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Alginate and DNA Gels Are Suitable Delivery Systems for Diabetic Wound Healing

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Abstract

Diabetic foot ulcers (DFU) represent a severe health problem and an unmet clinical challenge. In this study, we tested the efficacy of novel biomaterials in improving wound healing in mouse models of diabetes mellitus (DM). The biomaterials are composed of alginate- and deoxyribonucleic acid (DNA)-based gels that allow incorporation of effector cells, such as outgrowth endothelial cells (OEC), and provide sustained release of bioactive factors, such as neuropeptides and growth factors, which have been previously validated in experimental models of DM wound healing or hind limb ischemia. We tested these biomaterials in mice and demonstrate that they are biocompatible and can be injected into the wound margins without major adverse effects. In addition, we show that the combination of OEC and the neuropeptide Substance P has a better healing outcome than the delivery of OEC alone, while subtherapeutic doses of vascular endothelial growth factor (VEGF) are required for the transplanted cells to exert their beneficial effects in wound healing. In summary, alginate and DNA scaffolds could serve as potential delivery systems for the next-generation DFU therapies.

Keywords

biomaterials, diabetic foot ulcers, wound healing, neuropeptides, endothelial precursor cells

Diabetic foot ulceration (DFU) represents one of the major diabetes mellitus (DM) complications. It is the leading cause of nontraumatic lower extremity amputations[1] and carries a considerable socioeconomic burden.[2] Given the alarming rise of DM prevalence in the general population, it can only be expected that the number and cost of DFU will intensify in the future. Despite the severity of the problem, no new treatments have been licensed recently, and the only 3 commercially available products—Regranex (Becaplermin), Apligraf, and Dermagraft—are characterized by moderate efficacy.[3-5]

Studies from our groups have identified possible mechanistic interventions that can improve DFU management; that is, we have demonstrated a persistent inflammatory state in DM that is present at baseline[6-8] and throughout the impaired wound healing process.[7,9] In addition, we have found reduced levels of the neuropeptide Substance P (SP) in human and experimental DM.[9] Moreover, we have shown that both SP[9] and neurotensin (NT)[10,11] modulate inflammation and improve wound healing in DM mouse models. Finally, we have reported reduced numbers of endothelial precursor cells (EPC) in DM patients with complications[12] and shown beneficial effects of EPC transplantation in mouse models of DM wounds[13] and hind limb ischemia.[14]

Systemic delivery of drugs is associated with a number of limitations that controlled delivery systems may effectively address.[15] Topical administration is usually preferred for wound treatment, but also has its limitations. The short half-lives of many drug candidates creates challenges, and this is particularly true for neuropeptides, since they are rapidly inactivated in the protease-rich wound environment.[16] In addition, it is now clear that the ability of transplanted cells to orchestrate regeneration is highly dependent on their

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interaction with host cells. Also, the gene expression profile of the transplanted cells is crucial for their therapeutic effect.[17-19] Therefore, next-generation delivery systems should protect bioactive agents in the wound environment, provide sustained release of the bioactive molecules, and adequately modulate the fate, location, and phenotype of transplanted cells.

Biomaterials based on alginate can be used as a multitherapy delivery system for wound healing. Biodegradable, injectable alginate gels have already been developed[20,21] with positive outcomes for cell and/or drug delivery applications; that is, they demonstrated to protect cells following transplantation, promoting proliferation of the cells inside the material, and releasing them into the wound site over time.[14,22,23] These gels have also proved useful for sustained and localized delivery of both small molecule drugs and macromolecular drugs.[24]

Biomaterials based on deoxyribonucleic acid (DNA) are new, innovative, and promising candidates for cell and/or drug delivery. The construction of DNA composite materials offers much greater control and versatility than has been available previously. On one hand, scaffolds built solely from synthetic polymers typically do not display the biological motifs that guide normal cellular behavior in the extracellular matrix (eg, adhesion, matrix synthesis and degradation, motility). On the other hand, scaffolds built solely from biological materials lack control over mechanical properties and spatiotemporal organization. DNA building blocks are "programmable" structures that offer a far finer level of spatial and mechanical control compared with other self-assembling biomolecules currently used for tissue engineering applications, such as extracellular matrix self-assembly of collagen fibrils and gels.

In this study, we tested the effect of alginate gels encapsulating (*a*) neuropeptides (SP and NT), (*b*) human umbilical cord–derived outgrowth endothelial cells (OEC), a type of EPC,[14] or (*c*) a combination of OEC and SP in a mouse model of DM wound healing. We also evaluated the potential use of DNA hydrogels as delivery systems for wound therapy. These studies were intended to provide a screen of the above-mentioned materials and potential therapeutic agents, as a first step toward more in-depth experiments with those combinations that appear most promising.

Materials and Methods

Biomaterials

Alginate Gels. Alginate gels were prepared as previously described.[14,25] The release kinetics of vascular endothelial growth factor (VEGF)[21,25] as well as the ability of OEC to migrate outward from alginate scaffolds have already been demonstrated[14] with positive results for prolonged periods of time (over 2 weeks). Here, the release kinetics for SP and NT from the alginate gels was analyzed. SP and NT cumulative release into EBM Basal Medium (Lonza, Walkersville, MD) in which gels were soaked, at 37°C, was measured using commercially available ELISA kits for SP (R&D Systems, Minneapolis, MN) and NT (Bachem, Torrance, CA).

DNA Gels. DNA hydrogels were prepared in the laboratory of Dr. Shih. Two Y-scaffold DNA gels were designed based on an adaptation of the model by Xing et al.[26] Each Y-scaffold was assembled from 3 single-stranded DNA (ssDNA) strands. Each strand of one Y-scaffold was 46 nucleotides long with the following components: 16 complementary sequences and 14 nucleotide "sticky ends." The hybridization of "sticky ends" of the first Y-scaffold to the second Y-scaffold resulted in the formation of a hydrogel. Sequences were as follows (3WJ-F-1, 2, 3 for first junction; 3WJ-F-4, 5, 6 for second junction):

3WJ-F-1: GTTGAAGCTCAGGCCCCCAGGGAGCC TGGGGCGAGAGAGAGAGAGA
3WJ-F-2: GCCCCAGGCTCCCTGGTGGGTCTGCAG GGGGCGAGAGAGAGAGAGA
3WJ-F-3: GCCCCCTGCAGACCCAGGGCCTGAGCT TCAACGAGAGAGAGAGAGA
3WJ-F-4: GCCACCTCTTTATGGGTCAGGAACAG GGCAACTCTCTCTCTCTCTC
3WJ-F-5: GTTGCCCTGTTCCTGAGCAGCTTTG CTGGGGCTCTCTCTCTCTCTC
3WJ-F-6: GCCCCAGCAAAGCTGCCCCATAAAGA GGTGGCTCTCTCTCTCTCTC

Each strand was resuspended in distilled water to 3 mM final concentration. To form each junction, 3 equivalent volumes of each strand was mixed together along with 1 equivalent volume of 10× phosphate-buffered saline (100 mM disodium phosphate, 18 mM monopotassium phosphate, 1.37 M sodium chloride, 27 mM potassium chloride). Junction solutions were heated to 80°C for 5 minutes, and then cooled to room temperature over the course of 2 hours in an MJResearch thermal cycler. Then, the 2 junction solutions were mixed quickly by being drawn into a 25-gauge needle, and then immediately injected (60 µL per application) into the mouse, such that gelation occurred mostly *in vivo*.

Mouse Model

Wild-type (WT) C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were made diabetic (DM) by administering streptozotocin via intraperitoneal injection (50 mg/kg STZ, ip, daily for 5 consecutive days) in citrate buffer (0.1 M). In the non-DM groups, mice were treated with vehicle alone. One week after the

last STZ injection, fasting blood glucose was monitored and mice that had values over 250 mg/dL were considered DM.

Wound Creation, Monitoring, and Treatment

6 to 8 weeks after STZ or vehicle treatment, mice were anesthetized using ketamine (100 mg/kg ip) and xylazine (5 mg/kg ip), and 2 circular 6-mm diameter full-thickness wounds were created on the shaved dorsum of the mice using a punch biopsy tool. Wound closure kinetics was monitored daily over a 10-day period by manually measuring the wounds using acetate tracing followed by analysis with Image J software (NIH) to determine the wound size. Data were presented as percentage of original wound size (Day 0) over the study period.

On Day 0, immediately after wound creation, the gels were injected into the tissue surrounding the wounds. For the studies using alginate gels, only DM mice were used. Treatments were as follows: (1a) alginate gel only (60 μL/wound), (1b) alginate gel encapsulating a combination of SP (32 μg/60 μL/wound) and NT (50 μg/60 μL/wound); (2a) alginate gel encapsulating a subtherapeutic dose of bioactive VEGF (3 μg/60 μL/wound), (2b) alginate gel encapsulating VEGF (3 μg/60 μL/wound) and OEC (1×10^6 OEC/60 μL/wound), (2c) alginate gel encapsulating VEGF (3 μg/60 μL/wound), OEC (1×10^6 OEC/60 μL/wound) and SP (32 μg/60 μL/wound); (3a) alginate gel only (60 μL/wound), (3b) alginate gel encapsulating OEC (1×10^6 OEC/60 μL/wound). For the studies using DNA-based gels, both DM and non-DM mice were used. The study groups were divided as follows: (4a) non-DM untreated wounds, (4b) non-DM DNA gel treated wounds (60 μL/wound), (4c) DM untreated wounds, (4d) DM DNA gel treated wounds (60 μL/wound). All mouse studies were performed in accordance with Institutional Animal Care and Use Committee (IACUC)–approved protocols.

Histological Analysis

At the end of the study, Day 10 postwounding, mice were euthanized and 1 cm × 1 cm skin sections that included the wound margins were cut. For morphologic analysis and immunohistochemistry, tissue was fixed in 10% formalin and subsequently embedded in paraffin (FFPE). For morphologic analysis and evaluation of the inflammatory cell infiltrate, FFPE sections (5 μm) were subjected to routine histological processing with hematoxylin and eosin (H&E).

Statistical Analysis

The Minitab (Minitab, State College, PA) statistical package was employed. Differences among experimental groups were analyzed using either the *t* test or the one-way ANOVA test followed by Fisher's post hoc test. Statistical significance was defined for a *P* value lower than .05.

Results

Alginate Gels Offer Continuous Release of SP and NT

SP (Figure 1A) and NT (Figure 1B) release kinetics from the alginate hydrogels was evaluated for up to 1 month. Both neuropeptides were cumulatively released from the gels during the time period tested. At 1 week, the closest time point to the end of our *in vivo* wound healing studies, the cumulative release of SP and NT was 54% and 46%, respectively (Figure 1C).

Alginate Gels Releasing SP and NT Improve Wound Healing in DM Mice

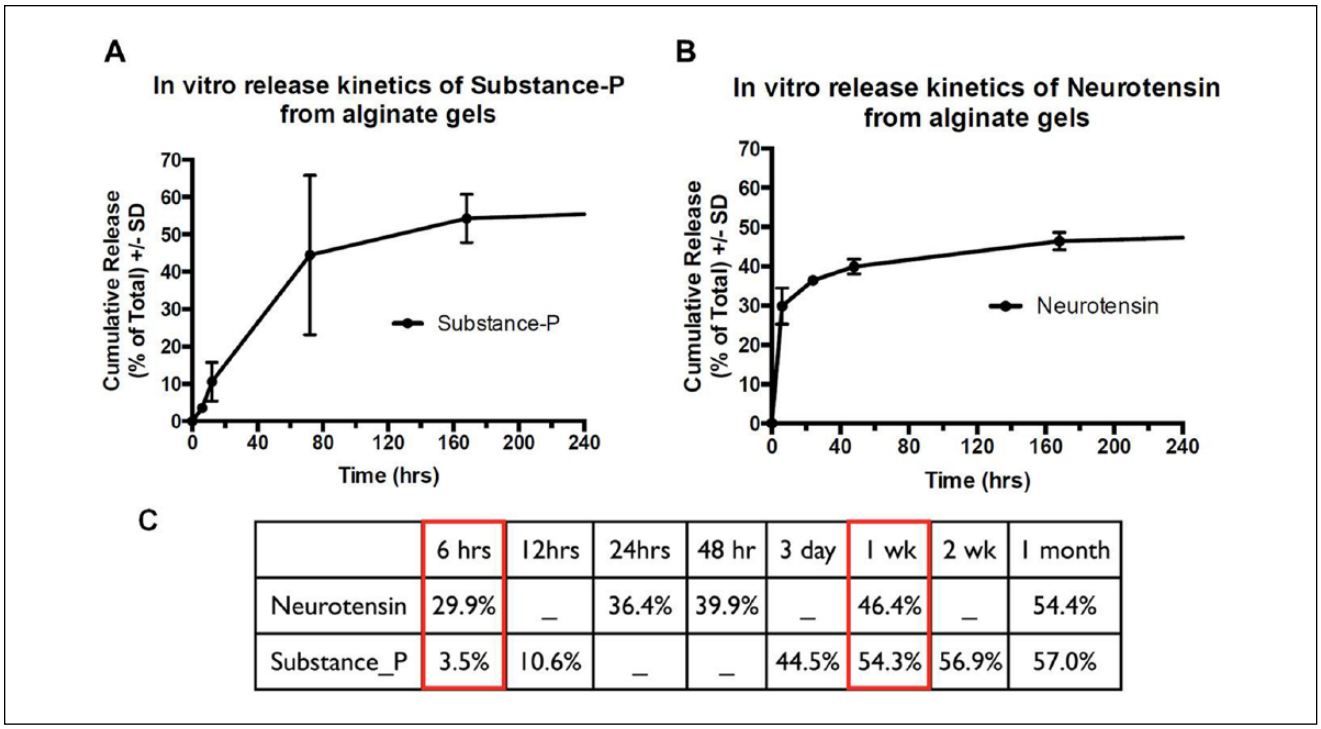
We evaluated the effect of alginate gel incorporating the neuropeptides SP and NT on wound healing in DM mice. A single injection of alginate gel with the neuropeptide combination into the wound margins of DM mice reduced wound size by 54% when compared with the wounds that received alginate gel only at Day10 postwounding ($P < .05$, Figure 2).

Alginate Gel Deployment of OEC Accelerates Wound Healing in DM Mice

We evaluated the effect of (*a*) alginate gel with VEGF, (*b*) alginate gel with VEGF + OEC, and (*c*) alginate gel with VEGF + OEC + SP on wound healing in DM mice. Unlike alginate gel with VEGF only, both VEGF + OEC and VEGF + OEC + SP delivery via alginate gel prevented wound size expansion in DM mice observed at Day 2 postwounding ($P < .05$, Figure 3A). In addition, at Day 4 postwounding, the wound size in group (*a*) was still approximately the same as at Day 0, whereas in groups (*b*) and (*c*), wound size had reduced significantly ($P < .05$, Figure 3A). In fact, healing was accelerated in the wounds that received alginate gel encapsulating VEGF + OEC or VEGF + OEC + SP compared to the ones that received alginate gel with VEGF only from Day 2 to Day 10 postwounding ($P < .05$, Figure 3B and C). In addition, healing was improved in the wounds that received alginate gel encapsulating VEGF + OEC + SP compared to wounds that received alginate gel encapsulating VEGF + OEC from Day 4 to Day 10 postwounding ($P < .05$, Figure 3B and C). These results suggest that the combination of OEC and SP has an additive, if not synergistic, effect in DM wound healing.

Subtherapeutic Doses of VEGF Are Needed to Assure OEC Function In Vivo

We also evaluated the effect of (*a*) alginate gel only (without VEGF) and (*b*) alginate gel with OEC (without VEGF) in our model of DM wound healing. No major



C

	6 hrs	12hrs	24hrs	48 hr	3 day	1 wk	2 wk	1 month
Neurotensin	29.9%	_	36.4%	39.9%	_	46.4%	_	54.4%
Substance_P	3.5%	10.6%	_	_	44.5%	54.3%	56.9%	57.0%

Figure 1. Alginate gels offer sustained release of SP and NT for more than 10 days.
In vitro release kinetic profiles of SP (A) and NT (B) from alginate hydrogels measured continuously for over 240 hours. Data represent mean ± SD. (C) Table showing the mean cumulative release (as percentage of total) of NT and SP at the studied time points.

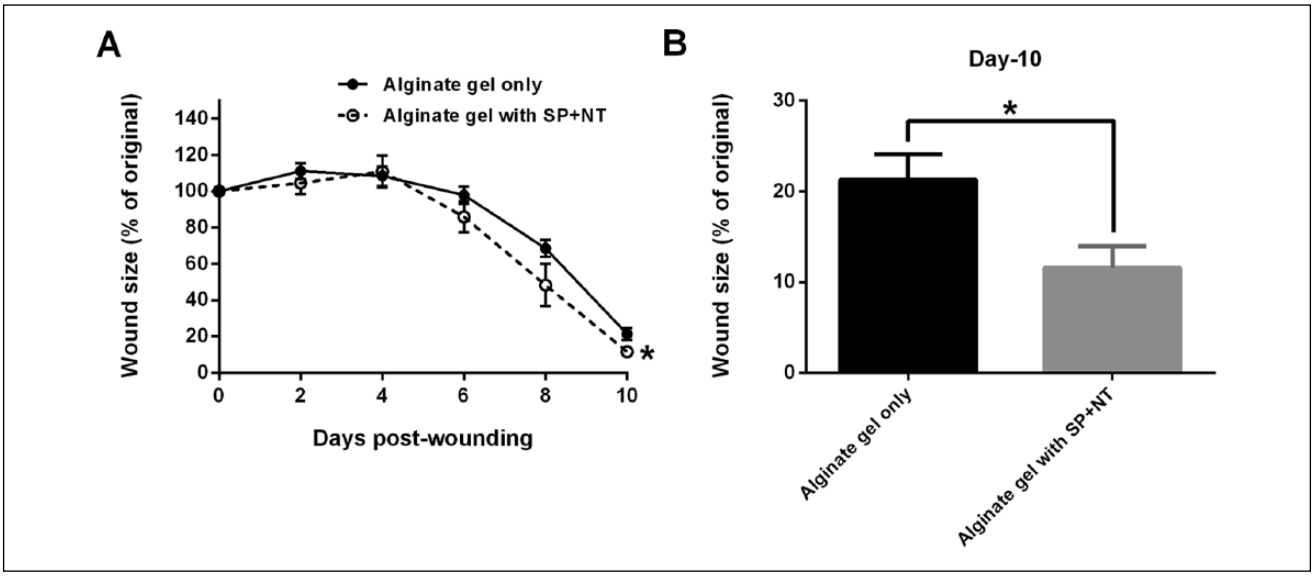


Figure 2. Topical treatment with alginate gel encapsulating SP and NT reduces wound size in DM mice.
(A) Wound healing progress of DM wounds treated with alginate gel only or with alginate gel with SP + NT over 10 days. (B) Day 10 wound size of DM wounds treated with alginate gel only or with alginate gel with SP + NT. Data represent mean ± SEM. *$P < .05$, compared to alginate gel only.

differences were observed in terms of healing kinetics over the studied 10-day period between the wounds that received the different treatments ($P = .84$, NS, Figure 4A and B). Taken together, these results suggest that subtherapeutic doses (3 µg) of VEGF have to be incorporated in the alginate gel encapsulating the OEC to observe a beneficial effect of the cells in wound healing.

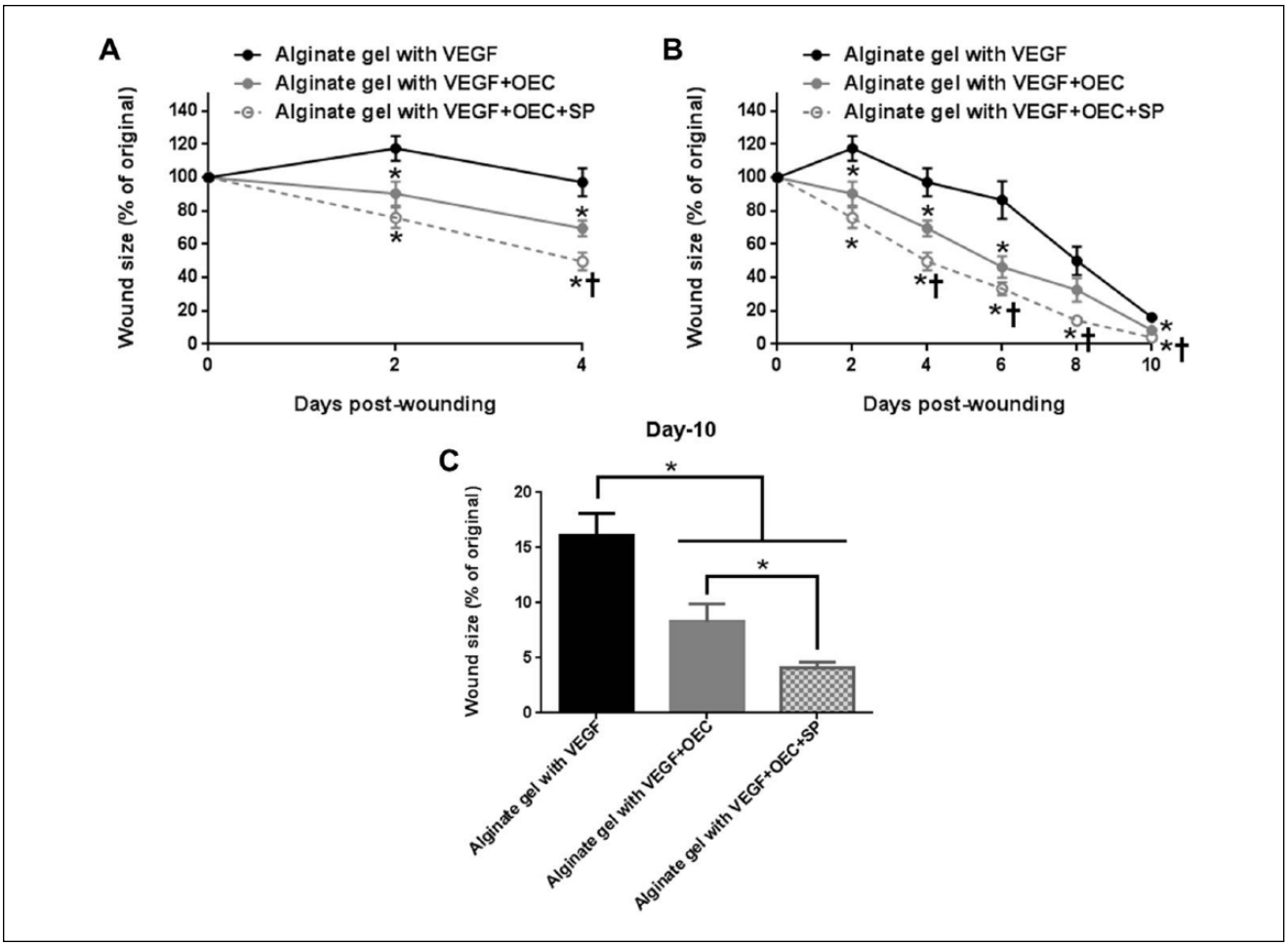


Figure 3. Topical treatment with alginate gels encapsulating VEGF and OEC improves wound healing in DM mice. The combination of VEGF and OEC with SP in the alginate gels further improved healing.
Wound healing progress of DM wounds treated with (*a*) alginate gel with VEGF, (*b*) alginate gel with VEGF + OEC, or (*c*) alginate gel with VEGF + OEC + SP over 4 days (A), showing differences in wound expansion at the early stages of healing or 10 days (B), showing that the wound healing improvement by treatments (*b*) and (*c*) continues until the end of the study. Day 10 wound size (C) of DM wounds treated with (*a*) alginate gel with VEGF, (*b*) alginate gel with VEGF + OEC, or (*c*) alginate gel with VEGF + OEC + SP. Data represent mean ± SEM. **P* < .05, compared to alginate gel with VEGF. †*P* < .05, compared to alginate gel with VEGF + OEC.

DNA-Based Hydrogels Are Suitable Material for Drug and/or Cell Delivery in Our Mouse Model of Wound Healing

We next evaluated the effect of DNA gels in wound healing using both non-DM and DM mice. As expected, and according to our previous studies, DM mice showed impaired healing when compared to non-DM mice ($P < .05$, Figure 5A and B). DNA gel did not significantly affect wound closure in our mouse models. If anything, injection of DNA gel in the tissue surrounding the wounds showed a tendency toward reduced wound size in non-DM mice at Day 10, but was not statistically significant (18 ± 3 vs 26 ± 6, $P = .24$, NS). No major differences were observed in DM mice (33 ± 4 vs 36 ± 4, $P = .69$, NS; Figure 5A and B).

There were no macroscopic signs of immune reaction to the hydrogel in the mouse wounds. In addition, histological analysis showed no difference between untreated and DNA gel treated wounds in terms of extent ($P > .99$ for non-DM mice; $P = .86$ for DM mice, NS) and intensity ($P = .84$ for non-DM mice; $P = .85$ for DM mice, NS) of inflammatory cell infiltrate (Figure 6A-C), indicating that the DNA-based material did not induce an immune or inflammatory reaction. These preliminary findings suggest that DNA-based gels are a suitable material to serve as scaffold for the delivery of cells, neuropeptides, growth factors, or other molecules to the wound area.

Discussion

Wound healing is a complex physiological process that is severely impaired in DM. Current consensus suggests that there are multiple mechanisms involved in DM-associated impaired wound healing, including neuropathy and associated

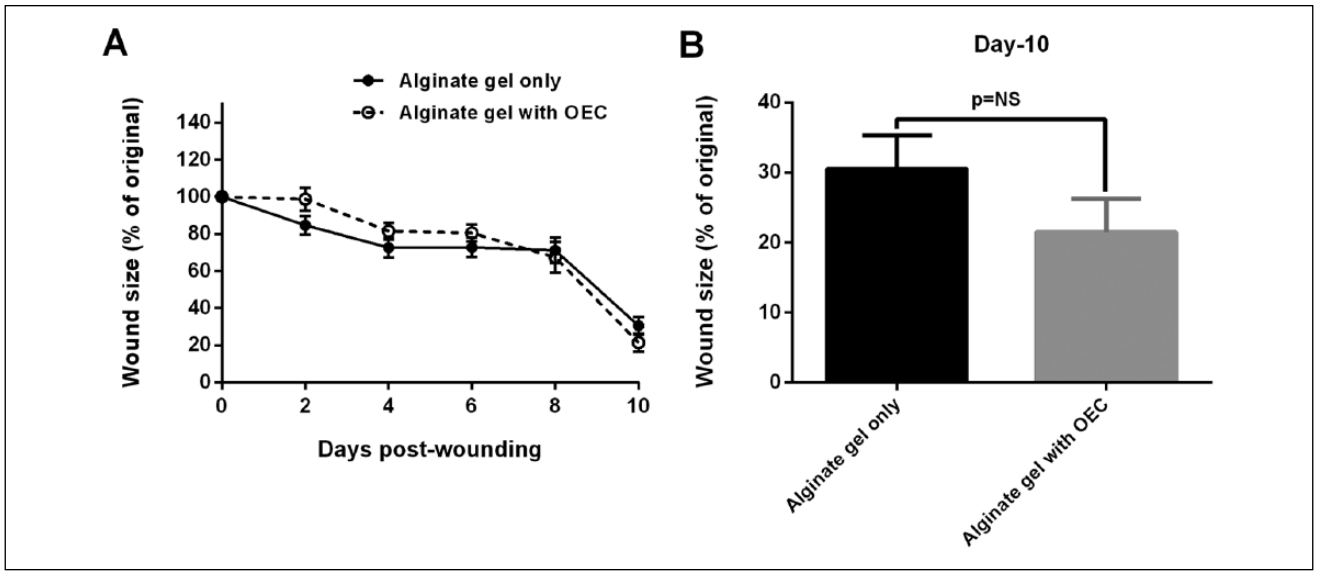


Figure 4. Topical treatment with alginate gels encapsulating OEC without VEGF does not affect wound healing in DM mice.
(A) Wound healing progress of DM wounds treated with (*a*) alginate gel only (without VEGF) and (*b*) alginate gel with OEC (without VEGF). (B) Day 10 wound size of DM wounds treated with (*a*) alginate gel only (without VEGF) and (*b*) alginate gel with OEC (without VEGF). Data represent mean ± SEM.

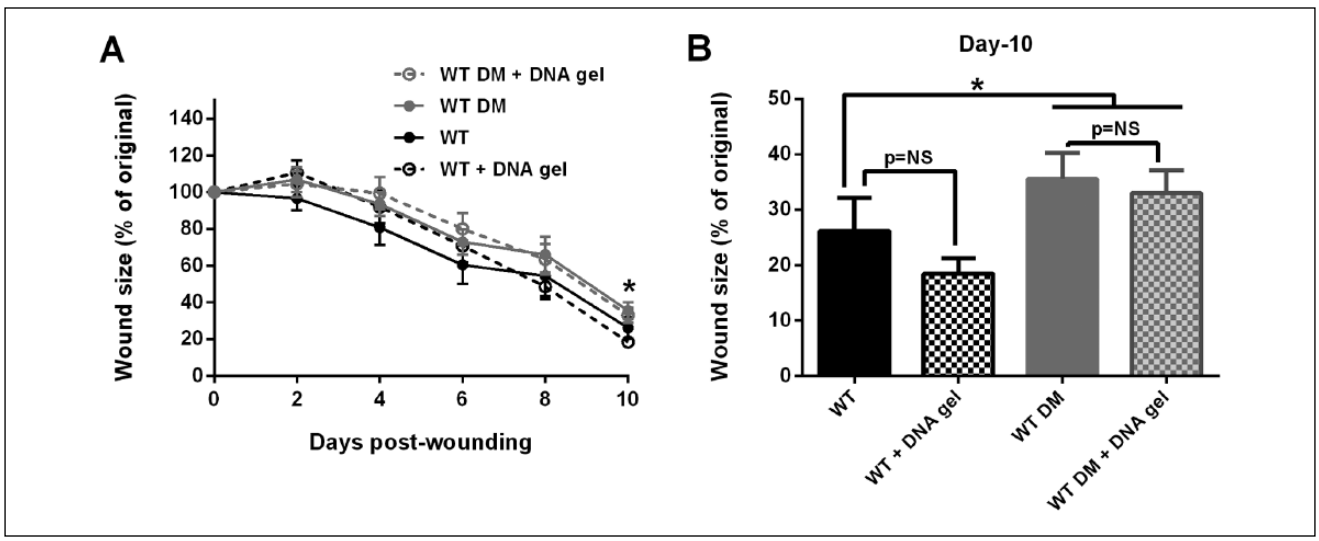


Figure 5. Topical treatment with DNA gels did not significantly affect wound healing in DM mice.
(A) Wound healing progress of non-DM and DM wounds, untreated or treated with DNA gels; (B) Day 10 wound size of non-DM and DM wounds, untreated or treated with DNA gels. Data represent mean ± SEM. *$P < .05$, WT DM versus WT.

neuropeptide-deficiency, chronic inflammation, reduced intracellular signaling of growth factors, and impaired neovascularization. Therefore, the development of new products that can affect multiple of the above-mentioned mechanisms is likely to be highly beneficial for DFU. In addition, it is well recognized that the cells and molecules that participate in wound repair interact with and influence each other and that many effectors act synergistically to promote healing. Moreover, the hyperglycemic and proteolytic environments that characterize DM wounds impair the viability and function of cells and reduce the bioavailability of active molecules. Hence, novel delivery systems providing protection and controlled release of multiple factors will likely be more efficacious than single or multiple bolus administrations for DFU treatment.

In this study, we tested new biomaterials as potential delivery systems for DM wound therapeutics. We used injectable alginate gels to release a combination of SP and NT, as these neuropeptides have shown to improve

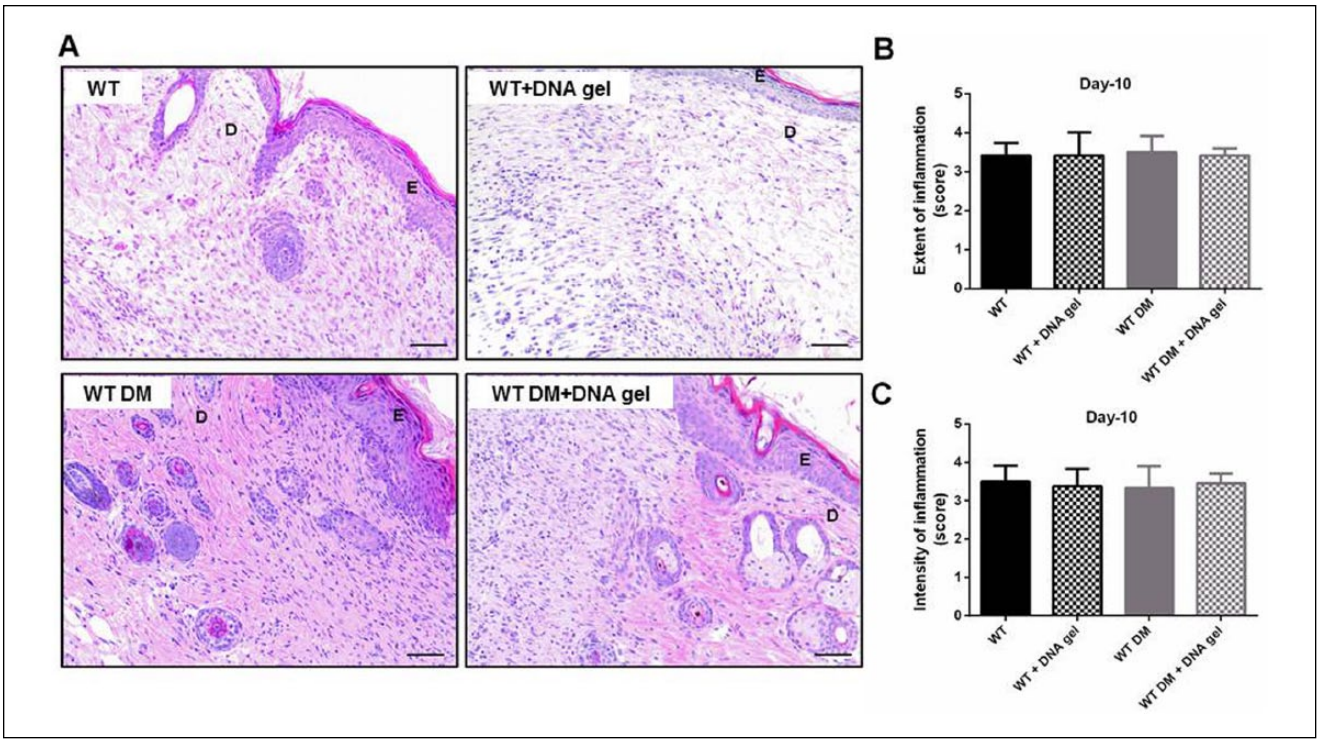


Figure 6. DNA gels did not increase wound inflammation in non-DM and DM mouse models.
(A) Representative H&E images of the peri-wound skin of (*a*) WT non-DM untreated, (*b*) WT non-DM DNA gel treated, (*c*) WT DM untreated, and (*d*) WT DM DNA gel treated wounds. E, epidermis; D, dermis. Scale bar = 100 µm. No differences were observed in terms of extent (B) or intensity (C) of inflammatory cell infiltrate between the different groups (*P* = NS). Data represent mean ± SEM.

DM-impaired healing when individually applied to the wounds.[10,11] We also used macroporous alginate polymer scaffolds to encapsulate and deliver OEC to the wounds, either in single therapy or in combination with SP. Finally, we applied a new DNA nanoparticle system to non-DM and DM mouse wounds to evaluate its potential adverse or beneficial effects in wound healing.

Our main finding is that a combination of multiple effectors, namely, OEC and SP, is more advantageous than single treatments for DM wounds. In addition, we show that OEC require a minimal dose of VEGF to have a beneficial effect in our mouse model of wound healing when administrated via alginate gels. This is in accordance with a previous study, which reported that the inclusion of low doses of VEGF increased cell migration from the gels.[14] The dose of VEGF incorporated in the gels was subtherapeutic—3 µg instead of the 10 µg or 20 µg commonly used for wound healing studies[27,28]—and there was a significant improvement in the wounds treated with OEC + VEGF when compared to the wounds that received VEGF only. Therefore, these results suggest that the beneficial effect on wound healing was mediated by the cells and not by the low dose of VEGF. In addition, we show that the alginate gels provide sustained release of SP and NT, which have been previously validated for DM wound healing in our mouse model.[10,11] We also show that peri-wound injection of the alginate gels incorporating SP and NT accelerates healing. Finally, we demonstrate safety and biocompatibility of DNA-based gels *in vivo*. The DNA gels are able to incorporate active molecules,[29,30] and our results suggest that they could serve as a delivery system for wound therapy. Although these data are preliminary, we believe that the above-mentioned systems are promising and could lead to the development of novel bioproducts for DFU treatment. Further investigation is needed to optimize the combination of biomaterials and effectors, evaluate the safety and efficacy of the products in additional experimental models of DM wound healing, and, in case of positive outcomes, explore their translational capabilities.

In summary, alginate gels were used to provide controlled delivery of neuropeptides, OEC, or a combination of both. Sustained release of the neuropeptides SP and NT for over 10 days was demonstrated *in vitro*. When treating DM mouse skin wounds, all treatments accelerated wound closure compared to alginate gel only, and the combination of OEC and SP proved to be the most effective. DNA-based gels were tested in DM and non-DM mouse models of wound healing and demonstrated to be secure. Taken together, our findings indicate that the new biomaterials tested are safe and efficacious for wound healing in our

mouse models and suggest that they could serve as novel delivery systems for wound therapeutics.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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