Original Research

Topical treatment with the opioid antagonist naltrexone accelerates the remodeling phase of full-thickness wound healing in type 1 diabetic rats

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Abstract

Wound repair involves a series of overlapping phases that include inflammation, proliferation, and tissue remodeling, with the latter phase requiring months for proper healing. Delays in any of these processes can result in infection, chronic ulceration, and possible amputation. Diabetes is a major risk factor for improper wound repair, and impaired wound healing is a major complication for more than 26 million people in the US diagnosed with diabetes. Previous studies have demonstrated that the opioid antagonist naltrexone (NTX) dissolved in moisturizing cream reverses delays in wound closure in streptozotocin-induced type 1 diabetic (T1D) rats. NTX accelerated DNA synthesis and increased the number of epithelial and mast cells, as well as new blood vessel formation. In this study, remodeling was evaluated in T1D rats up to eight weeks after initial wounding. Twenty days following wounding, diabetic rats treated with vehicle had elevated numbers of MMP-2+ fibroblasts, suggesting delayed healing processes; birefringence of granulation tissue stained with Sirius red revealed diminished collagen formation and maturation. Wound tissue from NTX-treated T1D rats had comparable numbers of MMP-2+ fibroblasts to control specimens, as well as accelerated maturation of granulation tissue. The integrity of wounded skin was evaluated by tensile strength measurements. T1D resulted in delayed wound healing, and wounded skin that displayed reduced tensile strength relative to normal rats. Topical NTX applied to wounds in T1D rats resulted in enhanced collagen formation and maturation over a 60-day period of time. Moreover, the force required to tear skin of NTX-treated T1D rats was elevated relative to the force necessary to tear the skin of vehicle-treated T1D rats, and comparable to that for normal rats. These data reveal that complications in wound healing associated with T1D involve the novel OGF-OGFr pathway, and that topical NTX is an effective treatment to enhance wound healing.

Keywords: Naltrexone, opioid antagonists, tensile strength, wound remodeling, opioid growth factor receptor, collagen formation, cutaneous wounds

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Introduction

A major complication of diabetes involves the impairment of proper and timely closure of excisional wounds.[1,2] Delayed wound healing can result in chronic lesions and ulcers, and may lead to limb amputation. More than 26 million persons in the United States have diabetes,[1] and more than 80 million other individuals have undiagnosed diabetes or prediabetes. Nearly 50% of all cases of diabetes are associated with at least one complication related to delayed cutaneous wound healing over their lifetime. With the rapid rise in diagnosis of persons with diabetes, as well as escalating medical expenses, there are financial and health-related needs for novel therapies that target the underlying biology of diabetic complications, and are effective, of low cost, and enhance patient compliance.

The process of wound healing is initiated by hemostasis followed by three overlapping phases including inflammation, proliferation, and tissue remodeling.[3] A series of studies have shown that daily topical application of the opioid antagonist naltrexone (NTX) enhances full-thickness wound closure in type 1 diabetic (T1D) rats, and does so by stimulation of epithelial proliferation[2] and angiogenesis.[4] Disruption of interactions between endogenous opioids and their receptors through invoking a total opioid receptor blockade with NTX applied topically or systemically

stimulates DNA synthesis in dorsal skin epithelium of T1D rats.[3]

The mechanistic pathways associated with enhanced wound repair are related to cell proliferation,[2] and offset or compensate for at least one of the underlying pathogenic characteristics of diabetic complications. Endogenous opioids including [Met^5]-enkephalin and β-endorphin have been shown to be elevated in individuals with diabetes,[5,6] as well as in animal models of both T1D rats and genetically obese (*db/db*) mice, a model for type 2 diabetes.[7] In addition to these neuropeptides playing a role in nociception and neuromodulation, the opioid peptide [Met^5]-enkephalin has been found to be a negative growth factor termed opioid growth factor (OGF). OGF regulates DNA synthesis and proliferation in cells and tissues by upregulation of p16 and/or p21 cyclin-dependent inhibitory kinases.[8] OGF and its receptor, OGFr, are present in epithelial tissue and have been shown to regulate normal homeostasis of corneal epithelium[9–11] and skin.[12–15] OGF is an inhibitory molecule that impedes proliferation, and thus, individuals and animals with elevated levels of OGF may experience delayed epithelialization and ultimately, wound closure. The last phase of wound healing encompasses a number of processes that involve collagen production, organization, and final remodeling of the wound bed. These phases in healing occur over a period of months in humans and weeks in animal models of diabetes,[16,17] and are often protracted under hyperglycemic conditions.[17–19]

The present study explores the long-lasting, residual effects of topical treatment with NTX. NTX was applied for 20 days to full-thickness cutaneous wounds in T1D rats, and effects evaluated up to 60 days later. Collagen deposition and maturation in wounded tissue from T1D and normal rats were compared using Sirius red staining. Extracellular matrix remodeling was assessed by measurement of the expression of metalloproteinase (i.e. MMP-2) expression, and final collagen remodeling and integrity of the wounded skin were evaluated by tensile strength measurements. Finally, because patients with diabetes often develop ulcers or epithelial erosion when a previously wounded area is reinjured, the same paradigm was established in T1D rats to evaluate whether wounded and healed skin that was topically treated with NTX developed hyperproliferative scars, or was more or less sensitive to rewounding.

Methods and materials

Animals and induction of diabetes

Adult male Sprague-Dawley rats (~150 g) were purchased from Charles River Laboratories, Wilmington, MA, and housed in an environmentally controlled animal room, with water and food (2018 Global Rodent Diet, Teklad®, Indianapolis, IN) provided *ad libitum*. All studies conformed to an approved protocol and the guidelines of The Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

At six weeks of age, rats received intraperitoneal (i.p.) injections of 40 mg/kg streptozotocin (STZ, Sigma, St. Louis, MO) or citrate buffer on two consecutive days. STZ at this concentration produces insulin-dependent T1D (DB) in 100% of the animals within seven days.[4] Control animals receiving i.p. injections of citrate buffer were considered normal (Normal, N). Blood glucose levels were monitored from the dorsal tail vein using a True Track Smart System glucometer (Home Diagnostics, Ft. Lauderdale, FL). Blood glucose measurements were taken prior to STZ injections and on weeks 1, 4, and 8 after the induction of hyperglycemia. Measurements of >350 mg/dL denoted hyperglycemic levels of the diabetic cohort. If the animal had sustained blood glucose measurements > 600 mg/dL, and appeared lethargic and unwilling to eat, it was removed from the study to eliminate confounding variables related to abnormal nutrient intake, systemic infection, or other hyperglycemic-related morbidity.

Cutaneous wounds and naltrexone application

Full thickness cutaneous wounds were created as described previously.[4] In brief, rats were anesthetized by i.p. injection of a mixture of ketamine (60 mg/kg), xylazine (10 mg/kg), and acepromazine (1 mg/kg), and four excisional circular (6 mm diameter) skin wounds were created 1 cm off the midline of the dorsum under sterile conditions; wounds were created to the level of the panniculus muscle. After surgery, wounds were swabbed with an antiseptic surgical scrub and left without a dressing. All surgeries were conducted between 0800 and 1100 h to alleviate the potential effects of diurnal rhythm. NTX or sterile saline was dissolved into Neutrogena moisturizing cream[3] and topically applied 3× a day for 20 days to the wounded area. For each rat, wound treatment was randomized, but two of the wounds received NTX and two wounds received sterile saline in moisturizing cream daily at 0800, 1200, and 1700 h.

Rewounding studies were initiated 13 weeks following induction of hyperglycemia and were conducted to determine the integrity of skin following topical NTX treatment. In subgroups of rats from both T1D and normal cohorts, a 6-mm diameter wound was created in the region previously wounded. Wounds were not treated and were allowed to heal without bandages. Photographs to monitor closure were taken immediately following wounding and on days 2, 4, 6, 8, 10, 12, 14, and 16.

Histopathology

Skin was harvested from animals for histopathology on days 20 and 60 following the initial surgery. Tissue specimens were collected from at least six wounds/treatment at each time point, representing 3–6 rats/treatment group. Animals were euthanized with a 0.5 mL i.p. injection of Euthasol® (Virbac AH, Inc, Fort Worth, TX), and subsequently exsanguinated. A 3-cm^2 region of skin encompassing the original (or rewounded) wound was removed and bisected, fixed in 10% neutral buffered formalin and processed for paraffin embedding.[4] In order to compare similar regions of dense irregular connective tissue, the deepest most peripheral region of the reticular dermis was analyzed. Skin sections (10 μm thick) were stained with hematoxylin-eosin or Sirius red to assess collagen formation and maturation[18]; some sections were immunohistochemically

stained with antibodies to MMP-2 (1 : 200 dilution, Abcam, Cambridge, MA) followed by goat anti-mouse (1 : 1000; Abcam) antibodies to detect MMP-2+ fibroblasts.[19–21]

Tensile strength

To assess collagen maturation and skin integrity, the tensile strength of wounded and unwounded tissue from T1D and Normal animals was recorded approximately four weeks (26–32 days) and seven weeks (55–60 days) following initial surgery using published protocols.[18] At each time point, the wounded area and surrounding tissue were dissected, and the specimen was dissected into strips (~5 mm wide × 15 mm long); the width was slightly smaller than the original biopsy diameter so that peripheral unwounded skin did not reinforce the granulation tissue during testing. This produced a specimen where failure would occur at the central wounded region and stress accumulation at the jaws of the grips was avoided. Specimens were dissected from the dorsum parallel to the vertebral column. For tensile testing, wounded skin samples were placed in two vertically oriented clamps with toothed-grips and were stretched at a low speed (0.5 mm/s) using a precision mechanical test machine (LMI Electroforce, Bose, Eden Prairie, MN). Clamps were positioned with a 5-mm gauge length. Testing of tensile strength utilized the Win-Test 4.0 logging software to record the load applied to the skin and the force that produced failure. Wounds were moistened lightly with Sorenson's phosphate buffer for no longer than 1 h prior to testing.

Statistical analysis

Data were analyzed using one- or two-way analyses of variance, with *post hoc* comparisons assessed with Newman-Keuls tests.

Results

Rats injected with STZ were rendered diabetic within 48 h, with their blood glucose levels being 5-fold that of Normal rats (~118 mg/dL) within seven days. T1D rats had blood glucose levels > 500 mg/dL throughout the eight-week study, and one T1D rat died over the course of the eight-week experiment presumably from complications related to an abscess at the injection site. Full-thickness wounds were created and monitored for 20 days. Comparable to earlier data,[3,4] wound closure was delayed for T1D rats relative to closure rates for Normal animals. By day 12 after surgery, mean residual wounds were 5% or less of their original area for both groups of Normal and T1D rats receiving NTX relative to animals in the Normal + Vehicle group, whereas T1D animals treated with topical saline had residual wounds that were ~15% of their original size. On day 14, the residual wound area was negligible for all groups except T1D rats receiving saline where an 8% residual wound was noted; by day 18 T1D wounds were closed.

MMP-2 expression

An initial process of the remodeling phase is secretion of MMPs to facilitate angiogenesis. Specifically, MMP-2 is secreted by fibroblasts and vascular endothelial cells.[19–21] MMP-2, also termed gelatinase A or 72 kD type IV collagenase, is active in the breakdown of extracellular matrix related to the basement membrane and maturing dermis, necessary for preparing the wound bed for vascularization and new collagen formation. Granulation tissue collected 20 days following wounding was stained (Figure 1(a)) with antibodies to MMP-2, and data revealed that T1D + Vehicle rats had 2.5-fold more MMP-2+ fibroblasts than observed in tissue sections from Normal + Vehicle controls. Moreover, treatment with topical NTX reduced the number of cells labeled by MMP-2 antibodies within 20 days of application to levels comparable to controls (Figure 1(b)).

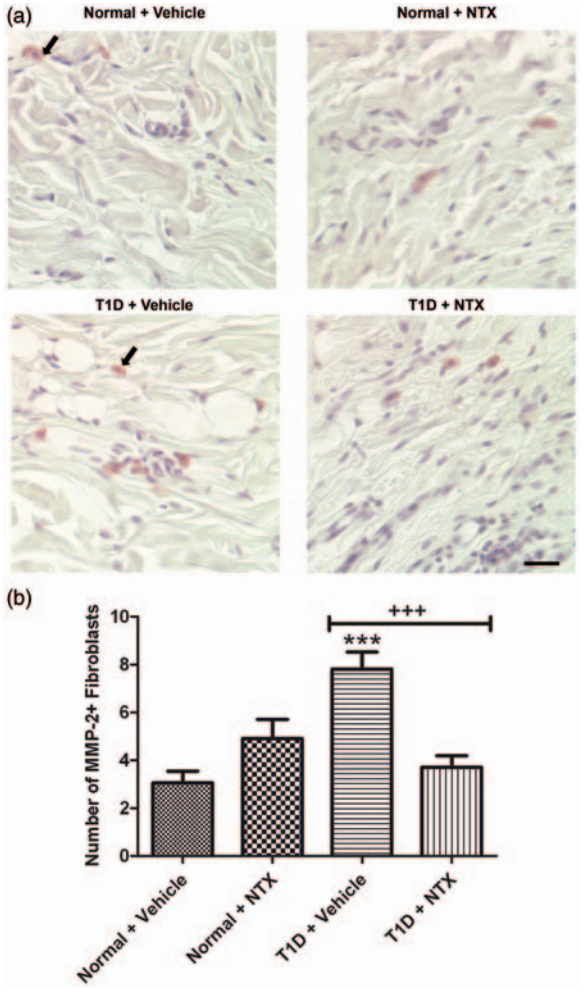

Figure 1 MMP-2+ fibroblast proliferation in granulation tissue following topical treatment with NTX. Full-thickness cutaneous wounds created in T1D and Normal rats were treated topically with 10^{-5} M NTX dissolved in moisturizing cream or cream alone (Vehicle). (a) Photomicrographs of granulation tissue in the reticular dermis of the wound bed at 20 days. Arrows indicate MM-2+ stained fibroblasts; bar = 25 μm. (b) Number of MMP-2+ fibroblasts per field in granulation tissue 20 days following wounding. Values represent means ± SEM. ***Significantly different from Normal + Vehicle values at $P < 0.001$. +++Significantly different between T1D + Vehicle and T1D + NTX at $P < 0.001$. (A color version of this figure is available in the online journal)

Collagen maturation – Sirius red staining

Collagen formation and maturation were evaluated by Sirius red staining that utilizes a polarized light microscope to determine the color of birefringence emitted from individual collagen fibers.[18] Red emissions indicate greater organization of collagen fibrils relative to the appearance of orange/yellow or green in other specimens. After 20 days following wounding, sections of wounded skin from Normal or NTX-treated T1D rats displayed wound images with mature collagen relative to the newly synthesized or immature collagen fibers (primarily green region) associated with T1D rat wounds receiving vehicle (Figure 2(a)). Higher magnification of the wound bed revealed disorganized collagen in comparison to more interwoven collagen fibers in the T1D + NTX specimen (Figure 2(b)). Analyses of surface area conducted with ImagePro 6.2 software indicated 20–30% mature collagen in Normal + Vehicle and T1D + NTX skin sections, and less than 2% in the T1D + Vehicle specimens.

Sixty days following wounding, and 40 days after termination of NTX treatment, Sirius red-stained sections in the Normal + Vehicle and T1D + NTX group had mature collagen across more than 40% of the wound region (Figure 3(a) and (b)), whereas specimens from T1D + Vehicle rats displayed 10% or less mature collagen fibers (i.e. emitting red birefringence under polarized light).

Wound integrity – tensile strength

Tensile strength was measured in unwounded skin of Normal and T1D rats at four and eight weeks following wounding; at the later time point, T1D rats were hyperglycemic for more than 14 weeks (Figure 4(a) and (b)). Data revealed that there were no differences in tensile strength between T1D and Normal rats, with tensile strength measuring 23.5 ± 2 N. Untreated, but wounded, skin demonstrated reduced tensile strength at both four weeks and eight weeks (Figure 4(c) and (d)) relative to that of unwounded skin ($P < 0.05$). The tensile strength of T1D + NTX rats was comparable to that for wounded skin from Normal + Vehicle rats at both four and eight weeks (Figure 4(e) and (f)). However, the tensile strength for T1D + Vehicle wounds was 7.3 ± 1.0 N, and significantly less ($P < 0.05$) relative to the force required to tear T1D + NTX treated specimens at four weeks (Figure 4(e)). By eight weeks following wounding, the integrity of diabetic skin as measured by tensile strength was markedly less in T1D rats receiving vehicle in comparison to T1D rats treated with NTX (Figure 4(f)).

Evaluation of stiffness (N/mm) suggested that no differences were noted between normal or T1D + Vehicle specimens. Stiffness measurements for T1D + NTX specimens demonstrated increased collagen at 60 days in this group.

Wound integrity following rewounding

To evaluate whether the skin of T1D rats that was previously wounded and treated with NTX exhibited any form of abnormal pathology two or three months following the surgery, wounded regions on T1D animals that were treated with either NTX or vehicle were rewounded using the same

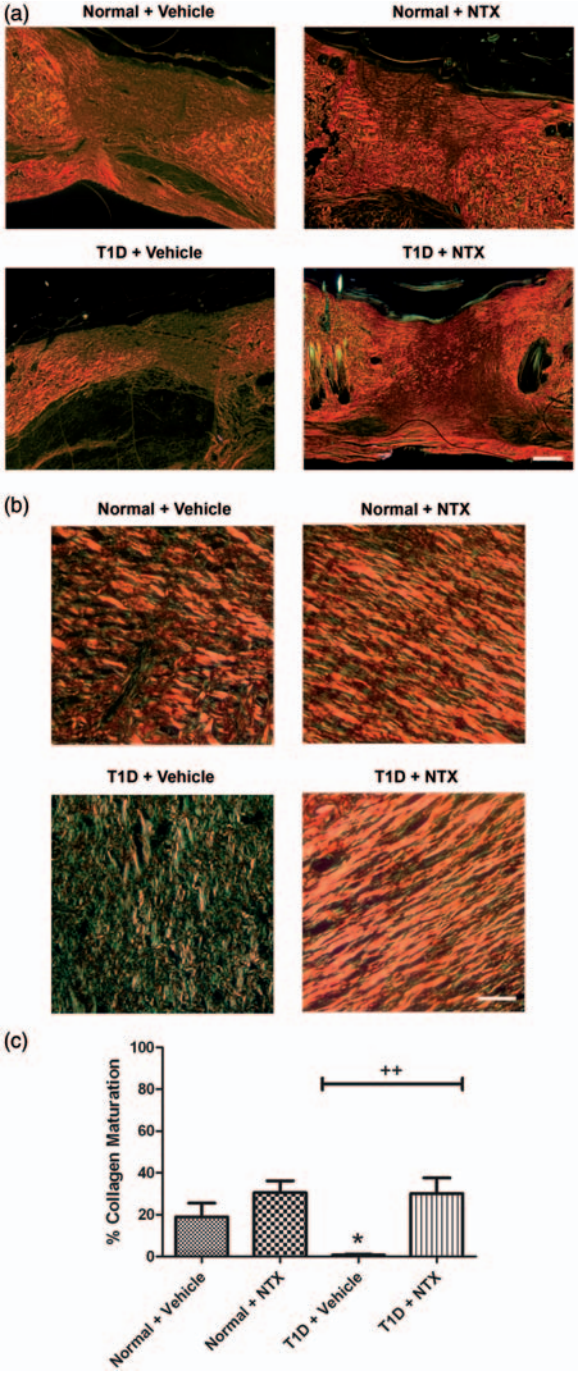

Figure 2 Remodeling of full-thickness wounds created in T1D and Normal rats as measured by collagen formation in the reticular dermis 20 days after wounding and treatment with NTX dissolved in moisturizing cream or cream alone (Vehicle). Wounds from Normal and T1D rats were treated three times daily with either 10^{-5} M NTX or sterile saline (Vehicle) dissolved in moisturizing cream. (a) Low magnification (4×) photomicrographs of Sirius red birefringence of skin sections encompassing the full-thickness wound and peripheral unwounded skin; bar = 100 μm. (b) High magnification of collagen maturation in Sirius red stained sections described in (a); bar = 25 μm. (c) Histograms of the percent collagen maturation analyzed by Image J at 20 days. Values represent means ± SEM. *Significantly different from Normal + Vehicle values at $P < 0.05$; ++significantly different between T1D + Vehicle and T1D + NTX at $P < 0.01$

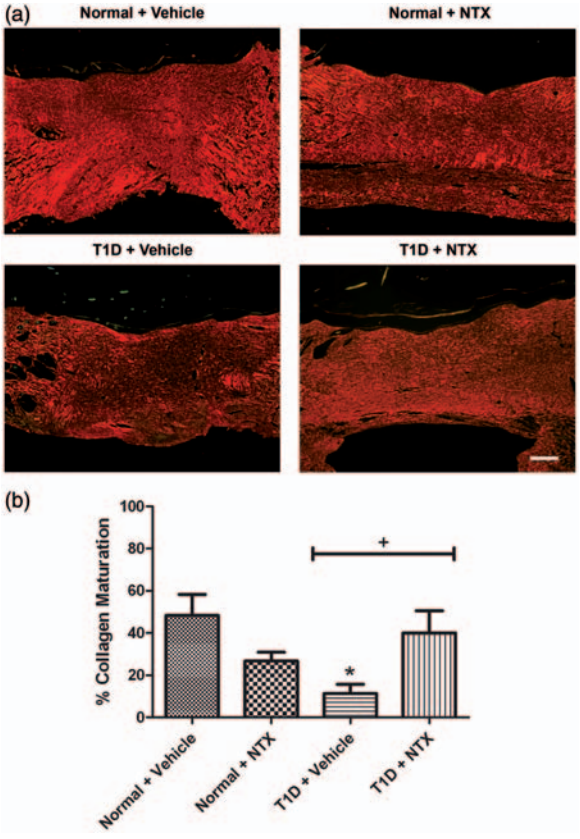

Figure 3 Collagen organization in wounded tissue two months following full-thickness cutaneous wounding in T1D and Normal rats. (a) Sirius red staining of dense irregular connective tissue in skin sections encompassing the full-thickness wound and peripheral unwounded skin. Wounds from Normal (N) and T1D rats were treated three times daily with either 10^{-5} M NTX or sterile saline (Vehicle) dissolved in moisturizing cream; bar = 100 μm. (b) Histograms of the percent collagen maturation analyzed by Image J at 20 days. Values represent means ± SEM. *Significantly different from Normal + Vehicle values at $P < 0.05$

protocol and examined 13 weeks after initiation of the studies. At the time of rewounding, T1D rats had a 28% reduction ($P < 0.001$) in body weight relative to Normal rats that weighed 498 ± 20 g.

Measurement of the residual defects following the initial wounding procedure in T1D animals with wounds treated with Vehicle showed delays in closure relative to wound closure in the T1D + NTX group (Figure 5(a) and (b)). After the second wounding, wounds created in skin that had previously been treated with NTX had closure times comparable to that of T1D + Vehicle wounds from both the first and second rounds of wounding (Figure 5(b)). Visual inspection of the skin did not reveal any histological changes between re-epithelialization of initial wounds or rewounded regions, indicating that epithelial tissues were not compromised by NTX treatment.

Tensile strength testing of rewounded tissue revealed that neither NTX nor topical application of Vehicle alone during the first round of wounding altered the integrity of the skin, suggesting that there were no long-term changes observed after 20 days of NTX treatment. Tensile strengths of Normal + NTX wounds were comparable to Normal + Vehicle wounds, and measurements for T1D + NTX wounds were comparable to T1D + Vehicle wounds (Figure 5(c)).

Discussion

Data from the present experiments, along with evaluation of wound repair during phases of proliferation[2] and angiogenesis,[4] demonstrate that wound healing is regulated by opioids and their receptors in both normal and abnormal (i.e. T1D) animal models. Both the pace of wound closure and the timetable of angiogenic events are modulated by blockade of opioid and opioid receptor interfacing.[4] Utilizing topical applications of NTX, a long-acting, potent opioid antagonist that block opioid receptors, cell proliferative events that culminated in collagen deposition and maturation were accelerated and/or enhanced. Topical NTX treatment of cutaneous full-thickness wounds in T1D rats reduced the presence of MMP-2+ cells that induce scar formation, and accelerated collagen formation and maturation. Tensile strength in T1D + NTX skin samples was increased relative to that reported for skin of T1D + Vehicle animals.

Diabetes is known to be accompanied by delayed wound closure,[1,2,4,11,18] and impaired wound healing is a major complication of diabetics.[1,22] Approximately 8% of elderly diabetic persons have foot ulcers, with 2% requiring amputation.[1] The cause of this complication is unknown but increased apoptosis, as well as reduced angiogenesis and decreased formation of collagen have been cited as major contributing factors to the delayed wound closure.[18,22–26]

Evidence supports the hypothesis that wound healing is aberrant in T1D, in part, because of elevated concentrations of circulating enkephalins[5–7] which are known to delay epithelial cell proliferation.[27,28] Studies confirm that blockade of at least one enkephalin, OGF, from interaction with its receptor, OGFr, upregulates DNA synthesis in epithelial tissues,[28] corneal surface abrasions,[11,29] and full-thickness wounds.[3,4] Thus, at least one peptide–receptor interaction appears to maintain the pace of wound closure and play a role in proliferation of mast cells, endothelial and smooth muscle cells necessary for angiogenesis, and fibroblasts for collagen formation. Previous studies have determined the presence and location of OGF and its receptor in the skin, indicating that the opioid-receptor signaling pathway is the OGF-OGFr axis.[30] Thus, the OGF-OGFr axis has a governing role in the epithelialization phase of wound closure, and data from these studies are consistent with the pathway being involved in proliferation and organization of connective tissue in the dermis.

Events in wound healing need to be regulated for their duration and level of activity. One example is inflammation, and several reports indicate that growth factors are an effective means for accomplishing this control.[22,23,26] Although inflammation is important for healing in order to bring cytokine-secreting cells to the area, excess inflammation may be detrimental to repair processes. Delayed cellular infiltration is not advantageous, just as hypertrophic scar formation is aberrant, thus the roles of proliferation and apoptosis are in a fine balance.[23]

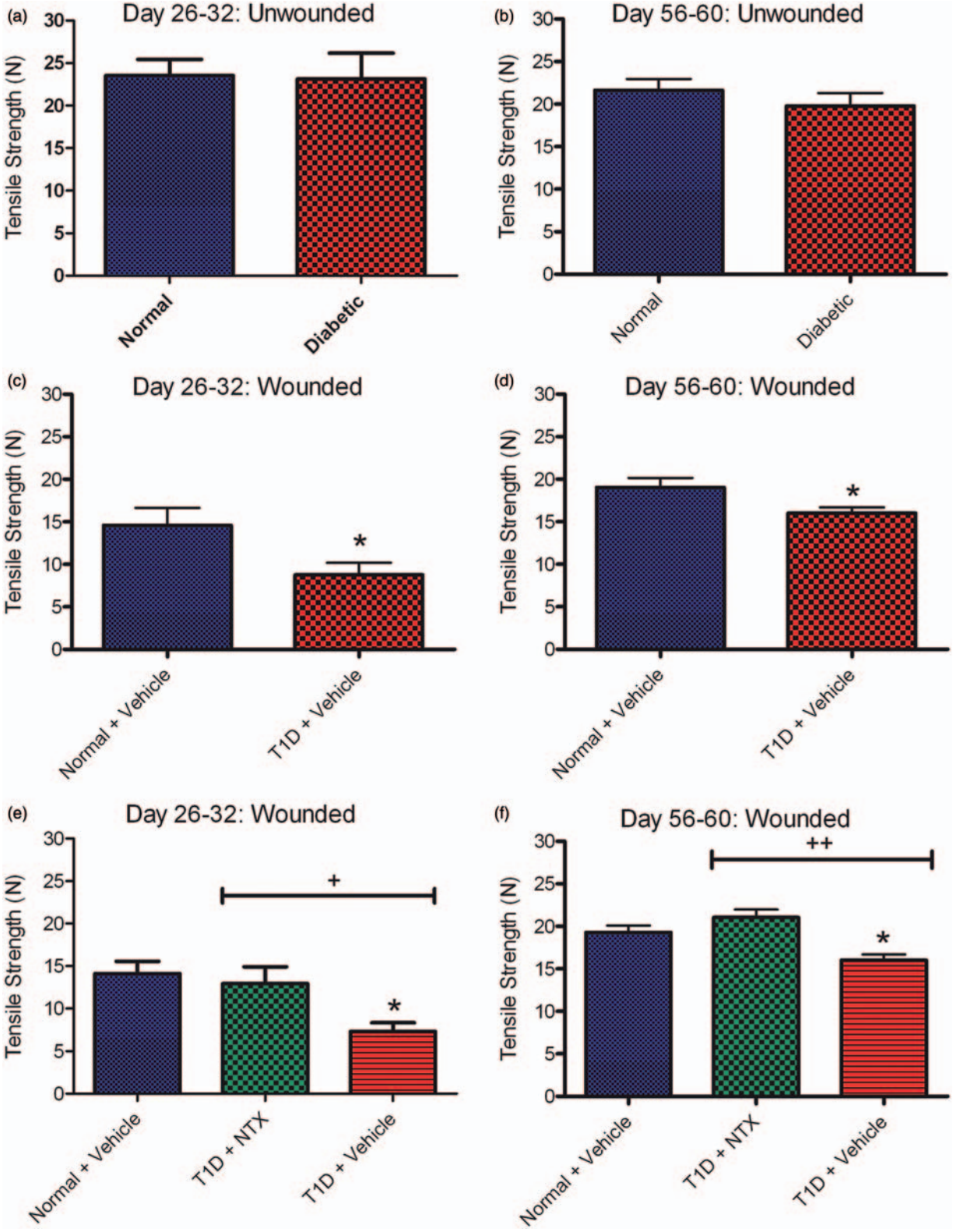

Figure 4 Tensile strength of cutaneous wounds treated for 20 days with either 10^{-5} M NTX or sterile saline dissolved in moisturizing cream. (a,b) Tensile strength (Newtons) of unwounded skin from Normal and T1D rats at 26–32 days (a) and 56–60 days (b). (c, d) Comparison of tensile strength in wounded skin from Normal (N) and T1D rats at 26–32 days (c) or 56–60 days (d) following creation of a 6 mm diameter full-thickness cutaneous wound. (e,f) Tensile strength of wounded skin from Normal or T1D rats treated with 10^{-5} M NTX or sterile saline dissolved in moisturizing cream at 26–32 days (e) and 56–60 days (f). Values represent means ± SEM for six or more wounds per group. *Significantly different from Normal + Vehicle at $P < 0.05$; significantly different from T1D + NTX at $P < 0.05$ (+). (A color version of this figure is available in the online journal)

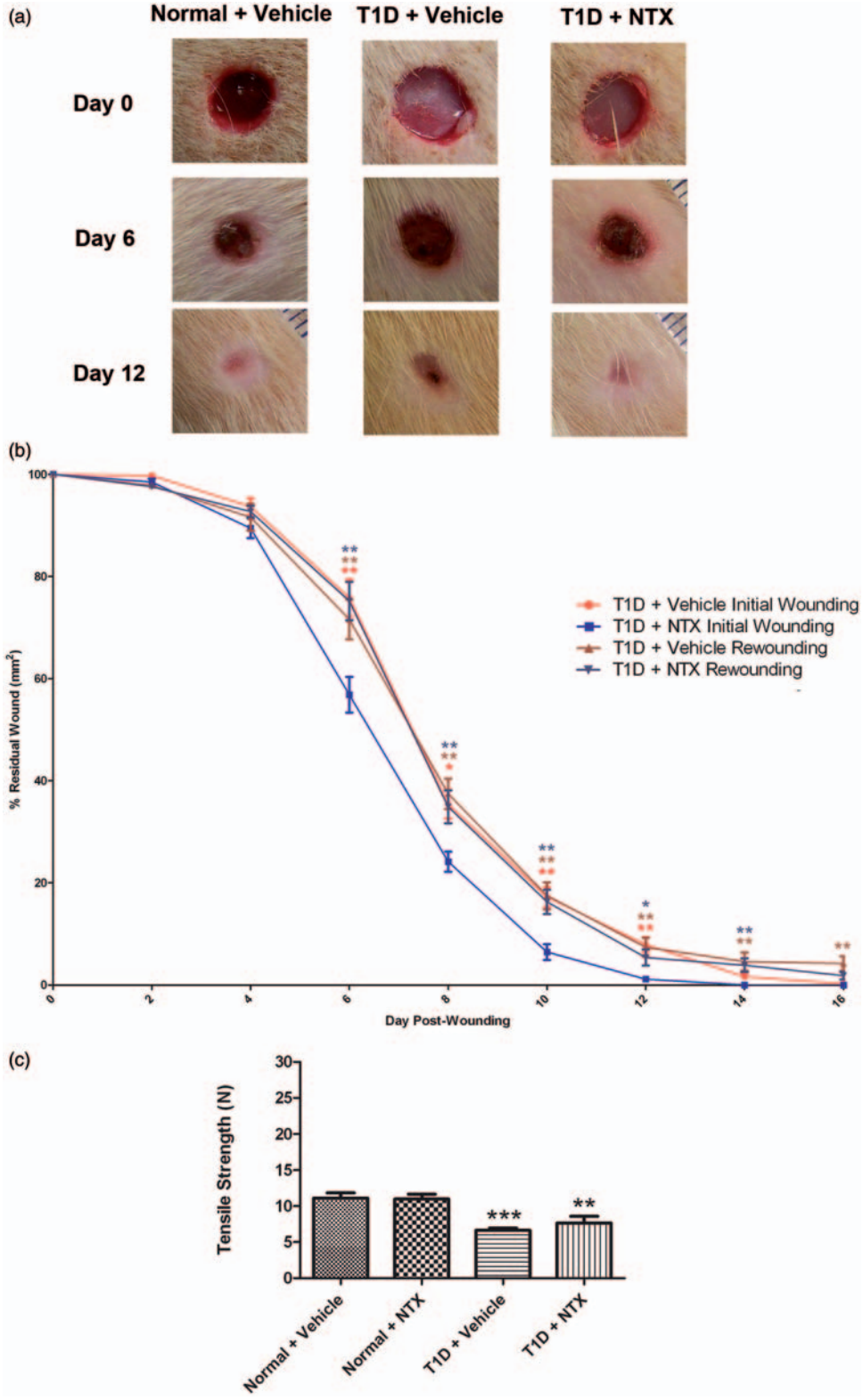

Figure 5 Residual wound closure in skin previously wounded and treated with 10^{-5} M NTX for 20 days. Wounds were 'recreated' 30 days following the initial wounding. (a) Photomicrographs of residual wounds from rats in the Normal + Vehicle, T1D + Vehicle, or T1D + NTX groups. (b) Comparison of re-epithelialization in T1D rats treated with topical NTX or saline dissolved in moisturizing cream during the initial wounding experiments and the rates of closure for the untreated 'rewounded' regions. Values represent means ± SEM for at least six wounds. The only significant differences were noted for original wounds treated with NTX, relative to all other groups at $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$. (c) Tensile strength of 'rewounded' skin. Values represent means ± SEM for six wounds. **Significantly different from values for Normal + Vehicle rats at $P < 0.01$ or $^{***}P < 0.001$. (A color version of this figure is available in the online journal)

Thus, topical application of NTX to full-thickness cutaneous wounds in T1D rats results in significant reductions in wound size that correlate with histopathological findings. No adverse pathology was associated with this short-term, but chronic (i.e. 20-day) treatment with the opioid antagonist. Epithelialization and DNA synthesis are increased,[2] mast cell infiltration and angiogenesis are accelerated[4] and collagen formation and maturation are advanced. This final phase of wound healing involves formation of collagen matrix that is vital to the overall homeostasis of the wound bed and is important in establishment of skin integrity.[23]

Reductions in cellular staining of MMP-2+ in skin specimens treated with NTX suggest the inhibition of gelatinase A that leads to the formation of glial scars and can subsequently impose a physical barrier to healing.[19,21] Elevated metalloproteinase levels, reported in wound fluid from chronic leg ulcers, exacerbates degradation of adhesion molecules that are important for retaining intact skin.[20] In T1D, the number of MMP-2+ cells are elevated, perhaps supporting the delayed healing observed in these patients, and reduced in T1D + NTX rat tissues. In summary, the data are consistent with the possibility that blockade of the OGF-OGFr axis through the use of topical NTX mediates the remodeling phase of wound healing, advances collagen maturation, and increases skin integrity in T1D. Additional studies are warranted to identify specific signaling pathways that may be involved in the observed effects on the dermis.

Author contributions: All authors participated in the design and data analyses of one or more sections of the manuscript, and have reviewed the manuscript. JAI and GPL performed the research. PJM, ISZ and JAI interpreted the data and wrote the manuscript.

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