Reversal of Diabetic Dry Eye by Topical Opioid Receptor Blockade Follows Dual Pathways

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Purpose. To determine pathways in the trigeminal ganglion and corneal epithelium that are targeted by topical naltrexone (NTX) treatment for dry eye.

Methods. NTX drops were administered topically daily for 15 days to the corneal surface of male and female adult type 1 diabetic rats. Schirmer scores and corneal sensitivity were measured at baseline, 5, 10, and 15 days. Trigeminal ganglion and corneal epithelium were processed for immunohistochemistry to detect expression of opioid growth factor receptor (OGFr), Ki67, nerve growth factor, insulin-like growth factor-1, calcitonin gene-related peptide, substance P, and TNF-α. A proteomic study determined protein changes in the cornea.

Results. Corneal sensitivity and tear production in diabetic rats were restored to normal levels within 5 days after topical NTX. Assessment of corneal tissue after 15 days of treatment revealed that defects in OGFr expression, epithelial cell number, and Ki67$^+$ expression were restored to normal by NTX. Inflammation markers (e.g., TNF-α) were reduced in tissue from diabetic rats treated with NTX. Proteomic data suggest diabetes causes dysregulation in inflammatory biological processes. The percentages of calcitonin gene-related peptide–positive neurons, but not substance P–positive neurons, in the trigeminal ganglion were increased after NTX treatment. Diabetic male and female rats responded to NTX in a comparable manner.

Conclusions. Type 1 diabetes results in decreased tear production and altered corneal surface sensitivity. These complications coincide with dysregulated OGFr that maintains ocular homeostasis. Reversal of dry eye and restoration of corneal sensitivity in diabetic male and female rats after 15 days of topical treatment with NTX occur following dual pathways of increased cellular proliferation and reduction of inflammation.

Keywords: naltrexone, dry eye, trigeminal ganglion, corneal epithelium, diabetes

Type 1 diabetes is a metabolic disease that involves elevated blood glucose levels, resulting in microvascular, macrovascular, and neuropathic abnormalities.[1] In 2021 the International Diabetes Federation estimated that over 536 million people globally live with diabetes, with the prevalence in 2045 projected to be more than 1 billion individuals.[2] Dry eye disease (DED) is a common complication in diabetes. The prevalence ranges from 30% to 50% of the diabetic population and increases with age.[2–4] Evidence now suggests that the duration of diabetes (e.g., >10 years) reduces Schirmer scores indicating dry eye and decreases corneal surface sensitivity even further in comparison with patients with diabetes less than 10 years.[5] DED is characterized by tear film instability, accompanied by dysregulation in the lacrimal functional unit (LFU) and corneal surface inflammation.[6] There are various etiologies that can contribute to DED, including age, lifestyle choices, genetics, metabolic conditions, and the environment. Because of the various causes, the treatment varies depending on severity of disease.[7] Previous findings reported from our laboratory show that the inhibitory growth peptide opioid growth factor (OGF) is elevated in diabetic rat corneal tissue and serum, as well as human plasma.[8–11] Our studies have shown that sustained blockade of the OGF receptor OGFr, using topical naltrexone (NTX), reversed complications related to type 1 diabetes (T1D) associated with LFU dysregulation.[12] In diabetic rats treated with topical NTX, there was an increase in tear production, restoration of corneal sensitivity, and increased limbal cell proliferation.[12–14] Topical treatment for 10 days with NTX impacted the lacrimal gland and resulted in increased expression of aquaporin-5, a water channel protein related to secretory function.[12]

The current investigation was undertaken to determine specific pathways associated with tear production that were altered in diabetes and that could be restored after a short exposure (i.e., 15 days) to topical NTX. Reports have implicated both inflammation and depressed cell proliferation as pathways that NTX alters within a short duration of treatment.[15,16] NTX has been shown to decrease production of inflammatory cytokines by way of Toll-like receptor (TLR)7, TLR8, and TLR9 receptors located on infiltrat-

ing myeloid cells.[15] NTX also has neuroprotective effects when given after traumatic brain injury in mice, enhancing recovery and reducing neuroinflammation.[16] In addition, diabetes is associated with alterations of the trigeminal ganglion, which are associated with altered neural responsiveness.[17,18]

In this study, we further examined the mechanisms of topical NTX on a type-1 diabetic rat model. We hypothesized that inflammation, caused by diabetes, dysregulates the corneal epithelium causing alterations to neuroprotective peptides and neurotrophins that contribute to the development of DED and corneal insensitivity, is reversed by twice daily topical administration of NTX. We report that topical NTX treatment reduced TNF-α and nerve growth factor (NGF) staining intensity, increased the number of proliferating basal corneal epithelial cells, and elevated the number of positive calcitonin gene-related peptide ($CGRP^+$) cells in the trigeminal ganglion. These changes in inflammation and growth factors indicate dual pathways by which topical NTX functions to reverse DED associated signs and symptoms.

Materials and Methods

Animals and Treatment

The study was approved by the Penn State College of Medicine Institutional Animal Use Committee and all experiments conformed to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male and female Sprague-Dawley rats weighing approximately 180 g and 160 g, respectively, were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in a humidity- and temperature-controlled environment, with 12 h light-dark cycles. Rats were placed two per cage, with food and water available ad libitum.

Hyperglycemia was induced following previously reported protocols.[12,13] Briefly, animals were fasted for four hours, then given a single intraperitoneal injection of streptozotocin (Sigma-Aldrich, St Louis, MO, USA) at 60 mg/kg dissolved in citrate buffer, pH 4.5. After 72 hours, tail vein blood was sampled using a glucometer, and readings greater than 300 mg/dL indicated successful induction of hyperglycemia. Hyperglycemic rats were housed two per cage to accommodate the frequent cage changes related to increased urination. Animals that did not become hyperglycemic after 5 days were removed from the study.

After 6 weeks of hyperglycemia, animals were assigned randomly to a cohort receiving either topical NTX drops or vehicle for 15 days. Animals receiving topical NTX were designated as T1D-NTX and administered a single drop (50 µL) of NTX to the right eye, containing 5×10^{-5} M NTX, twice a day for 15 days without anesthesia at 08:00 to 09:00 and 17:00 to 18:00. Hyperglycemic and nondiabetic rats received vehicle-containing drops (50 µL) and were designated as T1D and normal, respectively. Male and female rats were treated comparably.

To address rigor and reproducibility, multiple independent experiments were conducted. Tissue was sampled across multiple experiments. Ocular tissue from 17 diabetic males, 20 diabetic females, 14 nondiabetic males, and 15 nondiabetic females were used in this study.

Ocular Surface Complications: In Vivo Measurements

Tear production and corneal sensitivity were measured following published procedures[12,13] on both males and females before treatment at 6 weeks of hyperglycemia, and on days 5, 10, and 15 after NTX treatment, with measurements made before the morning application. Measurements were recorded from unanesthetized animals.

Schirmer Tests. Tear production was measured using Schirmer strips (TearFlo, HUB Pharmaceuticals, Farmington Hills, MI, USA) that were cut to 1 mm × 17 mm in length and placed in the lower eyelid cul-de-sac for 60 seconds.[12,13] The wetting distance was recorded to the nearest one-half millimeter using the manufacturer's provided scale.

Corneal Surface Sensitivity. Corneal surface sensitivity was recorded using a Cochet–Bonnet aesthesiometer (Boca Raton, FL, USA).[12,13] The amount of force (g/mm^2) required to prompt the blink reflex was considered indicative of corneal sensitivity, with higher values indicating greater corneal insensitivity. Each measurement was repeated three times and then averaged. The sensitivity (g/mm^2) was calculated using the length of the filament and the conversion scale provided by the manufacturer.

Morphological Alterations in the Cornea

The day after the conclusion of topical treatments, all rats were humanely euthanized using an Euthanex carbon dioxide chamber, followed by decapitation. The eyes were fixed as described by Sun et al.[19] The eyes were enucleated and immediately placed into a fixative solution consisting of 97% methanol and 3% acetic acid that was chilled using dry ice. The fixation solution plus tissue was stored at −80°C for 2 days, then step thawed for 4 hours at −20°C followed by 48 hours at 20°C and then embedded in paraffin. After enucleating eyes, trigeminal ganglia were excised and placed into 2% paraformaldehyde for 1 hour at 20°C, followed by cryoprotection in 30% sucrose for 12 hours at 4°C, and then embedded in optimal cutting temperature and stored at −80°C until cryosectioned.

Corneal morphology was assessed in hematoxylin and eosin–stained sections (4–6 µm). Basal cell number was measured as the number of cells within 200 µm. Images were photographed at 40× magnification on an Olympus BX50 microscope. Original images were analyzed using ImageJ and calibrated using the microscope scale bar. Basal cells were counted using at least three sections per eye and at least three eyes per sex for each condition.

Immunohistochemistry and Immunofluorescence

Frozen sections (10–14 µm) and paraffin sections (4–6 µm) of corneas and trigeminal ganglia were stained with validated primary antibodies for OGFr, Ki67, CGRP, substance P (SP), TNF-α, insulin-like growth factor 1 (IGF-1), and NGF followed by appropriate secondary antibodies and DAPI (Table). Procedures followed those published elsewhere.[12–14] Original images were obtained using an LSM-Zeiss Examiner.Z1 confocal microscope at 10× and 20× magnification. Images were processed with ZEN3.1 software before being exported to ImageJ.

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Table. Antibodies Used in Immunohistochemistry and Immunofluorescence

Antibody Name	Company	Dilution
Anti-OGFr	Bioss	1:200
Anti-Ki67	Thermo	1:200
Anti-CGRP	Thermo	1:500
Anti-SP	R&D Systems	1:800
Anti-TNF-α	Thermo	1:50
Anti-IGF-1	AlamoLabs	1:100
Anti-NGF	Abcam	1:200
DAPI	Sigma	1:5000
Alexafluor 488	Thermo	1:1000
Alexafluor 567	Thermo	1:1000
Alexafluor 647	Jackson Immuno Research Lab	1:200

Proteomic Study

Diabetic and nondiabetic male and female corneas were excised and flash frozen in liquid nitrogen and stored at −80°C. Individual corneas were lysed in 300 µL ice-cold 100 mM Tris-HCl pH 8.5, 8 M urea, and proteinase inhibitor cocktail (Roche, Mannheim, Germany) tablet with 2% SDS. Samples were mechanically lysed with a sonicator using three cycles of 30% power on/off every 30 seconds until homogenous. DTT was added to a final concentration of 10 mM, heated in a water bath to 95°C for 10 minutes and then cooled to 20°C. Iodoacetamide was added to a final concentration of 55 mM, and incubated at 20°C for 20 minutes in the dark. Protein was precipitated and the pellets were reconstituted in 100 µL 8 M urea and 100 mM Tris-HCl and quantified using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Pooled protein concentrations were analyzed by liquid chromatography mass spectrometry.

Raw data were processed using Proteome Discoverer 2.5 (ThermoFisher). Proteins found across all samples with a P value of less than 0.05 and a two-fold change compared with normals were examined. Proteins not recorded in at least one group were filtered out. P values were transformed into −Log10, and fold changes to Log2. Proteomic data were analyzed and depicted using modified methods from Ji et al.,[20] briefly sorting of differentially expressed proteins (DEPs) were analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID)[21] for gene ontology biological process (GOBP), and molecular function (GOMF).

Data Analysis

Immunofluorescence staining intensity was measured by optical density using ImageJ software following ImageJ protocols. Mean gray values were obtained by measuring 100 × 25 µm area of corneal epithelium. CGRP- and SP-positive ganglion cells were counted within a 500 µm^2 cross-section of the trigeminal ganglion. Total positive ganglion cells are expressed as a percentage against total ganglion cells counted from entire ganglion sections. Data from independent experiments were merged for each sex and analyzed using two-way ANOVAs between sex and condition (normal, $T1D_{vehicle}$, and $T1D_{NTX}$) with Tukey's post hoc test for multiple comparisons. Throughout the study, data are presented as means ± SEM. All analyses were performed using GraphPad Prism version 10.0 (GraphPad Software Inc.); A P value of less than 0.05 was considered statistically significant.

Results

Clinical Signs

Recorded weights for each condition and sex are presented in Figure 1A. Before the induction of hyperglycemia, male rats weighed approximately 178 ± 7 g and female rats weighed 241 ± 11 g. Eight weeks later, normal male rats weighed 473.3 ± 32.0 g and T1D males weighed significantly less ($P = 0.001$) than normals, at 334.8 ± 55.0 g. Eight weeks after induction, T1D female rats weighed significantly less than normals ($P < 0.0001$), at 343.3 ± 21.0 g and 235.0 ± 35.0 g for normal and T1D rats, respectively. Blood glucose levels recorded 72 hours after hyperglycemia induction were approximately 422.2 ± 77.8 and 383.0 ± 112.8 mg/dL for induced male and female rats, respectively, and 138.0 ± 26.0 and 131.0 ± 16.0 mg/dL for noninduced male and female rats (Fig. 1B). NTX treatment had no effect on blood glucose levels or weight in either sex.

Corneal Sensitivity and Mean Tear Production

Corneal sensitivity was analyzed using a two-factor ANVOA (Fig. 1C) between sex, treatment (vehicle, NTX), and time. There were no differences in sex or interactions between sex, treatment, and time. There was a significant interaction between time and treatment ($P < 0.0001$). Subsequent analyses with Tukey's multiple comparisons test found significant increases in corneal sensitivity in males treated with NTX on days 5 ($P = 0.0014$), 10 ($P = 0.0006$), and 15 ($P < 0.0001$). Similar analysis with females revealed significant increases in corneal sensitivity on days 10 ($P = 0.0002$) and 15 ($P = 0.0001$). Two-factor ANOVAs with repeated measures comparing normal males treated with vehicle compared with NTX-treated males showcased significant differences on day 5 ($P = 0.0007$), but not days 10 and 15. Normal females compared with NTX-treated females had significant differences at day 5 ($P = 0.0221$), but not on days 10 and 15.

After 6 weeks of hyperglycemia, tear production was recorded at baseline, and after days 5, 10, and 15 of NTX or vehicle treatment for all rats; values are presented in Figure 1D. Three-factor ANOVA between sex, condition (normal, $T1D_{vehicle,}$ and $T1D_{NTX}$), and time were performed. There was no significant interaction between sex and time or treatment. Three-factor ANOVA revealed a significant interaction between time and treatment ($P = 0.0001$). Subsequent two-factor ANOVAs indicated a significant increase in tear production in NTX-treated males when compared with vehicle at days 5 ($P = 0.009$), 10 ($P = 0.0074$), and 15 ($P = 0.0003$). ANOVAs performed on females indicated NTX treatment significantly increase in tear production when compared with vehicle at days 5 ($P < 0.0001$), 10 ($P = 0.0071$), and 15 ($P = 0.0001$). Two-way ANOVAs comparing normal males receiving vehicle with NTX-treated males found no significant differences at days 5, 10, and 15. A similar analysis comparing normal females receiving vehicle to NTX-treated females found significant differences at day 5 ($P = 0.0021$) and 10 ($P = 0.0027$), but not day 15.

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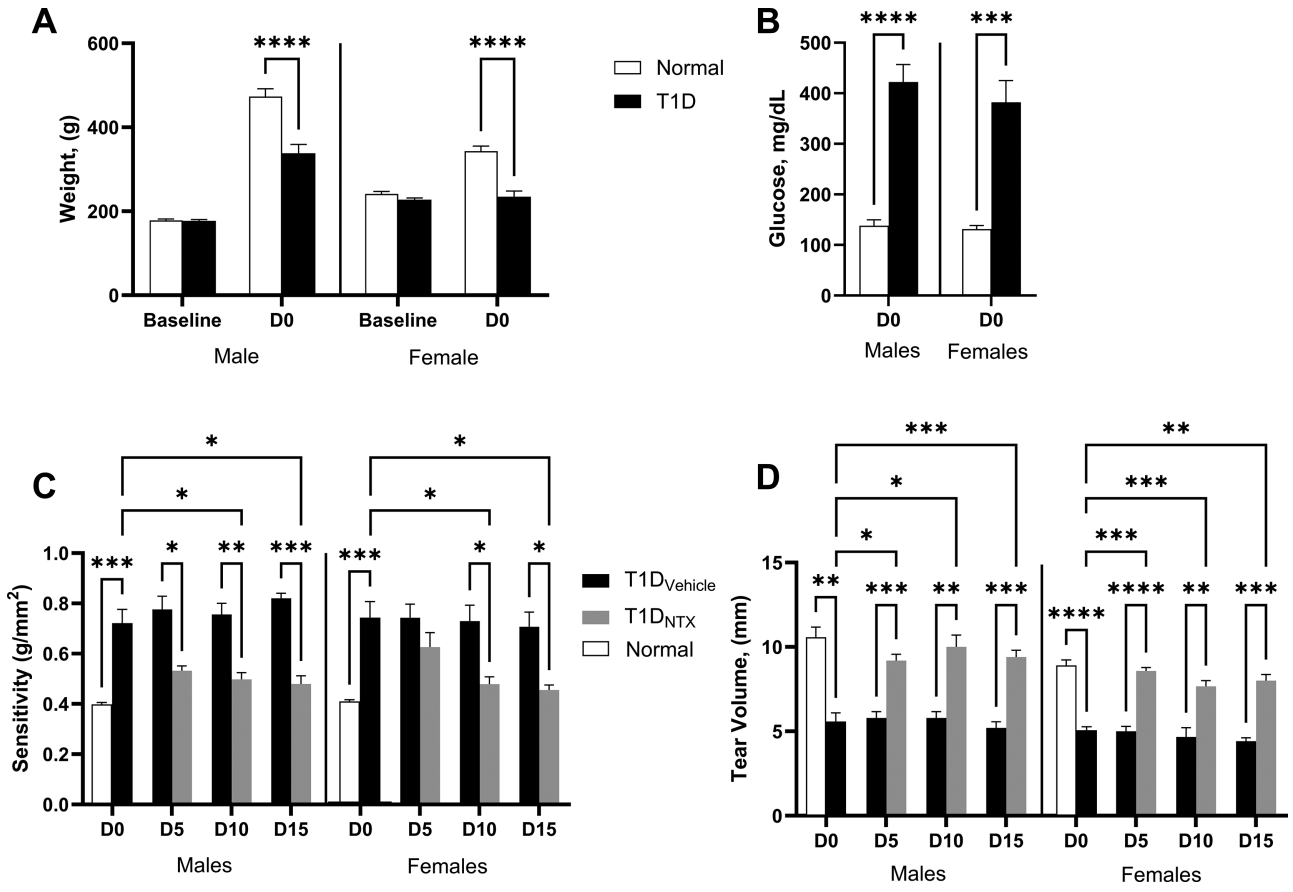

Figure 1. Clinical signs indicating disease and recovery. (**A**) Weights (g) and (**B**) blood glucose levels recorded at before induction (baseline) and at 6 weeks (baseline [D0]) after hyperglycemic induction in male and female rats. (**C**) Corneal surface sensitivity (g/mm^2) and (**D**) tear volume (mm) measured after 6 weeks of hyperglycemia. Values were collected before treatment (D0) from normals and T1D groups and on days 5, 10, and 15 days of NTX treatment in both sexes of T1D rats. Histograms represents means ± SEM for at least four rats per sex per group. Data were analyzed using a two-factor ANOVA (condition, sex), with the post hoc Tukey's multiple comparisons test. Significant differences on each day were noted as *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; or ****$P < 0.0001$.

Corneal Morphology and Immunohistochemistry

Corneal Epithelium. Analyses of hematoxylin and eosin–stained sections of female and male corneal epithelium demonstrate the effect of hyperglycemia on cell diameter and cell number (Fig. 2A). The numbers of basal corneal epithelial cells are presented in Figure 2B. Two-factor ANOVAs indicated no significant interaction between condition (normal, $T1D_{vehicle}$, and $T1D_{NTX}$) and sex ($P = 0.256$), but revealed that sex and condition significantly impacted treatment ($P < 0.0001$ and $P < 0.0001$, respectively). Post hoc multiple comparisons show diabetic females treated with vehicle had significantly fewer basal cells ($P < 0.0001$, n = 23.38 basal cells per 200 µm) compared with NTX-treated females. NTX treated females were significantly different from normal females ($P = 0.0082$). Data also showed significantly fewer basal cells in vehicle-treated diabetic males ($P < 0.0001$; $n = 27.27$ per 200 µm) when compared with NTX treated males ($n = 32.15$ per 200 µm). There was no significant difference between normal and NTX-treated diabetic males.

Decreased cell proliferation is reported in diabetic models.[22] Figure 2C demonstrates Ki67-positive staining in female and male corneal epithelium from all conditions. Figure 2D presents the mean number of replicating basal epithelial cells, counted within the paracentral region of the cornea in both sexes. Two-factor ANOVA revealed no significant interaction between condition (normal, $T1D_{vehicle}$, and $T1D_{NTX}$) and sex; however, analysis indicated treatment was a significant source of variation ($P < 0.0001$). Post hoc Tukey's multiple comparisons test found diabetic females treated with vehicle were significantly different from NTX-treated diabetic females ($P < 0.0001$) with respective means of 3.1 ± 0.99 and 5.9 ± 0.99 positive cells per 500 µm. $T1D_{vehicle}$ male rats were significantly different from normals ($P < 0.0001$) and $T1D_{NTX}$ male rats ($P < 0.0001$) with a mean of 2.769 ± 8.300 positive cells per 500 µm.

The optical density of OGFr was measured in corneal epithelium for each condition and for both sexes (Figs. 2E, 2F). A two-factor ANOVA of optical density measurements revealed there was no significant interaction between condition and sex. Analysis indicate that sex ($P = 0.025$) and treatment ($P < 0.0001$) are significant sources of variation. Post hoc Tukey's multiple comparisons show there is a significant decrease in OGFr optical density in NTX-treated females ($P < 0.0001$) and males ($P < 0.0001$) when compared with same sex vehicle-treated groups. Subsequent analysis indicate there is no significant difference between normal and NTX cohorts in both sexes.

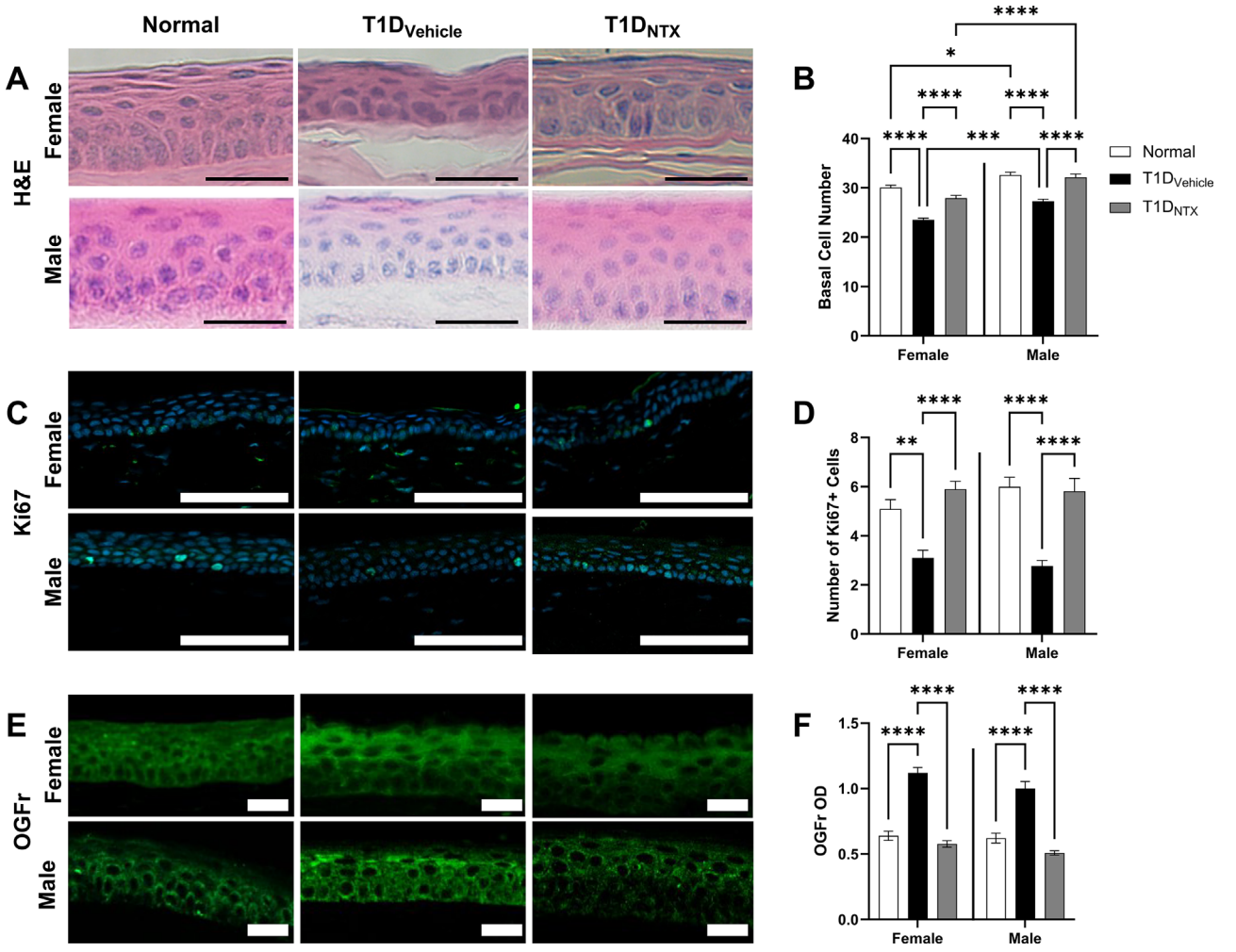

Figure 2. Morphology of corneal surface in normal and T1D animals. (**A**) Corneal basal cells were stained with hematoxylin and eosin and photographed using an Olympus BX50 microscope at an original magnification of ×40. Scale bar, 25 µm. (**B**) Histograms representing the number of basal corneal epithelial cells per 200 µm (mean ± SEM). (**C**) Representative images of Ki67^{+} staining of corneal epithelium after 15 days of NTX treatment. Scale bar, 50 µm. (**D**) Histograms represent the number per 500 µm (mean ± SEM) of Ki67^{+} cells in corneal epithelium. (**E**) Corneal tissue stained with anti-OGFr. Scale bar, 50 µm. (**F**) Histogram represents optical density for OGFr staining (mean ± SEM) in rats after 15 days of NTX treatment. Data were analyzed using a two-factor ANOVA (condition, sex), with the post hoc Tukey's multiple comparisons. Significant differences were noted as *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; or ****$P < 0.0001$.

A total of 1236 proteins were identified from the corneal samples of hyperglycemic animals (Fig. 3A). After 6 weeks post hyperglycemia induction, we detected 1235 and 1236 proteins in the normal and T1D cohorts, respectively, with 68 DEPs being identified. DEPs in corneal tissue after induction resulted in 27 upregulated DEPs and 41 downregulated DEPs (Fig. 3B, Supplemental Table S1A). To determine the effect of 6 weeks of hyperglycemia on the rat cornea, DEPs were analyzed using GOBP. For upregulated DEPs, the CRY gene family was excluded from further evaluation and assessments were focused on changes in biological processes not related to lens and visual development. GOBP found that downregulated DEPs were related to vesicle organization and localization, regulation of DNA-binding transcription factor activity, regulation of intracellular signal transduction, and cellular response to stress (Supplemental Table S2A). GOBP analysis for upregulated DEPs found T1D animals were enriched in biological processes involving immune response, intermediate filament organization, and cell killing (Supplemental Table S2B).

Further analysis interpreted molecular function using GOMF. Downregulated DEPs were associated with protein binding, enzyme inhibitor activity, molecular function inhibitor activity, cytoskeletal protein binding, and proton transmembrane transporter activity (Supplementary Table S3A). Upregulated DEPs were enriched in molecular functions related to peptidase activity, exopeptidase activity, nucleosomal DNA binding, and cysteine-type peptidase and endopeptidase activity (Supplementary Table S3B).

In our current experiments, TNF-α was measured in corneal epithelium using optical density in both sexes (Fig. 4A). Optical density measurements support current literature with NTX treatment decreasing staining intensity (Fig. 4B). A two-factor ANOVA between sex and treatment showcased no significant interactions between condition and sex; however, sex and condition were significant sources of variation ($P = 0.289$ and $P < 0.0001$, respectively). Subsequent Tukey's multiple comparisons indicated NTX-treated females had a significantly decreased optical density when compared with vehicle ($P < 0.0001$). Similar analysis

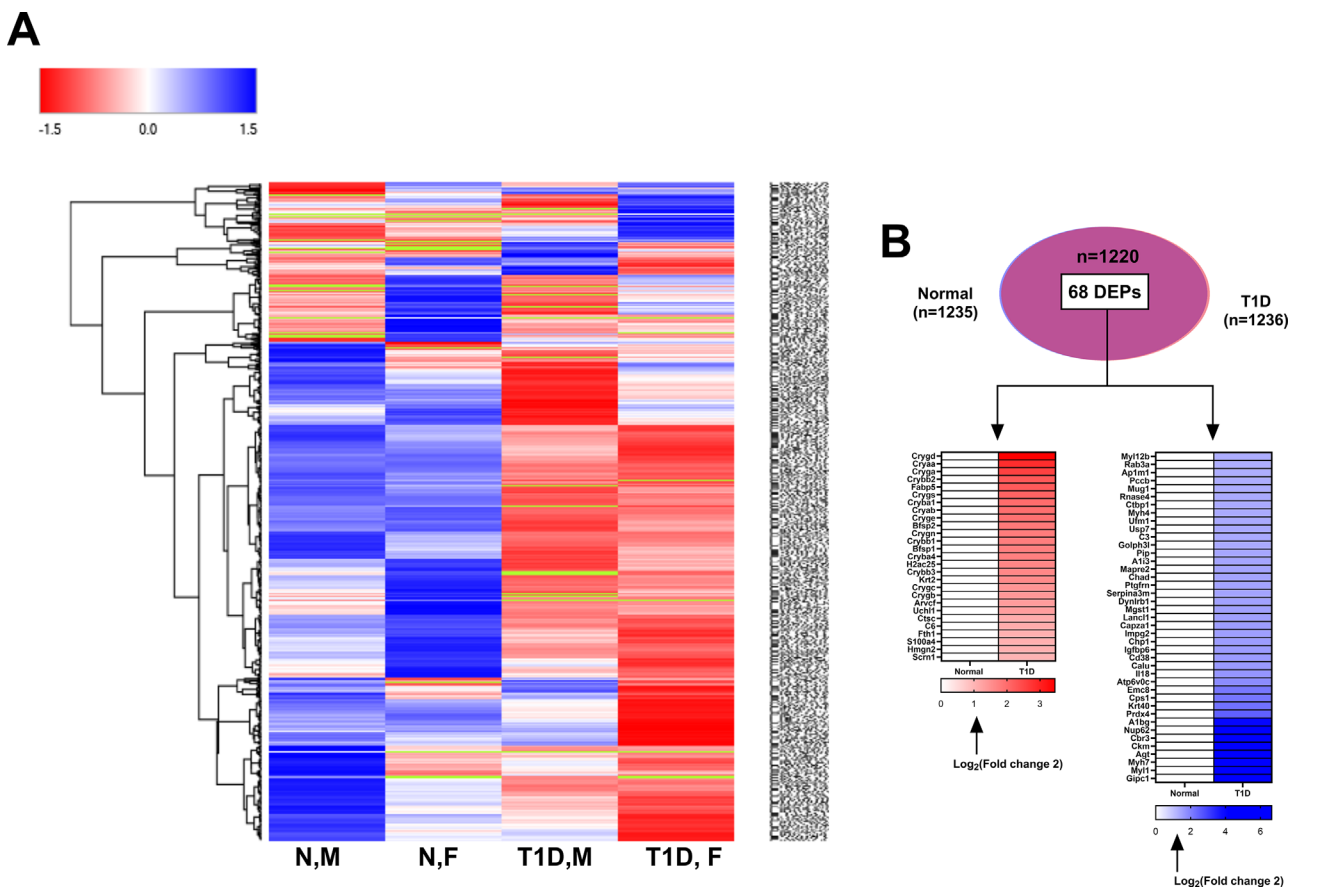

Figure 3. Isolation of DEPs in corneal tissue from normal and T1D animals in both sexes. (**A**) Heatmap of identified proteins among groups of all samples. N,F, normal female; N,M, normal male; T1D,F, T1D female; T1D,M, T1D male. (**B**) Total number of DEPs (log2 fold change: >1 or <−1; $P < 0.01$) in T1D animal groups. Heatmaps represent upregulated and downregulated DEPs in red and blue, respectively, in T1D animals compared with normals.

between NTX- and vehicle-treated males indicated significantly decreased intensity of TNF-α after NTX treatment ($P = 0.0029$). In both sexes there was no significant difference in TNF-α optical density between normal and NTX cohorts.

Representative images of corneal sections stained for NGF are shown in Figure 4C. Optical density measurements were analyzed using a two-factor ANOVA and presented as mean optical density ± SEM (Fig. 4D), which revealed no significant interaction between condition and sex. In the post hoc analysis of female normals and diabetics, elevated NGF staining intensity in T1D animals was evident when compared with normals ($P < 0.0001$) and NTX cohorts ($P < 0.0001$). Further analysis demonstrates a smaller significant difference between normal and NTX treated females ($P = 0.0187$). A similar trend is seen in males, with significantly elevated staining density in T1D groups when compared with normals ($P < 0.0001$) and NTX-treated groups ($P < 0.0001$). There was no significant difference between normal and NTX-treated males. Comparisons across sex show no significant differences.

Trigeminal Ganglion. Dysregulation of peptides in the rat trigeminal ganglion was analyzed in both sexes. For each sex and condition, CGRP was stained in rat trigeminal ganglion sections. Representative trigeminal ganglion sections containing positive CGRP ganglion cells are presented in Figure 5B. The number of positive cells was measured as a ratio of total ganglion cells, and this is presented as mean percentage ± SEM in Figure 5C. A two-factor ANOVA indicated no significant interaction between condition and sex; however, analysis shows that sex ($P = 0.0109$) and condition ($P < 0.0001$) are significant sources of variations. Post hoc analysis revealed that, in female animals, there is no significant difference between the NTX and vehicle cohorts; however, NTX animals are significantly different from normals ($P = 0.0190$). In males, a post hoc analysis indicates a significant difference between the NTX-treated and vehicle cohorts ($P = 0.0061$), but there was no difference between NTX-treated and normal groups.

Trigeminal ganglion tissue from each condition and sex were stained for SP positive ganglion cells. Sample images are presented in Figure 5D. The ratio of SP positive ganglion cells are represented in Figure 5E as the mean percentage ± SEM. A two-factor ANOVA indicated no significant interaction between condition and sex, and post hoc analysis found no significant differences between normals, and diabetic rats receiving vehicle or NTX treatment, or between the sexes.

Discussion

Diabetic dry eye is a multifactorial disease with numerous potential etiologies that may contribute to the dysregulation of ocular surface homeostasis and of the LFU.[7] Non–Sjögren syndrome aqueous deficiency dry eye is associated with reduced production and stability of tears, the develop-

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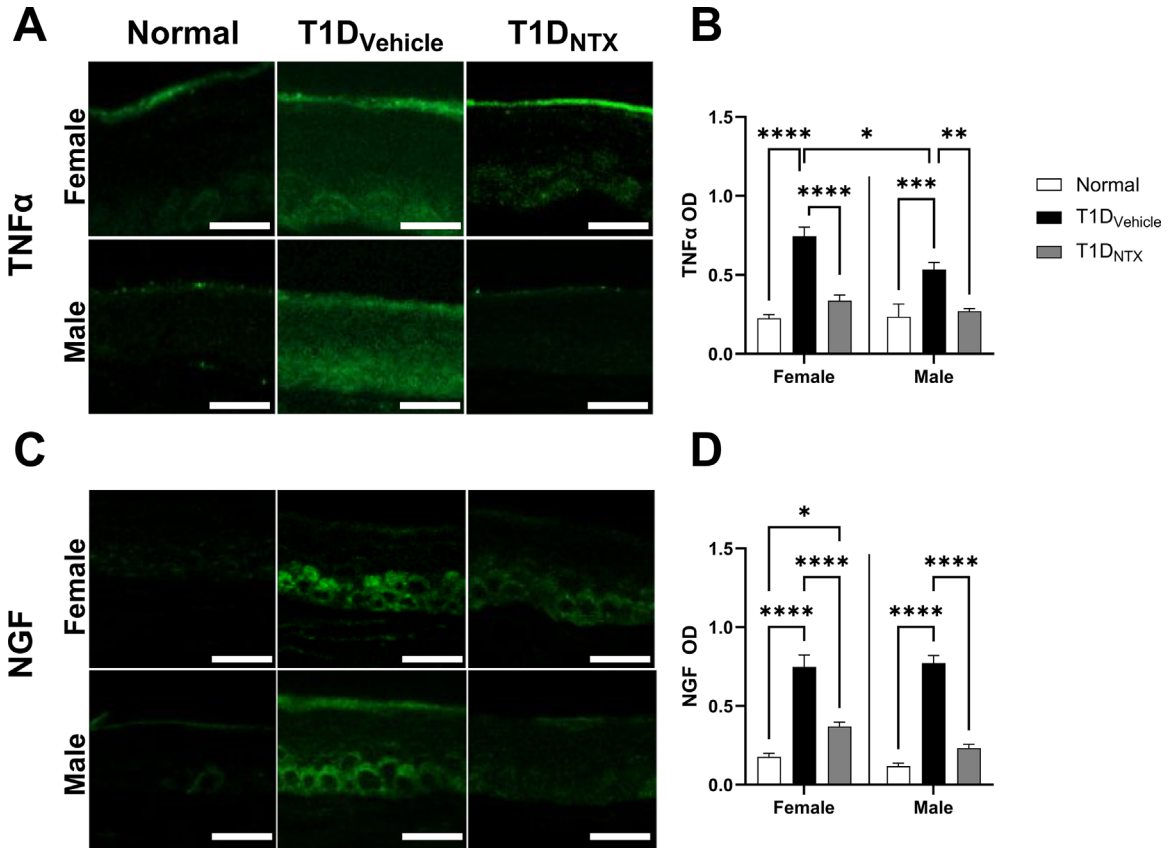

Figure 4. Representative images of normal and T1D corneal tissue from both sexes stained with (**A**) TNF-α or (**C**) NGF antibodies following 15 days of topical NTX treatment. Scale bar, 25 µm. Histograms represents optical density (OD) (mean ± SEM) for (**B**) TNF-α and (**D**) NGF. Data were analyzed using two-factor ANOVA between condition and sex, with post hoc Tukey's multiple comparisons. Significant differences were noted as *$P < 0.05$; **$P < 0.01$; ***)$P < 0.001$; ****$P < 0.0001$.

ment of corneal epithelium defects, and increased inflammatory markers.[7,23,24] Studies on the corneal structure and sensitivity in patients with type 1 diabetes showed that the corneal epithelium was thinner in diabetes with neuropathy in comparison with diabetic patients without neuropathy.[25] The interactions between corneal epithelium and the densely innervated corneal surface aid in maintaining ocular surface homeostasis.[26–28] Inflammation and dysregulation of neuromodulators on the corneal surface may play a role in the diminished corneal sensitivity, decreased reflexive tear production, and worsening of dry eye signs and symptoms.[29,30] Previous reports indicate that topical NTX treatment increased tear production in as little as 5 days, and reduced morphological and immunohistochemical alterations associated with diabetes in the rat LFU.[12–14] Owing to NTX's positive effect on cellular proliferation and wound healing, we hypothesized that topical NTX treatment would result in alterations to ocular surface growth factors, reduced inflammatory markers, and increase peptides associated with pain perception in the trigeminal ganglion of diabetic dry eye rats.

Neurotrophic factors have been identified in the corneal epithelium and are hypothesized to help maintain corneal surface homeostasis.[31–33] There is significant evidence linking diabetes with altered corneal morphology, including reduced basal cell packing density and reduced cell proliferation.[22,34,35] In support of our previous publications, the present study suggests that topical NTX reverses these morphological alterations by increasing basal cell number and increasing cell proliferation as indicated by elevated number of Ki67$^+$ cells. These results were interpreted as a return to normal after treatment with NTX.

Owing to the multiple postulated effects of diabetes and the multifactorial etiology of dry eye, including inflammation, we hypothesized that the proteomic data comparing T1D and normal rats would reveal changes to proteins associated with inflammation and immune function. The proteomic data interpreted using DAVID revealed a variable downregulation to biological processes related to cell response to stress. There was a decrease in the protein MGST1, a negative regulator of ferroptosis, which could contribute to decreased cell proliferation in the diabetic cornea.[34,36,37] Furthermore, there was a downregulation in processes related to vesicle organization and transportation, as well as synaptic vesicle cycle and clustering. We found a significant decrease in Atp6v0c in T1D animals, which supports previous studies identifying a similar reduction in diabetic mice, which is associated with a disruption in the autophagy lysosomal pathway.[38] Our study indicated the upregulation of several crystallin proteins. The effects of hyperglycemia on crystallin proteins is debated throughout various studies, but our results showcasing an increase in these proteins is supported in various publications.[39–42] We found an upregulation of alphaA- and alphaB-crystallin which are considered small heat shock proteins that support chaperoning damaged proteins to decrease

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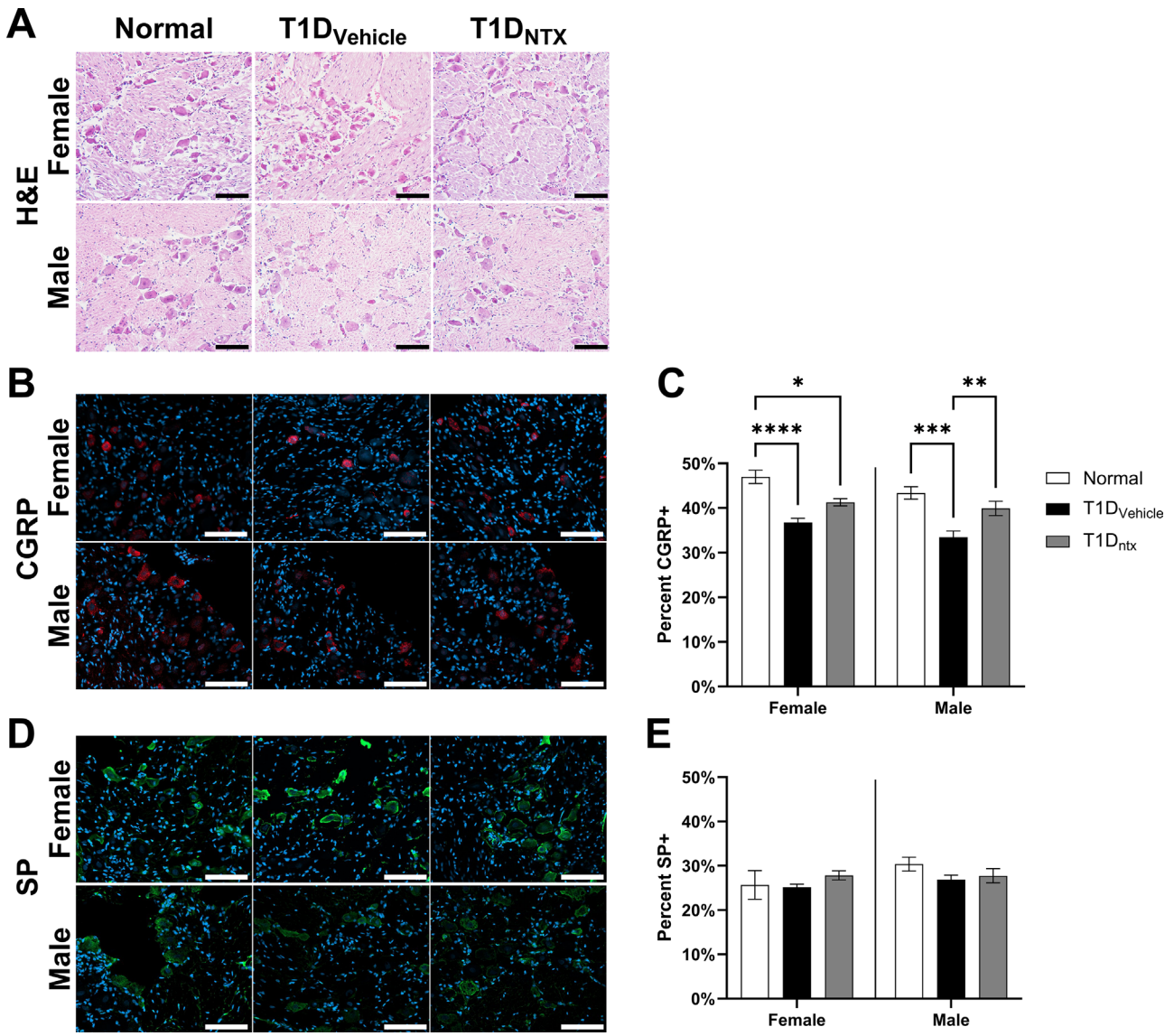

Figure 5. (**A**) Hematoxylin and eosin sections (10 µm) of the trigeminal ganglion. Representative images of the trigeminal ganglion from male and female normal and T1D rats treated with NTX or vehicle and immunohistochemically stained with (**B**) anti-CGRP or (**C**) anti-SP. Scale bar, 100 µm in (**A**) and 50 µm in (**B and C**). Red, CGRP; green, SP; blue, DAPI. Histograms represent the percent positive cells stained with CGRP (**B**) or SP (**D**); values are means ± SEM. . Significant differences were noted as $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

cellular stress.[43,44] Our proteomic results also demonstrate variability in factors related to cellular stress and inflammation. We believe this finding is due to the method of hyperglycemic induction and the length of hyperglycemia in these animals. Studies using streptozotocin to induce hyperglycemia have found protein expression to be varied, depending on the length of hyperglycemia, with publications showcasing marked elevations in proteins before 10 weeks and a significant decrease after 15 weeks.[18,45] Increasing the duration to 20 weeks has shown more significant macrophage infiltration and inflammation, as well as significant loss of nerves in the subepithelial nerve plexus.[45,46] These studies indicate that a longer time point may reveal more severe morphological and functional alterations associated with diabetes.

Based on the finding that GOBP and GOMF analyses indicated changes to inflammatory and stress processes, we explored inflammatory markers in diabetic rats. Inflammatory pathways can support corneal nerve regeneration; however, high levels of sustained inflammation can lead to further loss of nerves.[47] The importance of inflammation in the pathophysiology of DED has been extensively explored; however, data regarding the effects of NTX on these processes are sparse.[48–50] NTX was shown to attenuate inflammatory cytokines such as TNF-α and interleukin-6 after TLR4 stimulation in vitro studies.[15] Furthermore, Rodriguez et al.[16] recently found that the use of NTX reduced proinflammatory cytokines after traumatic brain injury in mice. To our knowledge, we are the first to describe topical NTX reducing TNF-α expression in the dry eye cornea. The reduction of this inflammatory marker indicates another pathway in which NTX reverses ocular surface dysregulation owing to diabetes. Decreasing inflammation is a common treatment for DED.[51] We believe this alternative pathway

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further supports the use of topical NTX to alleviate diabetic dry eye.

Altered expression of growth factors also has been reported in the diabetic cornea.[52–54] In this study, we explore peptides related to cell and neuronal proliferation. We have previously published that elevated levels of the OGF–OGFr axis are associated with diabetes.[12,13] Our current study reveals an upregulation of NGF, a neuropeptide related to neuronal regeneration, in short-term hyperglycemic animals with a significant decrease after topical NTX treatment. Studies involving the expression level of NGF in corneal tissue is sparse, with most studies indicating varied expression in tears depending on the severity of diabetes.[55–58] NGF is elevated in inflammatory diseases, such as dry eye and keratoconjunctivitis, and has been shown to be released by synovial fibroblasts after exposure to TNF-α.[59–62] Elevated NGF levels have been shown to reduce reactive oxygen species and are characteristics of apoptosis in cultured corneal epithelial cells grown under conditions of high glucose concentrations.[58] These data suggest that, independent of insulin levels, NGF ameliorates diabetic levels of inflammation and apoptosis. We believe that the increase in NGF seen in this study is compensatory mechanism owing to sustained hyperglycemia, and we postulate that the reduction in NGF seen after NTX treatment represents a return of homeostasis in the corneal epithelium and a reduction in corneal inflammation.

We explored other growth factors such as IGF-1 (data not shown). We found no significant changes in T1D animals when compared with normals regardless of NTX treatment. Studies have shown increased levels of IGF-1 in diabetic human corneas; however, it has been described that diabetic tears contain elevated levels of IGFBP-3, which significantly reduce the ability of IGF-1 to bind with IGF-1 receptor.[54,63]

We previously reported increased lacrimal gland secretory function after topical NTX treatment in diabetic rats.[12] Tear production relies on sensory innervation from the ophthalmic division (V1) of the trigeminal nerve. Afferent fibers in the cornea, conjunctiva, and eyelids transmit pain and temperature signals through various receptors.[64] As a result, these signals activate autonomic pathways that trigger the release of tears and lipids from the lacrimal and meibomian glands.[47,65,66] Corneal nerve impairment, particularly small fibers such as types C and A-δ, is considered to be impacted early in the course of diabetic keratopathy and even has been shown to be altered in human corneas before diabetes is diagnosed.[47,67–69] In this study, changes to the trigeminal ganglion associated with T1D have been explored. We have identified a significant decrease in the ratio of $CGRP^+$ trigeminal ganglion cells to all ganglion cells in T1D rats, which is reversed after 15 days of topical NTX treatment. CGRP is a peptide that is related to nociceptive systems in the cornea with studies showing a depletion after mechanical injury and an elevation during inflammatory states.[70,71] We also explored changes in SP^+ ganglions, but found no significant changes between normal and T1D groups. Studies have shown that 5 weeks of hyperglycemia is associated with a significant decrease in $CGRP^+$ and SP^+ ganglion cells, but there is a recovery by 12 weeks of hyperglycemia.[18] Our study used animals at 6 weeks of hyperglycemia at the time of treatment, which we believe allowed $CGRP^+$ ganglion cells to be increased owing to the return of homeostasis within the corneal epithelium, as seen by the increase in cell proliferation, cell density, and reduction of inflammatory cytokines.

In conclusion, our results suggest that NTX reverses dry eye through a dual pathway. One pathway promotes cell proliferation which can be seen by a blockade of OGFr, an increase in basal $Ki67^+$ cells, and a recovery in $CGRP^+$ ganglion cells. The second pathway decreases corneal inflammation as depicted by a decrease in TNF-α and NGF. Further study on corneal nerve density and other neurotropic factors will be necessary to reveal completely the mechanism(s) of NTX in reversing dry eye.

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References

1. Sapra A, Bhandari P. Diabetes. In: *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing; 2025. Available at: https://www.ncbi.nlm.nih.gov/books/NBK551501/.
2. Sun H, Saeedi P, Karuranga S, et al. IDF Diabetes Atlas: global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res Clin Pract*. 2022;183:109119.
3. Brar GK, Bawa M, Chadha C, et al. Proportion of dry eye in type II diabetics. *J Family Med Prim Care*. 2024;13:1311–1315.
4. De Freitas GR, Ferraz GAM, Gehlen M, et al. Dry eyes in patients with diabetes mellitus. *Prim Care Diabetes*. 2021;15:184–186.
5. Lyu Y, Zeng X, Li F, et al. The effect of the duration of diabetes on dry eye and corneal nerves. *Cont Lens Anterior Eye*. 2019;42:380–385.
6. Novack GD, Asbell P, Barabino S, et al. TFOS DEWS II clinical trial design report. *Ocul Surf*. 2017;15:629–649.
7. Golden MI, Meyer JJ, Zeppieri M, et al. Dry eye syndrome. In: *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing; 2025. Available at: https://www.ncbi.nlm.nih.gov/books/NBK470411/.
8. Zagon IS, Sassani JW, Purushothaman I, et al. Dysregulation of the OGF-OGFr pathway correlates with elevated serum OGF and ocular surface complications in the diabetic rat. *Exp Biol Med (Maywood)*. 2020;245:1414–1421.
9. Klocek MS, Sassani JW, McLaughlin PJ, et al. Naltrexone and insulin are independently effective but not additive in accelerating corneal epithelial healing in type I diabetic rats. *Exp Eye Res*. 2009;89:686–692.
10. Fallucca F, Tonnarini G, Di Biase N, et al. Plasma met-enkephalin levels in diabetic patients: influence of autonomic neuropathy. *Metabolism*. 1996;45:1065–1068.
11. Negri M, Tonnarini G, D'Alessandro M, et al. Plasma met-enkephalin in type I diabetes. *Metabolism*. 1992;41:460–461.
12. Diaz D, Sassani JP, Zagon IS, et al. Topical naltrexone increases aquaporin 5 production in the lacrimal gland and restores tear production in diabetic rats. *Exp Biol Med*. 2024;249:10175.

13. McLaughlin PJ, Sassani JW, Diaz D, et al. Elevated opioid growth factor alters the limbus in type 1 diabetic rats. *J Diabetes Clin Res*. 2023;5:1–10.
14. Zagon IS, Verderame MF, McLaughlin PJ. The biology of the opioid growth factor receptor (OGFr). *Brain Res Brain Res Rev*. 2002;38:351–76.
15. Cant R, Dalgleish AG, Allen RL. Naltrexone inhibits IL-6 and TNFα production in human immune cell subsets following stimulation with ligands for intracellular toll-like receptors. *Front Immunol*. 2017;8:809.
16. Rodriguez S, Sharma S, Tiarks G, et al. Neuroprotective effects of naltrexone in a mouse model of post-traumatic seizures. *Sci Rep*. 2024;14:13507.
17. Zhou Q, Yang L, Wang Q, et al. Mechanistic investigations of diabetic ocular surface diseases. *Front Endocrinol (Lausanne)*. 2022;13:1079541.
18. Troger J, Humpel C, Kremser B, et al. The effect of streptozotocin-induced diabetes mellitus on substance P and calcitonin gene-related peptide expression in the rat trigeminal ganglion. *Brain Res*. 1999;842:84–91.
19. Sun N, Shibata B, Hess JF, et al. An alternative means of retaining ocular structure and improving immunoreactivity for light microscopy studies. *Mol Vis*. 2015;21:428–442.
20. Ji YW, Kim HM, Ryu SY, et al. Changes in human tear proteome following topical treatment of dry eye disease: cyclosporine A versus diquafosol tetrasodium. *Invest Ophthalmol Vis Sci*. 2019;60:5035.
21. Sherman BT, Hao M, Qiu J, et al. DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). *Nucleic Acids Res*. 2022;50:W216–W221.
22. Nureen L, Di Girolamo N. Limbal epithelial stem cells in the diabetic cornea. *Cells*. 2023;12:2458.
23. Pflugfelder SC, de Paiva CS. The pathophysiology of dry eye disease: what we know and future directions for research. *Ophthalmology*. 2017;124:S4–S13.
24. Zhang X, Zhao L, Deng S, et al. Dry eye syndrome in patients with diabetes mellitus: prevalence, etiology, and clinical characteristics. *J Ophthalmol*. 2016;2016:8201053.
25. Rosenberg ME, Tervo TM, Immonen IJ, et al. Corneal structure and sensitivity in type 1 diabetes mellitus. *Invest Ophthalmol Vis Sci*. 2000;41:2915–2921.
26. Baker KS, Anderson SC, Romanowski EG, et al. Trigeminal ganglion neurons affect corneal epithelial phenotype. Influence on type VII collagen expression in vitro. *Invest Ophthalmol Vis Sci*. 1993;34:137–144.
27. Ko J-A, Mizuno Y, Ohki C, et al. Neuropeptides released from trigeminal neurons promote the stratification of human corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 2014;55:125–133.
28. Garcia-Hirschfeld J, Lopez-Briones LG, Belmonte C. Neurotrophic influences on corneal epithelial cells. *Exp Eye Res*. 1994;59:597–605.
29. Stepp MA, Pal-Ghosh S, Tadvalkar G, et al. Reduced intraepithelial corneal nerve density and sensitivity accompany desiccating stress and aging in C57BL/6 mice. *Exp Eye Res*. 2018;169:91–98.
30. Versura P, Giannaccare G, Pellegrini M, et al. Neurotrophic keratitis: current challenges and future prospects. *Eye Brain*. 2018;10:37–45.
31. Yu F-SX, Yin J, Xu K, et al. Growth factors and corneal epithelial wound healing. *Brain Res Bull*. 2010;81:229–235.
32. Chan KY, Jones RR, Bark DH, et al. Release of neuronotrophic factor from rabbit corneal epithelium during wound healing and nerve regeneration. *Exp Eye Res*. 1987;45:633–646.
33. You L, Kruse FE, Völcker HE. Neurotrophic factors in the human cornea. *Invest Ophthalmol Vis Sci*. 2000;41:692–702.
34. Cai D, Zhu M, Petroll WM, et al. The impact of type 1 diabetes mellitus on corneal epithelial nerve morphology and the corneal epithelium. *Am J Pathol*. 2014;184:2662–2670.
35. Cui X, Hong J, Wang F, et al. Assessment of corneal epithelial thickness in dry eye patients. *Optom Vis Sci*. 2014;91:1446–1454.
36. Zeng B, Ge C, Li R, et al. Knockdown of microsomal glutathione S-transferase 1 inhibits lung adenocarcinoma cell proliferation and induces apoptosis. *Biomed Pharmacother*. 2020;121:109562.
37. Kuang F, Liu J, Xie Y, et al. MGST1 is a redox-sensitive repressor of ferroptosis in pancreatic cancer cells. *Cell Chem Biol*. 2021;28:765–775.e5.
38. Huang X, Kuang S, Shen Z, et al. High glucose disrupts autophagy lysosomal pathway in gingival epithelial cells via ATP6V0C. *J Periodontol*. 2020;91:705–714.
39. Losiewicz MK, Fort PE. Diabetes impairs the neuroprotective properties of retinal alpha-crystallins. *Invest Ophthalmol Vis Sci*. 2011;52:5034–5042.
40. Kumar PA, Haseeb A, Suryanarayana P, et al. Elevated expression of alphaA- and alphaB-crystallins in streptozotocin-induced diabetic rat. *Arch Biochem Biophys*. 2005;444:77–83.
41. Fort PE, Freeman WM, Losiewicz MK, et al. The retinal proteome in experimental diabetic retinopathy: up-regulation of crystallins and reversal by systemic and periocular insulin. *Mol Cell Proteomics*. 2009;8:767–779.
42. Reddy VS, Reddy GB. Role of crystallins in diabetic complications. *Biochim Biophys Acta*. 2016;1860:269–277.
43. Arrigo A-P, Simon S, Gibert B, et al. Hsp27 (HspB1) and alphaB-crystallin (HspB5) as therapeutic targets. *FEBS Lett*. 2007;581:3665–3674.
44. Janowska MK, Baughman HER, Woods CN, et al. Mechanisms of small heat shock proteins. *Cold Spring Harb Perspect Biol*. 2019;11:a034025.
45. Yang H, Fan S, Song D, et al. Long-term streptozotocin-induced diabetes in rats leads to severe damage of brain blood vessels and neurons via enhanced oxidative stress. *Mol Med Rep*. 2013;7:431–440.
46. Yorek MS, Obrosov A, Shevalye H, et al. Effect of glycemic control on corneal nerves and peripheral neuropathy in streptozotocin-induced diabetic C57Bl/6J mice. *J Peripher Nerv Syst*. 2014;19:205–217.
47. Shaheen BS, Bakir M, Jain S. Corneal nerves in health and disease. *Surv Ophthalmol*. 2014;59:263–285.
48. Launay P-S, Reboussin E, Liang H, et al. Ocular inflammation induces trigeminal pain, peripheral and central neuroinflammatory mechanisms. *Neurobiol Dis*. 2016;88:16–28.
49. Ferrari G, Bignami F, Giacomini C, et al. Ocular surface injury induces inflammation in the brain: in vivo and ex vivo evidence of a corneal-trigeminal axis. *Invest Ophthalmol Vis Sci*. 2014;55:6289–6300.
50. Surico PL, Narimatsu A, Forouzanfar K, et al. Effects of diabetes mellitus on corneal immune cell activation and the development of keratopathy. *Cells*. 2024;13:532.
51. Mondal H, Kim H-J, Mohanto N, et al. A review on dry eye disease treatment: recent progress, diagnostics, and future perspectives. *Pharmaceutics*. 2023;15:990.
52. Xu K, Yu F-SX. Impaired epithelial wound healing and EGFR signaling pathways in the corneas of diabetic rats. *Invest Ophthalmol Vis Sci*. 2011;52:3301–3308.
53. Bettahi I, Sun H, Gao N, et al. Genome-wide transcriptional analysis of differentially expressed genes in diabetic, healing corneal epithelial cells: hyperglycemia-suppressed TGFβ3 expression contributes to the delay of epithelial wound healing in diabetic corneas. *Diabetes*. 2014;63:715–727.

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54. Saghizadeh M, Chwa M, Aoki A, et al. Altered expression of growth factors and cytokines in keratoconus, bullous keratopathy and diabetic human corneas. *Exp Eye Res*. 2001;73:179–189.
55. Mansoor H, Lee IXY, Lin MT-Y, et al. Topical and oral peroxisome proliferator-activated receptor-α agonist ameliorates diabetic corneal neuropathy. *Sci Rep*. 2024;14:13435.
56. Park KS, Kim SS, Kim JC, et al. Serum and tear levels of nerve growth factor in diabetic retinopathy patients. *Am J Ophthalmol*. 2008;145:432–437.
57. Kim HC, Cho YJ, Ahn CW, et al. Nerve growth factor and expression of its receptors in patients with diabetic neuropathy. *Diabet Med*. 2009;26:1228–1234.
58. Park JH, Kang S-S, Kim JY, et al. Nerve growth factor attenuates apoptosis and inflammation in the diabetic cornea. *Invest Ophthalmol Vis Sci*. 2016;57:6767.
59. Manni L, Lundeberg T, Fiorito S, et al. Nerve growth factor release by human synovial fibroblasts prior to and following exposure to tumor necrosis factor-alpha, interleukin-1 beta and cholecystokinin-8: the possible role of NGF in the inflammatory response. *Clin Exp Rheumatol*. 2003;21:617–624.
60. Lee HK, Ryu IH, Seo KY, et al. Topical 0.1% prednisolone lowers nerve growth factor expression in keratoconjunctivitis sicca patients. *Ophthalmology*. 2006;113:198–205.
61. Lambiase A, Bonini S, Bonini S, et al. Increased plasma levels of nerve growth factor in vernal keratoconjunctivitis and relationship to conjunctival mast cells. *Invest Ophthalmol Vis Sci*. 1995;36:2127–2132.
62. Minnone G, De Benedetti F, Bracci-Laudiero L. NGF and its receptors in the regulation of inflammatory response. *Int J Mol Sci*. 2017;18:1028.
63. Wu Y-C, Buckner BR, Zhu M, et al. Elevated IGFBP3 levels in diabetic tears: a negative regulator of IGF-1 signaling in the corneal epithelium. *Ocul Surf*. 2012;10:100–107.
64. Peterson DC, Hamel RN. Corneal reflex. In: *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing; 2025. Available at: https://www.ncbi.nlm.nih.gov/sites/books/NBK534247/.
65. Chang AY, Purt B. Biochemistry, tear film. In: *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing; 2025. Available at: https://www.ncbi.nlm.nih.gov/books/NBK572136/.
66. Meng ID, Kurose M. The role of corneal afferent neurons in regulating tears under normal and dry eye conditions. *Exp Eye Res*. 2013;117:79–87.
67. Mansoor H, Tan HC, Lin MT-Y, et al. Diabetic corneal neuropathy. *J Clin Med*. Epub December 6, 2020;9;3956.
68. Körei AE, Istenes I, Papanas N, et al. Small-fiber neuropathy: a diabetic microvascular complication of special clinical, diagnostic, and prognostic importance. *Angiology*. 2016;67:49–57.
69. Hossain P, Sachdev A, Malik RA. Early detection of diabetic peripheral neuropathy with corneal confocal microscopy. *Lancet*.;366:1340–1343.
70. Yuan K, Zheng J, Shen X, et al. Sensory nerves promote corneal inflammation resolution via CGRP mediated transformation of macrophages to the M2 phenotype through the PI3K/AKT signaling pathway. *Int Immunopharmacol*. 2022;102:108426.
71. Zidan AA, Zhu S, Elbasiony E, et al. Topical application of calcitonin gene-related peptide as a regenerative, antifibrotic, and immunomodulatory therapy for corneal injury. *Res Sq*. 2023;7:264.