknown was conducted using a standard curve constructed from NaNO₂ standards. The nitrite concentration in the buffer is directly related to how much NO has been released from the hydrogel. Nitrite alone was measured based on previous studies indicating that in the absence of heme-iron–containing compounds, most NO in solution forms nitrates and does not further oxidize to form nitrates. Use of nitrite in solution as an accurate measure of NO release is widely accepted. The amount of NO released from the hydrogels (cumulative μmoles NO released/g of polymer) was reported as percent released compared to the theoretical yield of NO from the polymer.

To evaluate possible effects of storage conditions and the effect of pH that may be relevant to the wound environment, release of NO was also measured following storage at 20 °C and at pH 6, which is closer to wound pH. Controls included PVA without amine modification and PVA with amine modification but without exposure to NO gas. Previous studies using NO-nucleophile complexes with amine-modified poly(ethylene glycol) showed that NO release was almost completely inhibited at pH 3.

Effects of hydrogels on fibroblast cultures
The effects of NO release from PVA-NO hydrogels on the growth and viability of human dermal fibroblasts (HDFs) were investigated in vitro. Cells were cultured on tissue culture polystyrene (TCPs) in the presence of a range of concentrations of NO-releasing hydrogels from 0.5 mM to 5 mM, which were suspended in the cell culture media 24 hours following cell seeding. After 4 days of culture, the cells were counted using a Coulter counter to assess the effect of NO release on cell growth. PVA-NO hydrogels prepared for the animal studies were assessed for their effect on fibroblast viability as outlined above. Viability at 4 days was assessed using trypan blue dye exclusion.

ECM production was assessed in cell culture through incorporation of ³H-glycine into glycoprotein, elastin, and collagen portions of the ECM as determined by sequential enzyme digestion. HDFs were seeded on TCPs, and PVA-NO hydrogels were formed in transwell inserts and added to the cell culture media 24 hours following cell seeding. The media was supplemented with 1μCi/ml ³H-glycine (Sigma-Aldrich, St. Louis, MO) at this time. The same procedure was followed for cells intended for counting, except that the media was not supplemented with
Two days following the addition of the hydrogels, the cells in nonradioactive plates were trypsinized and counted on a Coulter counter. The cells on the remaining plates were lysed in a solution of 25 mM ammonium hydroxide (Sigma-Aldrich, St. Louis, MO) for 30 minutes, and the plate was then dehydrated. A sequential digestion of ECM was performed by incubating the plates with 20 μg/ml trypsin for 4 hours, 58 U/ml elastase for 4 hours, and 76 U/ml collagenase for 8 hours, all at 37 °C in order to digest glycoproteins, elastin, and collagen, respectively. All enzyme solutions were made in digestion buffer, which is composed of 25 mM Tris-HCl and 5 mM calcium chloride, pH 8. The last step was the removal of any remaining labeled proteins with 1 N NaOH for 1 hour at room temperature. Radioactivity in samples from each digestion step was determined by scintigraphy and results were expressed as counts per minute (cpm)/cell.

Using the results from in vitro studies, as well as data from the literature, the doses of NO selected for in vivo studies were set at 0.5 and 5 mM.

**Effects of hydrogels on wound healing in vivo**

The animal model used for in vivo testing of the PVA-NO hydrogels was a full-thickness wound in the dorsal skin of genetically diabetic (C57BLK/J-m+/+Leprdb) (db/db) female mice, 8 weeks of age (Jackson Laboratories, Bar Harbor, ME). A total of 21 mice were used with equal numbers being assigned to each of three groups (two test groups and one control group). Test groups had either 0.5 mM or 5 mM hydrogels applied and the control group was dressed with PVA hydrogel without NO. At time = 0 days, a full-thickness, circular 15 mm diameter wound was created. Mice were anesthetized by isoflurane inhalation and the dorsal skin prepared for surgery using Betadine and 70% isopropanol. The wound was created using surgical scissors. At time = 0 days hydrogel dressings were placed, as prepared, directly on the wounds. As wound size decreased, a circular piece of sterile PVA-NO or control hydrogel was cut to match the size of the wounds using a sterile circular cork borer, and applied to the wound. Hydrogel dressings were transparent, soft, and flexible enough to fit to wound contours without mechanically interfering with the wound. The wounds with hydrogel dressings in place were covered with a transparent secondary dressing (Tegaderm, 3M, St. Paul, MN) adhered to the area surrounding the wound using tincture of Benzoin.

Every 2–3 days following surgery, wounds were redressed with fresh control or NO-releasing hydrogel dressings while the mice were under isoflurane inhalation anesthesia. The secondary dressing and the hydrogel were removed and the wounds were flushed with sterile saline to remove debris and to clean the wound area. A digital planimetric image of the wound was recorded using a Pixera video camera and stored on a PC using JPEG format. A calibration scale was recorded with each image. Once photographed, fresh dressings were placed on the wounds, and the wounds were covered again with fresh Tegaderm.

Wound areas were assessed by image analysis using ImagePro Plus 3.0 image analysis software. Using this software, the perimeters of the open wounds were defined and measured and the open wound area determined. Means and standard deviations of wound perimeters and areas at each time point were calculated.

For histological analyses, one animal from each group was euthanized at each time point of 8, 15, and 22 days and the final animals from each group were euthanized at 29 days. Wounds were excised at the time of euthanasia with about 2 mm of tissue surrounding the wound, fixed in Streck tissue fixative and embedded in paraffin. The specimens were oriented to provide cross-sections of the full width of the wounds. Sections from each wound were stained with hematoxylin and eosin and with Masson’s Trichrome stains. Granulation tissue thickness was measured at days 8 and 15 and scar tissue thickness was measured in sections from the final time point (29 days). Tissue thickness was measured using ImagePro Plus 3.0 image analysis software on images captured using an Olympus BX50WI microscope and SONY DKS 5000 camera.

Control of bias was achieved by assigning a color code to each of the test groups and the control group. Investigators were blinded to the identity of each of the groups and the test and control hydrogels have a similar appearance. The code was broken following completion of the final 4-week analysis. All animal experimentation was conducted under appropriate procedures approved by the UMDNJ IACUC review committee.

**Statistical analysis**

Where appropriate, data are expressed as mean values with their associated standard deviations. Statistical analysis of data was conducted using unpaired two-tailed t-tests, assuming equal variances, unless otherwise stated.

**RESULTS**

Release of NO from PVA-NO hydrogels was observed over a period of 48 hours at pH 7.4, as determined by the Griess assay. Because a slightly acidic pH is often observed in the wound environment, NO release from these hydrogels at pH 6 was also evaluated. Figure 1 shows the percent release over time at pH 7.4 and pH 6. No inhibition of NO release was observed when hydrogels were exposed to...
slightly acidic conditions. The difference between release at pH 7.4 and pH 6 was smaller than expected based on the work of Davies et al.\(^23\) who noted that NO release from diazeniumdiolates can vary dramatically with pH as well as with the structure of the compound. However, extensive kinetic studies to determine the pH dependence of the decomposition of the diazeniumdiolate were not the subject of this paper. The major reason for testing release at different pH was to determine the likelihood of inhibition of NO release in the presence of wound fluid, and further studies are required to determine whether this finding relates to the chemical structure of the diazeniumdiolate or other variables.

Hydrogels were tailored to obtain a range of NO concentrations by blending with aminated PVA that had not been reacted with NO prior to polymerization, allowing the preparation of both 0.5 mM and 5 mM NO-loaded hydrogels. The total amount of NO released at pH 7.4 and ~48 hours was 0.055 ± 0.002 μmoles for 0.5 mM NO hydrogels and for 5 mM NO hydrogels was 0.48 ± 0.05 μmoles. This translates to a release rate of approximately 0.33 pmol/second and 2.8 pmol/second for 0.5 and 5 mM NO hydrogels, respectively.

Control hydrogels for release kinetics studies consisted of aminated PVA that had not been exposed to NO, as well as unmodified PVA that had been exposed to NO but did not contain any reactive groups with which to form NO donors. No significant NO release was observed with either control group.

Effects of hydrogels on fibroblast cultures

Exposure of HDFs to a range of concentrations up to 5 mM of PVA-NO hydrogels did not affect cell growth, as shown in Figure 2. There was also no change in cell viability, as cells in all conditions remained > 90% viable, even at the highest NO concentration of 5 mM. Based on this finding, the concentrations selected for further cell studies and for the animal study were 0.5 mM and 5 mM NO hydrogels.

Analysis of ECM synthesis by HDFs cultured in the presence of PVA-NO hydrogels for 2 days displayed a significant increase in collagen production with increasing NO concentration (\(p < 0.01\)). Collagen production and total matrix production in control and 5 mM NO hydrogels are shown in Figures 3 and 4. Collagen production, expressed as cpm/cell, was corrected to account for the total amount of proteins produced. When expressed as a percentage of total matrix proteins (glycoproteins, collagen, and elastin), collagen accounted for approximately 80% of total matrix in the case of 5 mM NO hydrogels and approximately 40% of the total matrix for control hydrogels. There was also a slight increase in overall matrix production by cells exposed to 5 mM PVA-NO, although this difference was not significant.

Effects of hydrogels on wound healing in vivo

Wound area and perimeter were similar in test and control groups; however, there was a tendency for more rapid closure in the control group, particularly at day 8 where the difference in wound area and perimeter was most
pronounced. Figure 5 shows the wound area over time. Time to complete closure was similar in both control and NO-releasing hydrogels; however, there were some differences in wound area noted at day 8. In control wounds, wound area began to decrease at day 6 and complete wound closure first occurred by day 15 (one out of six mice). By day 22, control wounds were essentially closed (Figure 5). Wound area began to decrease slightly later at day 8 in the 0.5 and 5 mM PVA-NO groups. By day 24, wounds in the 0.5 and 5 mM were essentially closed.

Granulation tissue, characterized by proliferating fibroblasts and newly formed microvasculature, was present in the open wound at days 8 and 15 in all groups. Figure 6 shows a graph of the granulation tissue thickness comparing the test and control groups. The results reflect the mean thickness and standard deviation of three measurements taken on each of two serial sections (total of six measurements) within the open wounds. The three measurements were at a central point and 0.5 mm on either side of this point. Granulation tissue tended to be thicker with increasing NO concentration; however, this difference was not significant. Figure 7 (A–D) shows photomicrographs of granulation tissue at days 8 and 15 in the control group and the 5 mM PVA-NO group in the region adjacent to the edge of the wound where epithelial migration was occurring. There was no significant difference between test and control groups in the overall cellular composition of the healing tissue.

Scar tissue was assessed in histology sections at day 29 in the control and 5 mM groups only because the wound in the 0.5 mM animal was not completely closed. Scar tissue was measured in a manner similar to granulation
tissue with a central point and two points on either side 0.5 mm of the central point. Measurements were conducted on four serial sections from each animal resulting in a total of 12 measurements. Figure 8 (A and B) shows the histological appearance of scar tissue in control and 5 mM test groups. The mean scar tissue thickness (± SD) in the control animal was significantly smaller at 0.14 ± 0.03 mm compared with 0.28 ± 0.08 mm in the 5 mM test animal \((p < 0.001)\).

**DISCUSSION**

Approaches to chronic wound healing that are common today are typically based on simple wound care regimens involving debridement, cleaning, and application of moist dressings. More advanced dressings such as topical gels containing growth factors have resulted in enhanced healing rates in some clinical studies,\textsuperscript{24,25} however, on the whole these treatments are difficult to apply and are often too expensive for application to large, chronic wounds.

The results of this study suggest that exogenous NO released from a hydrogel wound dressing has potential to enhance wound healing. However, rather than accelerating wound closure, NO in this study appears to improve the quality of the tissue in the healing wound. Chronic wounds are not only characterized by untimely healing but also by the inability to remain closed following healing, thus time to closure may not be the only relevant end point or sole basis for efficacy of the treatment. When considering wounds that do not remain healed, the formation of scar and granulation tissue may play an important role in creating a more mechanically stable wound. Similar findings have been reported in studies examining the effect of growth factors on wound healing in animals. A review of multiple animal models for assessing efficacy of platelet-derived growth factor-BB concluded that epithelialization and wound contraction were not significantly altered, whereas in most models, including the diabetic mouse model, granulation tissue thickness was consistently increased following application of the growth factor.\textsuperscript{26}

That similar improvements in wound healing appear to occur following application of either growth factors or NO is not surprising and may be attributed to their interrelated mechanisms of action. The positive NO effect has been attributed to many factors, including encouragement of fibroblast and keratinocyte migration via growth factor up-regulation,\textsuperscript{10,27} and indeed a proposed mechanism for impaired healing in diabetic patients is reduced fibroblast proliferation caused by impaired growth factor receptor expression.\textsuperscript{27} These linked growth factor-NO mechanisms may also affect other cells important in wound healing such as endothelial cells and keratinocytes, and some studies have suggested that growth of the latter cell type is modulated by NO in a manner similar to that observed in fibroblasts.\textsuperscript{28,29} This study suggests that in the range of NO doses used, fibroblast proliferation is not significantly altered. Whether there is any direct effect on keratinocytes and endothelial cells (EC) was not studied in vitro; however, in vivo results showing increased granulation tissue response suggest that EC migration and angiogenesis may be increased. Reports of the effects of NO on keratinocytes are conflicting because
inflammatory stimuli have been implicated for induction of NO production by keratinocytes via iNOS and, conversely, epidermal growth factor, which promotes the proliferation of keratinocytes and suppresses the production of NO by an unknown mechanism. Future studies will focus on the differential effects of exogenous NO on other cell types in vitro.

One of the most significant positive effects attributed to NO is increasing collagen production, which enhances the quality of granulation tissue and can increase wound strength. Although in vitro studies reported here showed that fibroblast growth rates were not increased when exposed to therapeutic levels of NO, collagen production did increase. These in vitro results translated to equivalent in vivo findings of enhanced wound granulation tissue formation and scar tissue thickness. Similarly, increased in vivo NO synthesis has been shown to enhance collagen synthesis by fibroblasts in the wound environment. Cells transfected with the iNOS gene seeded onto PVA sponges implanted subcutaneously in rats also resulted in significantly increased collagen accumulation and inhibition of NO synthesis by competitive inhibitors resulted in decreased wound mechanical strength and wound collagen accumulation.

Lee et al. observed a 38% decrease in wound tensile strength in mice deficient in eNOS when compared to wild-type controls, suggesting that collagen deposition in wounds is influenced by NO production. Inhibition of NO synthesis by competitive inhibitors such as S-methyl isothiouronium and N\(^{\text{6}}\)-L-monomethyl-arginine acetate also resulted in decreased wound mechanical strength and wound collagen accumulation.

NO clearly plays a role in the wound healing process, most probably via multiple mechanisms including increased cell proliferation via up-regulation of growth factor receptors and up-regulation of matrix synthesis. That growth factors alone also enhance wound healing suggests that combining NO with growth factors may lead to synergistic effects. In fact it appears that there may be subpopulations of diabetic patients who would not respond to growth factor application without adjunct treatment for impaired NO production. Further studies expanding the preliminary animal results in the present study are required in order to examine the effects of NO and NO–growth factor combinations on granulation tissue formation and ultimate wound strength.

As previous studies have suggested, it is not enough to simply develop a material that releases NO for enhancing wound healing. It must be ensured that the material is appropriate for wound healing and can be conveniently applied to a wound in a clinical setting. This study has produced preliminary in vitro and in vivo evidence of efficacy of a NO-releasing PVA hydrogel wound dressing.

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