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RESEARCH

A NOVEL NITRIC OXIDE-RELEASING GEL FOR DIABETIC WOUNDS

Yukun Liu[1,2], Songxue Guo[1,3], Shuyi Wei[1,4], Huan Wang[1,4], Yong Liu[1,5], Andrea V. Moscoso[1], Zina Ribkovskaia[6], Tsvetelina Lazarova[6], Steven Riesinger[6], Dennis P. Orgill[1], Mihail Climov[1,7]

[1] Division of Plastic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA; [2] Department of Plastic and Cosmetic Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; [3] Department of Plastic Surgery, Second Affiliated Hospital, Zhejiang University, College of Medicine, Hangzhou, Zhejiang, China; [4] Plastic Surgery Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Shi-Jing-Shan District, Beijing, China; [5] Department of Burn and Plastic Surgery, West China Hospital, Sichuan University, Chengdu, China; [6] MedChem Partners LLC. Lexington, MA, USA; [7] Division of Plastic Surgery, Ruby Memorial Hospital, West Virginia University, Morgantown, WV, USA

Summary

Background. Diabetic foot ulcers (DFUs) are a serious health problem. Nitric Oxide (NO) has been found to be an effective modulator in wound healing if administered externally. While NO is a promising therapy for DFUs, the current delivery of gaseous NO is cumbersome. The present work explores a novel gel that releases NO when in contact with wound exudates.

Material and methods. Efficacy of the gel was assessed using a 1 cm^2 full-thickness dorsal wound model on diabetic type 2 mice (Lepr db/db). The wounds were treated with NO-releasing gel at two concentrations: 100 μM (Gel 100) and 1000 μM (Gel 1000). A Gel-control group and an untreated group were included for comparison. Planimetry was employed for wound size dynamics up to day seven. H&E and IHC staining were used to quantify granulation tissue, CD31, and α-SMA expressions.

Results. While the current experiment found no difference in wound healing kinetics between the groups, histological evaluation showed a significant increase in granulation tissue thickness in both Gel 100 and Gel-control groups compared to the other two groups ($p < 0.05$). Furthermore, Gel 100 significantly impacted the expression of angiogenesis marker CD31 and α-SMA compared to the other groups ($p < 0.05$).

Conclusions. This preliminary study showed that the NO gel used has a dose-dependent effect on CD31, α-SMA resulting in regulation of granulation tissue formation during wound healing in diabetic murine models. While these preliminary studies show the potential of this treatment, further studies to optimize these compounds are needed.

Key words: nitric oxide, wound healing, diabetic wounds

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Correspondence

Mihail Climov
Division of Plastic Surgery, Brigham and Women's Hospital, Harvard Medical School 75 Francis St, 02115 Boston, MA, USA.
E-mail: mihailclimov@gmail.com

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INTRODUCTION

The global incidence of diabetes is estimated to reach 552 million by 2030, posing a threat to public health worldwide [1]. Diabetic foot ulcers (DFUs) are the leading cause of amputations and one of the most feared complications associated with diabetes [2]. The recurrent, nonhealing wounds put an overwhelming burden on patients, their families, and society [2]. Current management for DFUs relies on a multidisciplinary team where wound care specialists are essential [3]. Advanced wound dressing materials are widely used as adjuvant therapy for standard treatment; however, the high cost of these materials results in a significant financial burden to the patient [4]. Due to the exponential growth of DFUs, there is a critical need to develop a low cost, highly efficient treatment to accelerate healing of diabetic and other nonhealing wounds [5].

Nitric oxide (NO) is an endogenous free radical with a short half-life produced from L-arginine by nitric oxide synthases (NOSs) [6]. During the wound healing process, NO is known to promote angiogenesis, and modulate inflammatory cell proliferation and collagen deposition [7]. Compelling studies have demonstrated that biosynthesis of NO is related to the outcome of wound healing [8,9]. Of note, the peak production of endogenous NO was found to take place in the early stages of wound healing [10]. In diabetic patients, NO production is known to be negatively affected, and is partially responsible for both impaired wound healing and a decreased immune response [11,12].

Considering the importance of NO in the regulation of wound healing, investigators have developed methods for using NO clinically [13]. The early development of gaseous NO was hampered by the use of gas tanks and the danger associated with manipulating this toxic gas. Side effects of exposure to the colorless, odorless gas include cytotoxicity to healthy cells and injury to tissue [14-17]. Nitric oxide-releasing nanoparticles encountered several safety problems [18]. Alternatives such as NO hybrid drugs and acidified nitrate have been under explored before; however, they are not widely used due to a variety of shortcomings (Tab. I) [19]. Therefore, an optimal delivery method of NO with low toxicity and high efficiency would be highly desirable.

We propose a novel NO releasing compound formulated in a PVA (polyvinyl alcohol) hydrogel. The NO releasing compounds are shelf-stable small molecules capable of releasing nitric oxide upon activation by ubiquitous esterases such as those found in wound tissues. In addition to the release of NO, the compounds also release an equivalent of formaldehyde and an equivalent of diethylamine. This is a well-known degradation process for NO compounds and has been used in the synthesis of NO containing non-steroidal anti-inflammatory drugs (NSAIDS) [20]. The diethylamine, as well as its nitrosamine intermediates, have been shown to have no significant toxicity when subjected to long term toxicity studies in animals [21]. For this study, a library of NO releasing compounds was generated with different NO release kinetics. These compounds were pre-screened *in vitro* using the Griess reaction in the presence of plasma to rank the compounds. For *in vitro* experiments, compound 12, which is an analog of compound 2, was selected with consistent NO release kinetics (Tab. II). We hypothesize that by administering this gel to the wound, a controlled release of NO overtime could be achieved, thus inducing a more efficient and predictable therapeutic effect. We evaluated the selected compound 12 for its ability to release NO *in vivo* in a well-established diabetic murine model.

MATERIAL AND METHODS

Animals

Forty-eight, 12-week-old C57BL/KsJ-Lepr (db/db) (strain # 000642) male mice were obtained from The Jackson Laboratory, Bar Harbor, MI, USA). Experimental procedures were carried out according to the Guide for Care and Use of Laboratory Animals (Eighth edition, National Academy of Sciences Press, 2011). Protocols were approved by the Institutional Animal Care and Use Committees of the Brigham and Women's Hospital.

Preparation of nitric oxide releasing compounds and hydrogel

We synthesized 20 compounds using the general scheme shown in Figure 1. Our goal was to prepare a library of compounds and to select them based off of formulation ease and having the proper NO release kinetics for wound healing applications. Once prepared, the compounds were tested for NO release using the Griess reaction which uses colorimetric measurement of nitrite levels (the primary breakdown product of NO). To determine stability and NO release behavior in a biological environment, the compounds were tested for NO release in both phosphate buffered saline (PBS) and rat plasma at 37 °C. NO release was recorded at 0, 30 minutes and 3 hours. Compounds were then ranked based on their NO release behavior as shown in Table 2. Based on the ranking, structure and chemical stability, compound 12 was chosen to be formulated into the hydrogel. A polyvinyl alcohol (PVA) based hydrogel was chosen, which has been widely used for previous biomedical applications [22]. The hydrogel was prepared by mixing 30 g of PVA (MW~61,000, Aldrich Cat#10852)

Table I. Current NO delivery methods for wound healing purposes.

Types of NO delivery	Examples	Advantages	Disadvantage	Clinical or experimental result
Gaseous NO	Air-plasma generator "Plason" [38]	Antimicrobial effects [39]	Cytotoxic effects [39]	Accelerate wound healing in rat model [38]
Nitric oxide-releasing nanoparticles	Silica nanoparticles [40] Gold nanoparticles [41]	Sustained and targeted delivery of NO without the unwanted systemic side effects [42]	Safety issue associated with nanoparticles [42] Not clinically tested	Accelerates wound healing and kills bacteria in hamster, rat and pig models [43]
Nitric oxide-releasing polymers	Releasing poly(vinyl alcohol) film/F127 [13]	Controlled release and low toxicity [45] polymer solutions reduced the cytotoxicity [46]	Fast release [44]	Accelerate wound closure, enhance re-epithelialization, collagen deposition, and blood vessel formation in mouse model [44]
Diazeniumdiolates (NONO-ates*)	N-Diazeniumdiolates (N-NONO-ate) [45]	Generate NO spontaneously [46]	Challenge for targeted delivery [46]	Treats many medical disorders in animal models [47]
S-nitrosothiol	S-nitroso-L-glutathione (GSNO) [48] S-nitroso-L-cysteine (CysNO)	Longer half-life than NO [49]	The need to keep it refrigerated until mixed for application [49]	Increase dermal vasodilation, accelerate wound healing, kills infectious microorganisms, relief inflammatory pain in diabetic rat [50]
NO hybrid platform	Nitric oxide-releasing poly (lactic-co-glycolic acid)-polyethylenimine nanoparticles [50]	Slow release of NO Improve effectiveness of the drug compared to pure NO donor [49]	No details reported Not tested clinically	Accelerate wound healing and epithelialization, mediates bactericidal efficacy in mouse model [50]
Zeolites	Nitric-oxide-loaded zeolites [51]	Stable when dry, only released when exposed to moisture [52]	Fast release [53]	Improve wound healing in Zucker obese rats, antibacterial effects and inhibition of platelet aggregation *in vitro* [51-53]
Acidified nitrate	Nitrite-based nitric oxide [54]	Easily produced, quick burst of NO [53]	Inflammatory and toxic effects on the treated skin [54]	Protection against Pathogens, beneficial to chronic wound [53,54] in diabetic mice model [55]
Probiotic patch	Nitric oxide producing probiotic patch [56]	Produces concentrations of gaseous NO instead of gNO from tank [56]	It requires modifications to achieve consistent release rates [53]	Antimicrobial and antifungal efficacy *in vitro* [56] and a New Zealand white rabbit model [57]
Other	Semi-permeable [58] membrane, nitrobenzene, NO metal-nitrosyl complexes [58]	No details reported	No details reported	No details reported

*NONO-ates discussed in this table are different from the one's generated within this study

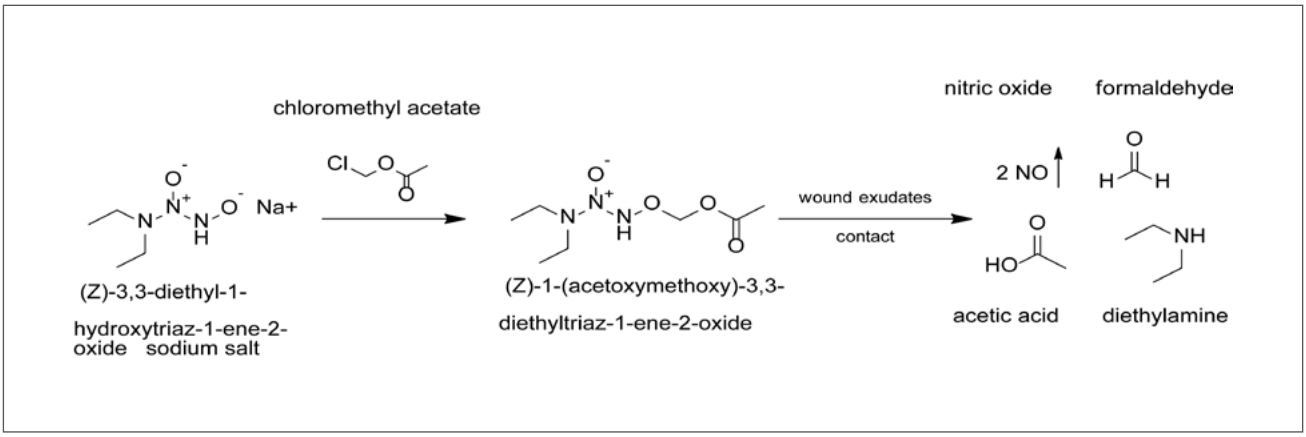

Figure 1. Synthesis of NONO-ate species. During enzymatic hydrolysis NO, formaldehyde, acetic acid and diethylamine are formed (figure provided by MedChem Partners LLC.).

Table II. Library of NO releasing compounds generated for this study. Compound #12 was chosen for the pilot experiments due to its stability and NO release kinetics. (Table provided by MedChem Partners LLC.)

Compound	Release behavior
1	Unstable
2	Slow/low release
3	Slow/low release
4	**Slow onset**
5	Steady
6	Slow/low release
7	Steady/low
8	**Slow onset**
9	**Steady**
10	Slow/low release
11	Slow onset
12	**Quick/long release**
13	Unstable
14	Steady/low
15	Unstable
16	Slow/low release
17	Slow/low release
18	Slow onset
19	Intermediate
20	**Steady**

with 200 ml of water and heating to 98 °C for 2 hours. The gel was cooled to room temperature and the NO releasing compound, dissolved in a minimum of DMSO, was added with stirring. As a positive control, the medicated gel was stored at 4 °C and tested for stability. We mixed the gel with a drop of 1N NaOH to chemically initiate NO production before NO assessment. The formulated material proved stable over several days without activation; however, NO was readily evolved upon NaOH introduction.

Diabetic wound model and grouping

On the day of surgery, animals were anesthetized with Isoflurane 2-3% with 100% oxygen. A 1-cm^2 full-thickness dorsal wound was excised and photographed [23]. 0.4-0.6 ml of the gel was applied to the wound immediately after surgery and once daily thereafter. Animals were divided into four groups (n = 12 per group). Two NO treatment groups, Gel 100 and Gel 1000, were administrated with the gel product containing different concentrations of the NO releasing compound (100μM and 1000μM respectively). A blank control group (Gel control) was treated with the unmediated gel (Gel without the NO releasing compound) and an untreated group without gel (Tegaderm control). To avoid cross contamination and mimic the clinical scenario, gel was applied to each animal with a different 1 ml syringe. Dressing changes were performed daily along with gel application and Tegaderm replacement. Photos of the wounds were taken daily for planimetry. On day 7, all the animals were euthanized and the wound area with its surrounding skin and underlying tissue was excised en-bloc. Tissue samples were fixed in 10% neutral-buffered formalin solution for 24 hours, transferred and kept in 70% ethyl alcohol until paraffin embedment was performed. Paraffin-embedded samples were cut into 3μm sections for further staining and observation. The wound size was measured by blinded assessors using Image J (1.48V, NIH, USA) and by using individual scale of each photograph. Surgeries, dressing changes and tissue harvesting were performed by the same team of operators.

HE staining and thickness assessment

To assess granulation tissue formation and wound morphology, hematoxylin and eosin (H&E) staining was performed for histological observation. Six slides were randomly selected from the four groups and were observed and recorded under a light microscope (Olympus, BX53, Japan) with 4X magnification. Thickness of the granulation tissue and full thickness were measured using Image J (1.48V, NIH, USA). The relative ratio was calculated by dividing the thickness of the wound bed by the thickness of the granulation tissue (Fig. 2). Three microscopic fields from each slide were randomly selected and measured twice in a blinded fashion by three assessors.

Immunohistochemistry staining and analysis

The immunohistochemistry (IHC) staining was

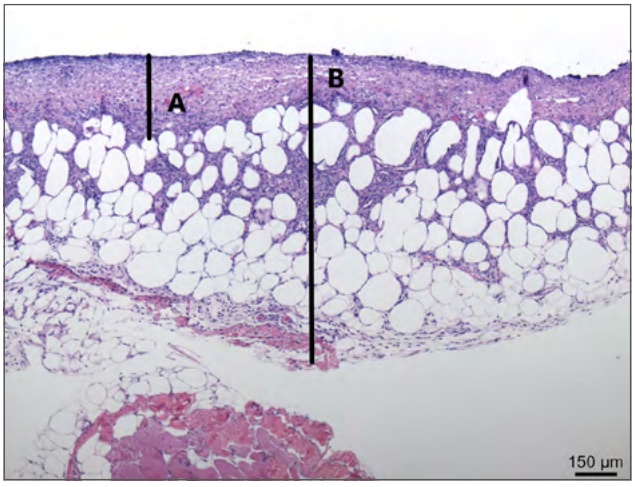

Figure 2. Granulation tissue ratio calculation, A/B. A) granulation tissue thickness; B) wound bed thickness. HE staining, scale bar 100 μm.

performed for analysis of angiogenesis and contractility. Slides were warmed on a hot plate for one hour then deparaffinized and rehydrated. Endogenous peroxidase was eliminated by 3% H_2O_2 methanol mixture. After washing with PBS, we performed antigen retrieval with Proteinase K bath for CD31 or Sodium Citrate (pH = 6) microwave heating for alpha-smooth muscle actin (α-SMA). After cooling, non-serum TNB (1,3,5-Trinitrobenzene) was applied to block the background (100 µl in each slide for one hour). Then, the slides were incubated with primary anti-CD31 antibody (1:100) (ab28364, Abcam, Cambridge, MA) or anti-αSMA (1:300) (ab5694, Abcam, Cambridge, MA) overnight at 4°C. The slides were rinsed with PBS Tween (0.5%) and incubated with anti-rabbit secondary antibodies (BA-1000, Vector, USA) for one hour for one hour in the dark at 37 °C. Then Streptavidin-HRP (1:100) (Dako North America, USA) was added, 100 µl on each slide for 30 min to amplify the signal. A 3,3-diaminobenzidine (DAB) kit (K3468, Dako North America, CA) was then used for visualization. Counter-staining was performed with hematoxylin. Photos of the mounted slides were taken under the light microscope, and the relative density of CD31 and α-SMA was analyzed using Image-Pro Premier (IPP) 9.2 (Media Cybernetics, MD, USA). The stained vessel number was counted in a blinded fashion by three assessors. Three fields were randomly selected from each slide for analysis.

Statistical analysis

Data were presented as mean ± standard deviation. GraphPad Prism 7 (GraphPad Software, USA) was used for statistical analysis. Statistical differences between multiple groups were determined by one-way analysis of variance (ANOVA) followed by a Turkey post-test. Statistical significance was considered when $p < 0.05$.

RESULTS

Wound dynamics-gross observation

Clinical observation and daily planimetry failed to show statistically significant difference in wound contraction between the groups. Daily planimetry did not show statistically significant difference in wound contraction between tested groups ($p > 0.05$) (Figs. 3,4).

Histological examination

Light microscopy with HE staining showed inflammatory cells infiltrating the wound bed in all groups. Moreover, the granulation tissue was thicker in Gel 100 and Gel control groups (Fig. 5A). To ensure the accuracy of the results, we also quantified the thickness of

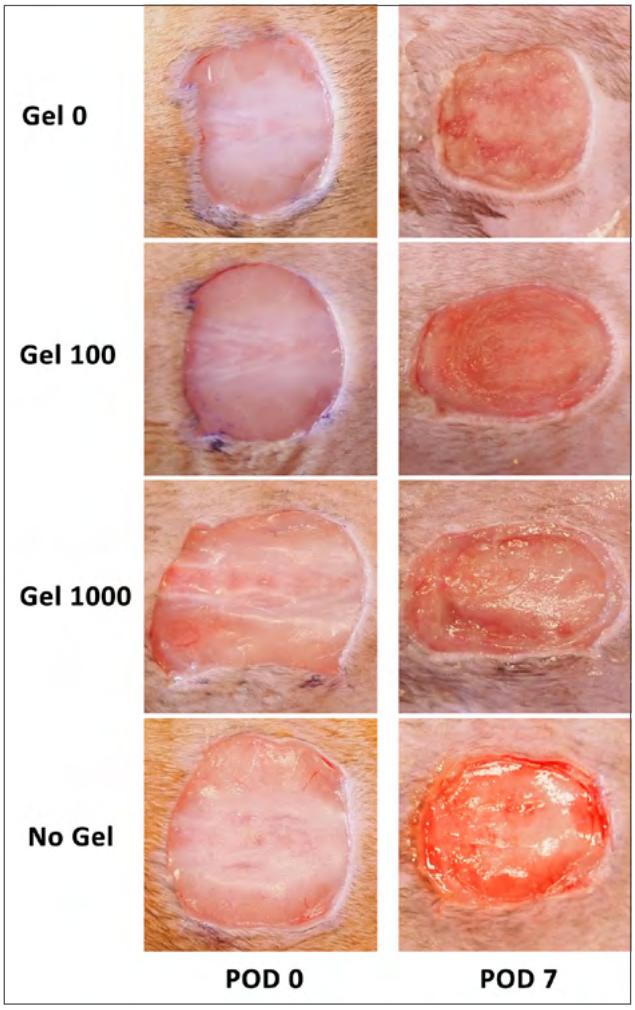

Figure 3. Gross comparison of wound size dynamics. Wound area reduction was observed in all groups with wound healing tendency.

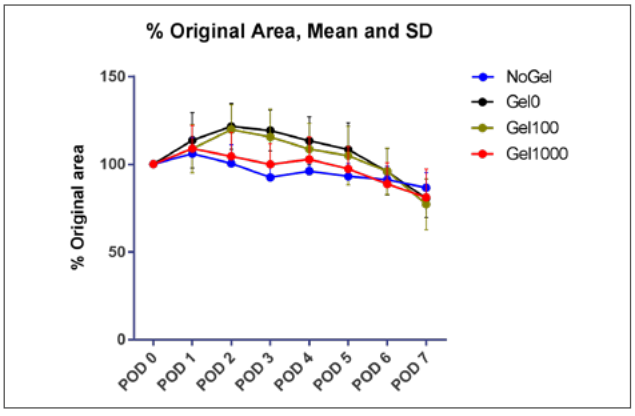

Figure 4. Planimetry of wound size dynamics. Wound size reduction was found in all groups. Although it appears that the Tegaderm and Gel 1000 groups *vs* Gel control and Gel 100 have different wound dynamics, the variability was not statistically significant.

granulation tissue using the ratio method described in materials and methods. Both Gel 100 and Gel-control groups showed more granulation tissue than the other groups, but there was no statistically significant difference between them. In addition, the thickness of granulation tissue in Gel 1000 group displayed a remarkable decrease compared to that of Gel 100 group. Tegaderm control group showed the least granulation tissue increase. The granulation tissue in Gel 100 was slightly thicker than the Gel-control group, although there was no significant difference between them (Fig. 5B).

Angiogenesis assessment

The IHC staining revealed that CD31 positive expression on endothelial cells, represented by brown circles and dots, were mainly distributed in the upper layer of the wound bed, indicating more vessels through this layer in the wound center (Fig. 6A). Based on the vessel counting method, Gel 100 showed better vascularization when compared to Tegaderm control and Gel-control groups ($p < 0.05$) (Fig. 6B). Although the Gel 100 group showed better vascularization per manual vessel counting, the relative area density of CD31 did not show any difference between groups ($p > 0.05$) (Fig. 6C).

The positive expression of α-SMA was easily distinguished from other structures by a dark brown spindle-shaped or agglomerate-like appearance within the granulation tissue in the wound bed (Fig. 7A). Widespread α-SMA staining was found in all the groups and mainly distributed in the basal dermis adjacent to the adipose layer within the wound center (Fig. 7A). There was a remarkable increase of α-SMA expression in the Gel 100 group in comparison to the other groups ($p < 0.05$) (Fig. 7B).

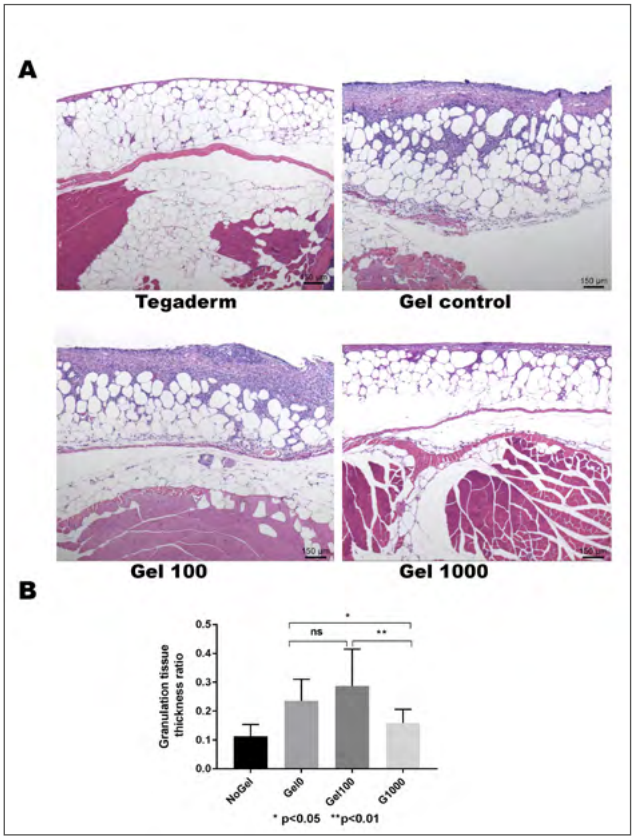

Figure 5. A) representative histology (HE staining) shows newly formed vessels and collagen deposition (pink) with inflammatory cell infiltration (purple). Gel 100 and Gel control groups present more granulation than the other two groups ($p < 0.05$); B) no significant difference was observed between Gel 100 and Gel-control groups. Gel 1000 shows less granulation tissue as compared to Gel 100 ($p < 0.05$). Scale bar 100 µm. Indicator key: * < 0.05, ** < 0.01 *vs* Tegaderm control; # < 0.05, # # < 0.01; ns > 0.05.

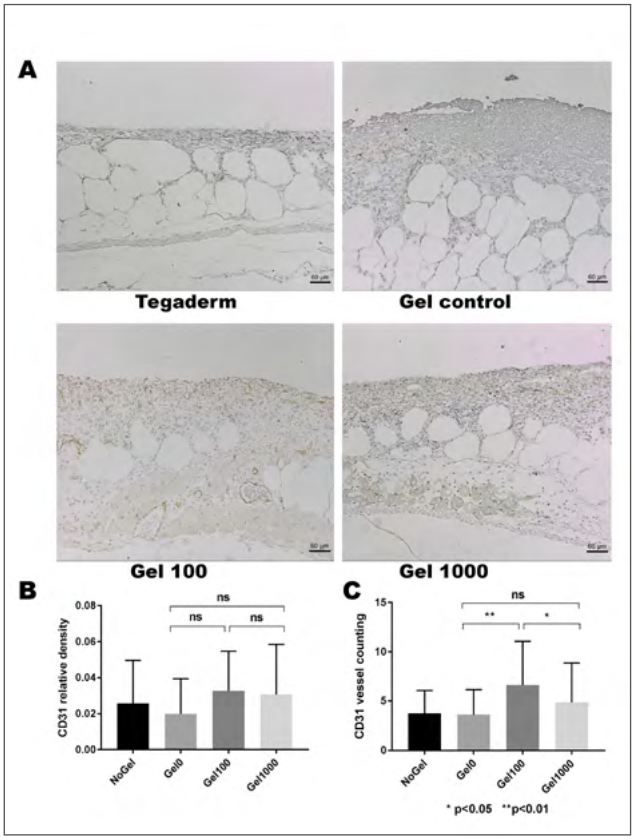

Figure 6. Representative microphotographs of CD31 IHC staining showed a brown color distributed along the cross section of vessels which represents positive staining. Statistical analysis showed no significant difference between the four groups ($p > 0.05$) but a significant increase of vessels on Gel 100 group was shown here ($p < 0.05$). Scale bar 100 µm. Indicator key: * < 0.05, ** < 0.01 *vs* Tegaderm control; # < 0.05, # # < 0.01; ns > 0.05.

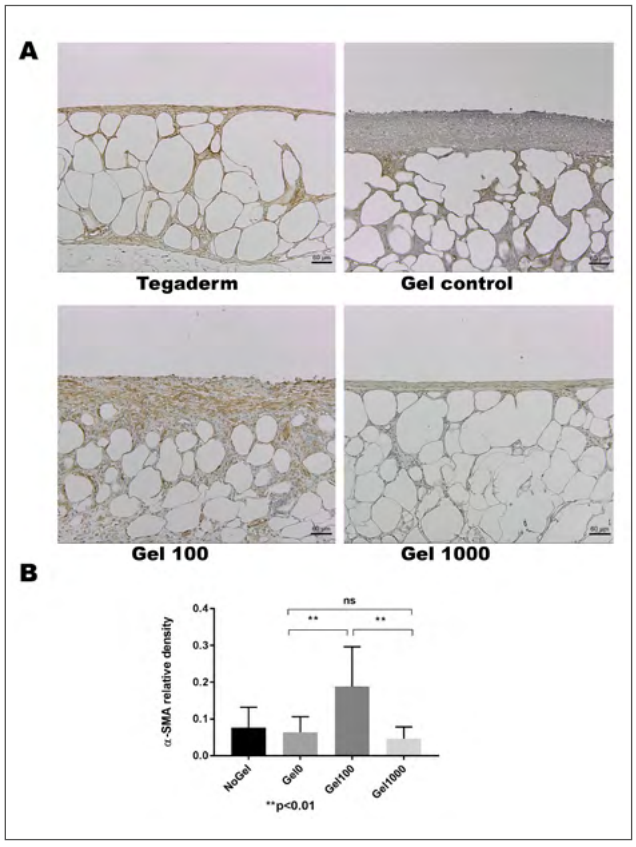

Figure 7. A) α-SMA positive expression is shown by brown stained areas on IHC stained slides; B) Gel 100 group shows an increase in ratio of positively stained areas than the other groups ($p < 0.01$). Indicator key: Scale bar 100 µm. ** < 0.01 *vs* Tegaderm control; # # < 0.01; ns > 0.05.

DISCUSSION

Nonhealing wounds have a profound effect on the quality of life for patients afflicted by them. A better method of treating these wounds would greatly improve patient outcomes. Previous studies have revealed that NO can mediate vasodilation, inhibit platelet aggregation, prevent invading pathogens, stimulate fibroblast proliferation, and modulate collagen deposition in wound healing [24]. The current study found beneficial effects of treatment with a NO releasing gel on a variety of wound healing markers including vascularization, granulation tissue, and angiogenesis [25].

A significant component of wound healing involves granulation tissue, which usually begins to form and fill at the bottom of the wound approximately four days post-wounding [26]. It has been reported that granulation tissue is capable of stimulating re-epithelialization [27], and thus is an early component of wound healing. In our study, histological examination showed more granulation tissue in Gel 100 and Gel-control groups, but decreases in Gel 1000 group. It is known that hydrogel, when used as a wound dressing, acts as a buffer to negate the toxic effect of enzymes, cytokines and oxygen residues formed in chronic wounds [28]. Our result indicates that the positive effects of hydrogel may be enhanced when combined with NO in lower dosages such as Gel 100. While it is unclear why the higher dose Gel 1000 group did not show a beneficial effect – since it is known that NO levels in the wound must be tightly controlled to ensure positive effects – it it may be that the gel 100 represents the optimal concentration of NO for wound healing.

Vascularization plays an important role in supporting granulation tissue formation. Recent reports have shown that CD31 expresses on the surface of several cell-types and is a good indicator of angiogenesis [29]. From IHC staining of CD31, we observed a significant difference in vessel density, but not in vessel area density between Gel 100 and other groups. The difference in results could be the outcome of vessels growing in a different dendritic pattern. Since the stimulation that the gel provides can orient the vessels towards the wound, and the lack thereof toward the wound margins, a different spatial arrangement of vessels could occur. In addition, this could affect the vessel shape and thickness evidenced on the slides since they were cut at the same perpendicular angle. Therefore, we conclude on an approach consistent with other studies: that it is more beneficial to assess the vessel density instead of the vessel area density [30].

Expression of α-SMA, a protein which is predominantly restricted to vascular smooth muscle cells, is a good indicator of the regrowth of vessels into the wound area [31]. It is used as a marker for angiogenesis and serves as an indicator in muscular fibrogenesis [32]. Because myofibroblasts participate in granulation tissue contraction, α-SMA indirectly represents the extent of contractile granulation tissue formation as a result [33]. In this study, greater expression of α-SMA was observed in Gel 100 group, indicating more angiogenesis and granulation tissue formation. However, the expression of α-SMA in Gel 1000 group was suppressed, indicating that a higher dose of NO may have an inhibitory effect.

Based on the increase in α-SMA and CD31 expression in Gel 100 and the decrease in Gel 1000, we speculate that the NO gel has a dose-dependent modulatory effect on angiogenesis, contractility and granulation tissue formation. In our study, Gel 100 group and Gel control group showed more granulation than the other groups, and Gel 100 group also showed more angiogenesis than Gel 1000 group. Thus, lower concentrations of NO showed an increased effect and higher concentrations exhibited an inhibitory effect on wound healing. These

results are consistent with previous studies which have shown that exogenous NO can have a differential effect on wound healing based on concentration. Low dosage of exogenous NO was capable of stimulating cell proliferation and collagen deposition, whereas high concentrations had the opposite effect [34,35]. Previous studies also found that high concentrations of NO inhibit the proliferation of fibroblasts, smooth muscle cells and endothelial cells [36,37].

These findings are encouraging and may provide new treatments for diabetic wounds, although further investigation is needed. Our study was limited by the short follow up period (7 days) and by having only two assessment angles-histology and planimetry. To fully assess this approach, further investigation is needed in the following: involving more functional and genetic assays; experiments testing longer treatment and varying frequencies of drug administration; and the effects of other NO releasing compounds or modes.

CONCLUSIONS

This study has demonstrated that exogenous nitric oxide has a significant effect on modulating wound healing. The NO-releasing materials used here demonstrated positive effects on wound healing. These results re-enforce the importance of NO and suggest that an NO releasing gel can increase angiogenesis and α-SMA expression, thus improving wound closure. Our results suggest that this novel gel exerted a modulatory effect in diabetic wound healing by affecting angiogenesis, cellular ingrowth and granulation tissue formation. Furthermore, wound healing effects are critically dependent on the proper concentration of NO delivered to the wound, with too much being detrimental, and too little showing a reduced effect.

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Conflict of interest statement

Zina Ribkovskaia, Tsvetelina Lazarova, Steven Riesinger are employees at MedChem Partners LLC.

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Authors' contributions

Yukun Liu: D, S, W
Songxue Guo: D, DT
Shuyi Wei: D, S
Huan Wang: D, DT, S
Yong Liu: D, DT, S
Andrea V. Moscoso: DT, W
Zina Ribkovskaia: A (helped determine proper formulation and dosing regime), O (prepared nitric oxide releasing compounds for use)
Tsvetelina Lazarova: A (helped determine proper formulation and dosing regime), O (prepared nitric oxide releasing compounds for use)
Steven Riesinger: A (helped determine proper formulation and dosing regime), O (prepared nitric oxide releasing compounds for use)
Dennis P. Orgill: A, W, O
Mihail Climov: A, D, DT, S, W

Abbreviations

A: conceived and designed the analysis
D: collected the data
DT: contributed data or analysis tool
S: performed the analysis
W: wrote the paper
O: other contribution (specify contribution in more detail)

Ethical consideration

All the experiments were carrried out according to the Guide for Care and Use of Laboratory Animals (Eighth edition, National Academy of Sciences Press, 2011). The protocols were approved by the Institutional Animal Care and Use Committees of the Brigham and Women's Hospital.

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