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Therapeutic Efficacy of Topical Epigallocatechin Gallate (EGCG) in Murine Dry Eye

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Abstract

Objective—To study the efficacy of topical epigallocatechin gallate (EGCG) for treatment of dry eye disease (DED).

Methods—Female 7–8 week old C57BL/6 mice were housed in the controlled environment chamber to induce DED. Topical 0.01% or 0.1% EGCG, or vehicle, was applied to the eyes of DED mice. Corneal fluorescein staining and the number of corneal CD11b+ cells were assessed in the different groups. Expression of IL-1β, tumor necrosis factor (TNF)-α, Chemokine ligand 2 (CCL2) and VEGF-A/C/D were evaluated by real-time PCR in the corneas at day 9. Corneas were stained for LYVE-1 to evaluate lympangiogenesis, and the TUNEL assay was used to evaluate apoptosis of corneal epithelial cells.

Results—Treatment with 0.1% EGCG showed a significant decrease in corneal fluorescein staining compared with the vehicle (24.6%, P=0.001), and untreated controls (41.9%, P<0.001). A significant decrease in the number of CD11b+ cells was observed in 0.1% EGCG treated eyes, compared with the vehicle in the peripheral (23.3%, P=0.001) and central (26.1%, P=0.009) corneas. Treatment with 0.1% EGCG was associated with a significant decrease in the corneal expression of IL-1β (P=0.029), and CCL2 (P=0.001) compared to the vehicle, and in VEGF-A and -D levels compared to the untreated group (P=0.002, P=0.005, respectively). 0.01% EGCG also showed a decrease in inflammation at the molecular level, but no significant changes in the clinical signs of DED. No cellular toxicity to the corneal epithelium was observed with 0.01% or 0.1% EGCG.

Conclusions—Topical EGCG treatment is able to reduce the clinical signs and inflammatory changes in DED through suppressing the inflammatory cytokines expression and infiltration of CD11b+ cells in the cornea.

Keywords

Cornea; Dry eye disease; Epigallocatechin gallate (EGCG); Lymphangiogenesis

INTRODUCTION

Dry eye disease (DED) is one of the most common ophthalmic pathologies with about 10 million people in the United States are affected by it.[1,2] Recently, it has become widely

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recognized that DED is associated with inflammation of the ocular surface, although its precise immunopathogenesis is still not well understood.[2–5]

Recent studies show evidence for the overexpression of proinflammatory cytokines in the ocular surface of patients and animals with DED.[3, 5–9] Several inflammatory mediators such as IL-1β, IL-6, IL-17, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, Chemokine (C-C motif) ligand 2 (CCL2) and a number of matrix metalloproteinases (MMPs) have been implicated in the ocular surface inflammation associated with DED.[3,5–8] In addition, the overexpression of chemokines such as CCL2, CCL5, and Chemokine (C-X-C motif) ligand (CXCL) 9–11 could mediate leukocyte and autoreactive T cell recruitment to the ocular surface in DED. DED induces the generation of autoimmune T cells, predominantly Th17 in the draining lymphoid tissues which antagonize the regulatory T cell, and also peripheralize to the ocular surface and cause epithelial damage.[3,4,9]

Several investigations have demonstrated that epigallocatechin gallate (EGCG), one of the principal extracts of green tea, has an inhibitory effect on the inflammation related to autoimmune disorders.[10–12] In addition, some studies have demonstrated that green tea extract reduces the expression of IL-1, IL-6 and TNF-α through inhibition of NF-kB activation, and have suggested that green tea may be useful to prevent or ameliorate diseases associated with inflammatory cytokine overexpression.[13–16]

In this study, we hypothesized that topical EGCG administration may have a therapeutic effect for the treatment of dry eyes by inhibiting the proinflammatory mediators. To validate our hypothesis, we used a well characterized murine model of dry eye disease.

MATERIALS and METHODS

Mouse Model of Dry Eye

Female 7–8 week-old C57BL/6 mice (Taconic Farms, and Charles River Lab) were used in these experiments. The protocol was approved by the Institutional Animal Care and Use Committee, and all animals were managed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

DED was induced in the controlled-environment chamber (CEC) and mice also received topical application of 1% atropine sulfate (Falcon Pharmaceuticals) twice daily for the first 48 hours and subcutaneous administration of scopolamine to maximize ocular dryness for 9 days as described previously.[7,17] In brief, 0.5 mg/0.2 ml scopolamine hydrobromide (Sigma-Aldrich) was injected subcutaneously in the dorsal skin of mice three times daily. The mice placed in the CEC were continuously exposed to a relative humidity < 30%, a constant temperature of 21°C to 23°C, and airflow of 15 L/min, 24 hours a day. Age and sex-matched mice placed in the standard vivarium were used as normal controls. After 48 hours, induction of dry eye was confirmed by corneal fluorescein staining.

Corneal Fluorescein Staining

Corneal fluorescein staining was performed at baseline (day 0), 48 hours after induction of dry eye (before topical administration), day 4, and day 9. One microliter of 1% fluorescein (Sigma-Aldrich) was applied to the inferior-lateral conjunctival sac of the mice, and after 3 minutes, corneal fluorescein staining was examined with a slit lamp biomicroscope. Punctate staining was evaluated in a masked fashion using the National Eye Institute grading system, giving a score from 0 to 3 for each of the five areas of the cornea.[18]

Topical EGCG Formulations and Treatment Regimen

Topical EGCG (Sigma-Aldrich) was diluted with 1% DMSO in phosphate-buffered saline (PBS). 1% DMSO in PBS was used as the vehicle control. Both concentrations of 0.1% and 0.01% EGCG were used. Forty-eight hours after the induction of dry eye, the mice in the CEC were randomly divided into the following 4 different groups (n=3 in each group): an untreated group, a group receiving topical vehicle control, and each of two groups receiving topical 0.1% or 0.01% EGCG. Two microliters of the topical agent was applied to both eyes of each mouse twice a day (9 AM and 5 PM) from day 3 to day 9. Mice were then euthanized on day 9 for cellular and molecular studies.

RNA Isolation and Real-time Polymerase Chain Reaction

Total RNA was isolated from the dissected corneal tissue using Trizol (Invitrogen) and RNeasy Microkit (Qiagen). The first strand of complementary DNA (cDNA) was synthesized with random hexamers using SuperScript III™ reverse transcriptase (Invitrogen) and quantitative real-time polymerase chain reaction was performed using Taqman Universal PCR Mastermix and FAM-MGB dye-labeled predesigned primers (Applied Biosystems) for IL-1β (Mm00434228_m1), TNF-α (Mm99999068_m1), CCL2 (Mm00439620_m1), VEGF-A (Mm00437304_ml), VEGF-C (Mm00437313_ml), VEGF-D (Mm00438965_ml), and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1). One microliter of cDNA was loaded in each well, and the assays were performed in duplicate. The GAPDH gene was used as the endogenous reference for each reaction. The results were analyzed by the comparative threshold cycle (CT) method and the relative expression level of each sample was expressed as fold change from normal control.

Analysis of Cellular Infiltration by Immunohistochemical Staining

The following primary antibodies were used for immunohistochemical staining: fluorescein isothiocyanate (FITC)–conjugated rat anti–mouse CD11b (1:100; monocyte/macrophage marker, BD Pharmingen, San Diego, CA) and FITC-conjugated rat IgG2bk (isotype control; BD Pharmingen). For whole-mount corneal staining for CD11b cells, excised corneas were fixed in acetone for 15 minutes at day 9. The immunostaining was performed as described previously.[7,19] Nonspecific staining was blocked with anti-FcR CD16/CD32 antibody (BD Pharmingen) and the specimens were immunostained with primary or isotype antibodies for overnight, washed with PBS, incubated with secondary antibodies, and mounted using Vector Shield mounting medium with DAPI (4,6 diamidino-2-phenylindole, Vector Laboratories).

Three corneas from 3 mice per group were taken, and $CD11b^+$ cells were counted in 4–6 areas in the periphery (0.5-μm area from the limbus) and 2 areas in the center (central 2-μm area) of each cornea in a masked fashion by using an epifluorescence microscope (model E800; Nikon) at 40× magnification. The mean number of cells was obtained by averaging the cell number in each area examined.

Analysis of Corneal Lymphangiogenesis by Immunohistochemical Staining

The following primary antibodies were used for immunohistochemical staining: FITC-conjugated rat anti–mouse CD31/PECAM-1 (1:100; Santa Cruz Biotechnology, Santa Cruz), goat-anti–rabbit LYVE-1 antibody (1:200; Abcam), rhodamine-conjugated secondary antibody (1:100; Santa Cruz), For whole-mount corneal staining for CD31/LYVE-1 cell, freshly excised corneas were fixed in acetone for 15 minutes at day 9. The specimens were stained with primary antibodies for overnight, incubated with secondary antibodies, and mounted using Vector Shield mounting medium with DAPI. To quantify corneal lymphangiogenesis, pictures of whole corneal flat-mounts were taken under epifluorescence

microscope at 20× magnification. The lymphatic areas (LA) covered by $CD31^{low}$ $LYVE\text{-}1^{high}$ vessels were analyzed using Metlab™ (The Mathworks Inc, Natrick, MA) and expressed as a percentage of the whole corneal area outlined using the inner-most vessel of the limbal arcade as the border.[20]

TdT-mediated dUTP nick end labeling (TUNEL) Assay

As an alternative to clinical assessment, we evaluated surface epitheliopathy by means of TUNEL assay. To evaluate corneal epitheliopathy, 7-μm cryostat cross sections were fixed in 4% paraformaldehyde in PBS at day 9, and TUNEL staining was performed according to manufacturer's protocol (TUNEL Kit, Roche). One cryosection was incubated with 1 μg/mL DNase I for 10 minutes at room temperature prior labeling procedures as a positive control and one cryosection was incubated without terminal transferase as a negative control. Each section was counterstained with Vectashield mounting medium with DAPI. Images were obtained at central cornea under an epifluorescence microscope with 40 × magnification. TUNEL-postive and DAPI-negative areas were excluded as artifacts. Both TUNEL-positive cells and DAPI-positive cells were counted in the 100-μm width × 40-μm depth areas of central cornea epithelial layer in a masked fashion as previous study.[21,22]

Statistical Analyses

Data are expressed as means ± SEM of three independently repeated experiments. Statistical significance among the groups was analyzed by the two-tailed t-test using Prism software (version 5.0; GraphPad, San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Topical EGCG on Clinical Signs of DED

Mice were placed in the CEC at day 0. Two days after dry eye induction, mice were randomized into 4 groups; i) group 1: topical application of 0.01% EGCG, ii) group 2: 0.1% EGCG, iii) group 3: vehicle, and iv) group 4: no eye drops (untreated controls). At day 4, all treatment groups including vehicle showed a significant decrease in corneal staining compared with the untreated group, but there was no difference in staining amongst these treatment groups. However, by day 9, 0.1% EGCG treated eyes showed significantly decreased corneal staining compared to the untreated (41.9% of decrease, $P < 0.001$) and vehicle treated groups (24.6%, $P = 0.001$). No significant difference was noted between the 0.01% EGCG and vehicle treated groups ($P = 0.356$) (Figure 1).

Effect of Topical EGCG on Corneal Infiltration of Inflammatory Cells

The number of central corneal CD11b+ cells in the EGCG treatment groups was found to be significantly decreased compared to the untreated group (36.2% of decrease for 0.1% EGCG, $P<0.001$; 33.1% of decrease for 0.01% EGCG, $P=0.001$) and to the vehicle treated group (26.1% for 0.1% EGCG, $P=0.009$; 22.6% for 0.01% EGCG, $P=0.025$) (Figure 2-A and 2-B). In the corneal periphery, 0.1% EGCG treatment also showed a significant decrease in the number of CD11b+ cells compared to the untreated group (39.9%, $P<0.001$) and the vehicle treated group (23.3%, $P=0.001$), whereas 0.01% EGCG treatment led only to a decrease in CD11b+ cells compared to the untreated group (25.3%, $P<0.001$), not to the vehicle treated group ($P=0.517$).

Effect of EGCG on Inflammatory Cytokine Expression in the Corneas

Real-time polymerase chain reaction was used to quantify the transcripts encoding IL-1β, TNF-α, and CCL2 in the corneas of the different groups (Figure 3). Treatment with topical 0.1% EGCG significantly decreased relative expression of IL-1β ($P=0.015$ vs untreated, and

P=0.029 vs vehicle), and CCL2 transcripts (*P*=0.017 vs untreated, and *P*=0.001 vs vehicle). 0.01% EGCG decreased relative expression of IL-1β (*P*=0.024 vs untreated), and CCL2 transcripts (*P*=0.039 vs untreated, and *P*=0.006 vs vehicle, respectively). However, there was no association between TNF-α transcripts with any of the treatments.

Effect of EGCG on DED–induced Corneal Lymphangiogenesis

Real-time polymerase chain reaction was used to quantify the transcripts encoding the proangiogenic and prolymphangiogenic growth factors (VEGF-A, VEGF-C, and VEGF-D) in the cornea of different groups (Figure 4). At day 9, the relative expressions of all these VEGF species had increased in the untreated group to 2 – 5 folds compared to those seen in the normal control group. 0.1% EGCG treatment significantly decreased the relative expression of VEGF-A (*P*=0.007 vs untreated) and VEGF-D transcripts (*P*=0.048 vs untreated). 0.1% EGCG treatment showed considerable decrease in VEGF-C levels compared to the untreated controls, though this was not statistically significant (*P*=0.129).

The area of corneal lymphangiogenesis was significantly increased in the dry eye state (2.4 times, that of normal control, *P*=0.023). Though there was a very modest decrease with topical therapy in the corneal area spanned by lymphatics, the decrease induced by EGCG was not significant (*P*=0.194, 0.1% EGCG vs untreated control) (Figure 5-A and 5-B).

Effect of EGCG on Apoptosis of the Corneal Epithelial Cells

The numbers of apoptotic cells were significantly increased in dry eye corneas compared with normal controls (*P*<0.0001). Both 0.01% and 0.1% EGCG treatments showed significant decrease in the number of apoptotic epithelial cells compared to the untreated group (*P*=0.009 and *P*=0.003, respectively). The vehicle treated group also showed a significant reduction in epithelial apoptosis compared to the untreated group; there was no significant difference between vehicle and EGCG treated groups in the level of apoptosis (Figure 6).

DISCUSSION

We present herein data demonstrating that topical 0.1% EGCG can decrease the clinical signs and inflammatory responses of the ocular surface in dry eye disease (DED). The therapeutic and prophylactic uses of green tea have been confined to “alternative medicine”, but recent studies clearly suggest that EGCG, a major active component of the polyphenolic fraction of green tea, has anticarcinogenic, anti-inflammatory, antioxidant, and anti-angiogenic effects both *in vitro* and *in vivo*.[10,11, 23–25]

DED is a complex and multifactorial inflammatory condition. In our study, we present that some inflammatory mediators such as IL-1β (7.1 times), tumor necrosis factor (TNF)-α (2.1 times), and Chemokine (C-C motif) ligand 2 (CCL2) (2.9 times) are increased at dry eye induced cornea, compared with normal control. These mediators can induce immune mediated inflammation such as the activation of antigen presenting cells (APC) and aggravate ocular surface cell damage.[19,26,27] Previous studies have shown that the corneal infiltration by $CD11b^+$ APCs is increased in dry eye, and IL-1β induces the loss of corneal epithelial barrier function associated with ocular inflammation in a NF-kB dependent manner.[19,26,27]

In this study, we demonstrate that topical 0.1% EGCG significantly reduces the ocular surface inflammation by decreasing the expression levels of IL-1β and CCL2. Moreover our data showed that topical EGCG decreases the number of CD11b+ cells in dry eye corneas, which may be associated with the reduced expression of inflammatory mediators such as IL-1β and CCL2. Although, our data clearly demonstrate decreased numbers of CD11b+

cells in DED corneas with EGCG treatment, the mere presence or absence of these CD11b+ cells may not reflect their functional status. Therefore, further studies may be needed to confirm the effect of EGCG on CD11b+ activation and function. CCL2 is the important chemokine regulating the migration and infiltration of monocytes and macrophages which play a key role in initiating the inflammatory process in dry eye.[24,28,29] Previous studies have reported that EGCG treatment decreases the expression of CCL2, resulting in the decreased number of monocytes and EGCG could induce T-cell tolerance during dendritic cell–T-cell interaction by inhibiting the maturation of dendritic cells.[12,29]

Our previous study has shown that corneal lympangiogenesis, but not angiogenesis, is significantly induced in DED.[30] Thus, lymphatics could play a key role in the induction of autoimmunity in DED by providing peripheral APC access to the lymphoid compartment to activate T cell. In this study, we also found that the area of corneal lymphatic and the expressions of mRNA VEGF-A, VEGF-C, and VEGF-D are significantly increased in dry eye model compared with normal control. Previous studies reported that EGCG can reduce VEGF expression at the transcriptional level in cell culture as well as animal models and anti-angiogenic effect of green tea is important for cancer prevention considering its low side-effects.[25, 31–33] In the current study, topical EGCG showed a significant decrease in the expression of pro-lymphangiogenic VEGF-D in dry eye corneas but overall reduction in the corneal lymphatic growth was modest.

Yeh S et al. demonstrated that proinflammatory cytokines such as TNF-α and IL-1 can increase the apoptosis of ocular surface epithelial cell in dry eyes, and the punctate epithelial erosions that are observed in the cornea and conjunctiva of patients with DED could be attributed to sloughing of apoptotic epithelial cells.[21] Similarly in our study, we also found an increase in epithelial cell apoptosis in dry eye induced cornea. Using TUNEL assay, we evaluated the effect of EGCG on prevention of dry eye and/or drug induced epithelial cell apoptosis. Topical 0.01% and 0.1% EGCG showed no toxicity, and significantly reduced the epithelial cell apoptosis. However, this decrease in apoptosis was not significantly different from vehicle treated dry eyes.

In conclusion, we demonstrate that topical application of EGCG in experimental dry eyes leads to significant improvement in clinical signs and inflammation-associated ocular surface pathologies.

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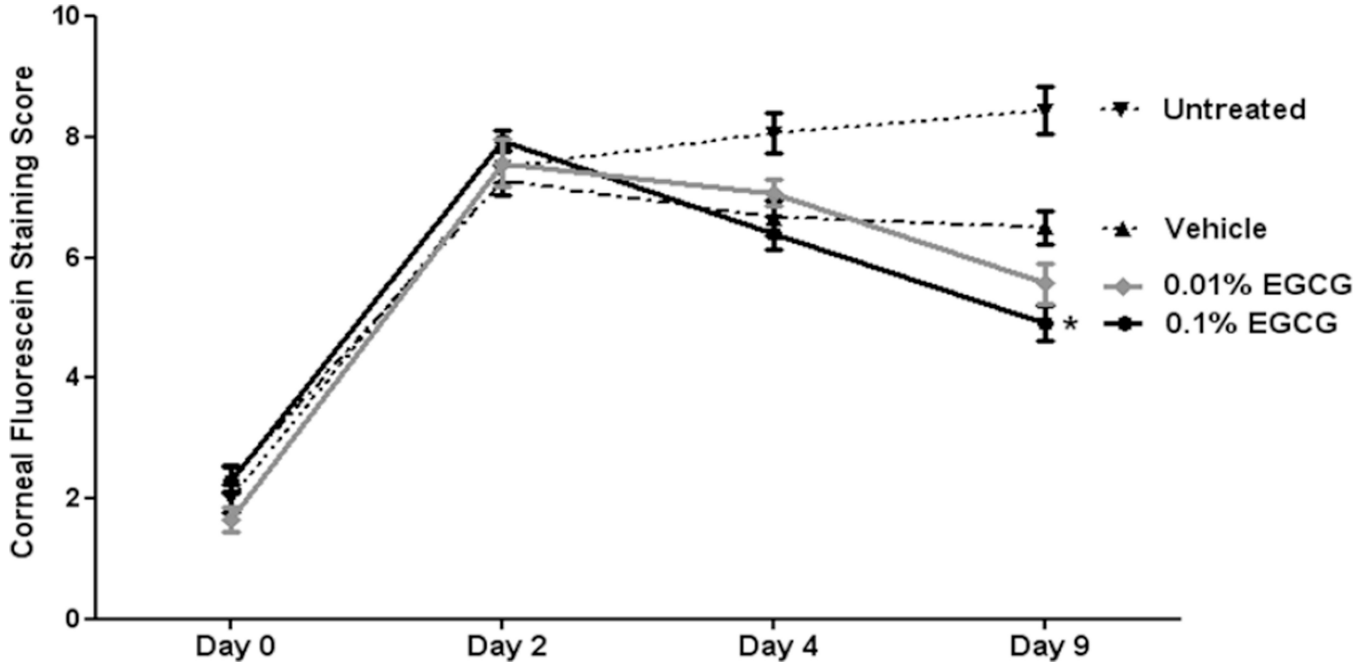

FIGURE 1.

Topical 0.1% epigallocatechin gallate (EGCG) treatment produced a significant decrease in corneal fluorescein staining score compared to the vehicle treated and untreated groups at day 9. But there was no significant difference between 0.01% EGCG and vehicle treated groups. * indicates $P<0.0001$ for 0.1% EGCG vs untreated and $P=0.001$ for 0.1% EGCG vs vehicle. Data are presented as mean ± SEM of three experiments. Each experiment consisted of 3 animals per group.

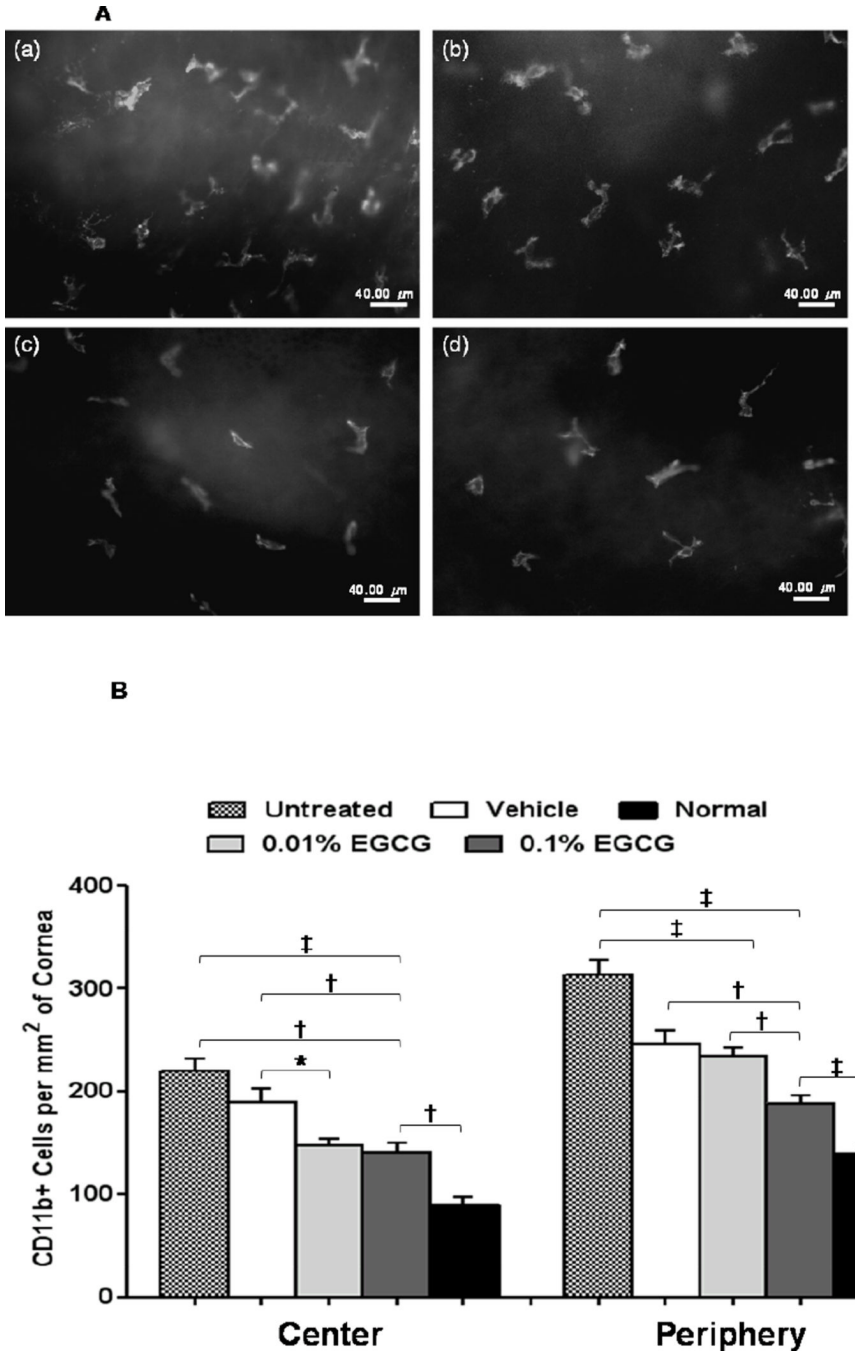

FIGURE 2.

A. Representative epifluorescence micrographs showing CD11b+ cells (grayscale) in whole-mount of central corneas. Images represent **(a)** untreated eyes; and eyes topically treated with **(b)** vehicle, **(c)** 0.01% EGCG, and **(d)** 0.1% EGCG (scale bar: 40 μm). **B**. Both 0.01% and 0.1% EGCG treatments decreased the number of $CD11b^+$ cells in the center and periphery of dry eye induced corneas compared to untreated and vehicle treated groups. P value signs indicate: * $P<0.05$;† $P<0.01$; and‡ $P<0.001$. Data are presented as mean ± SEM of three experiments. Each experiment consisted of 3 corneas per group.

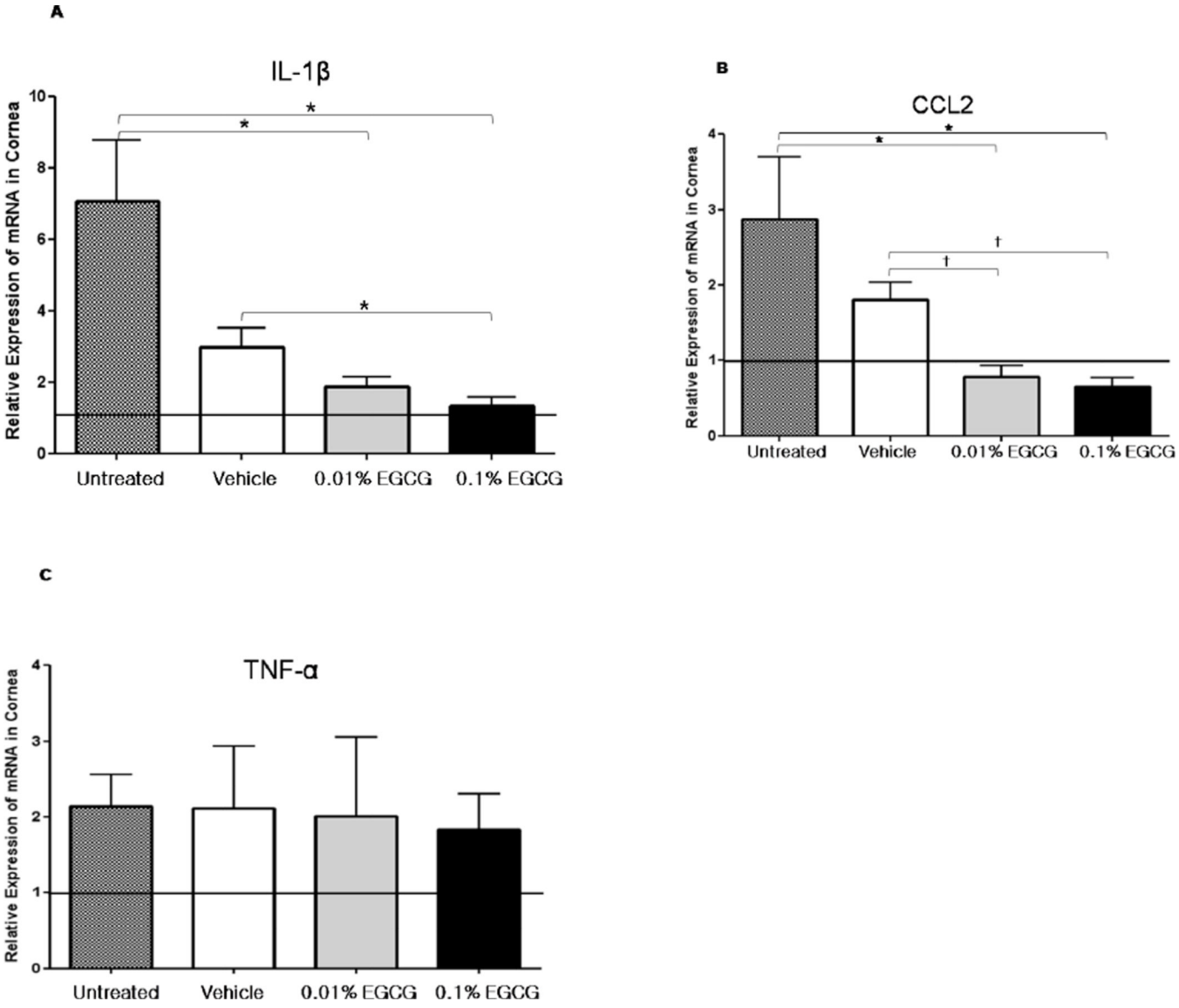

FIGURE 3.

Real-time polymerase chain reaction analysis showing that 0.1% and 0.01% EGCG significantly decreased relative expressions of **(A)** IL-1β (* $P<0.05$) and **(B)** Chemokine (C-C motif) ligand 2 (CCL2) (* $P<0.05$ and† $P<0.01$) but not of **(C)** TNF-α transcripts in dry eye corneas. Data were normalized to GAPDH mRNA as internal control and then values were expressed as the fold change over the normal control corneas. Data are presented as mean ± SEM of three experiments. Each experiment consisted of 2 to 3 corneas per group.

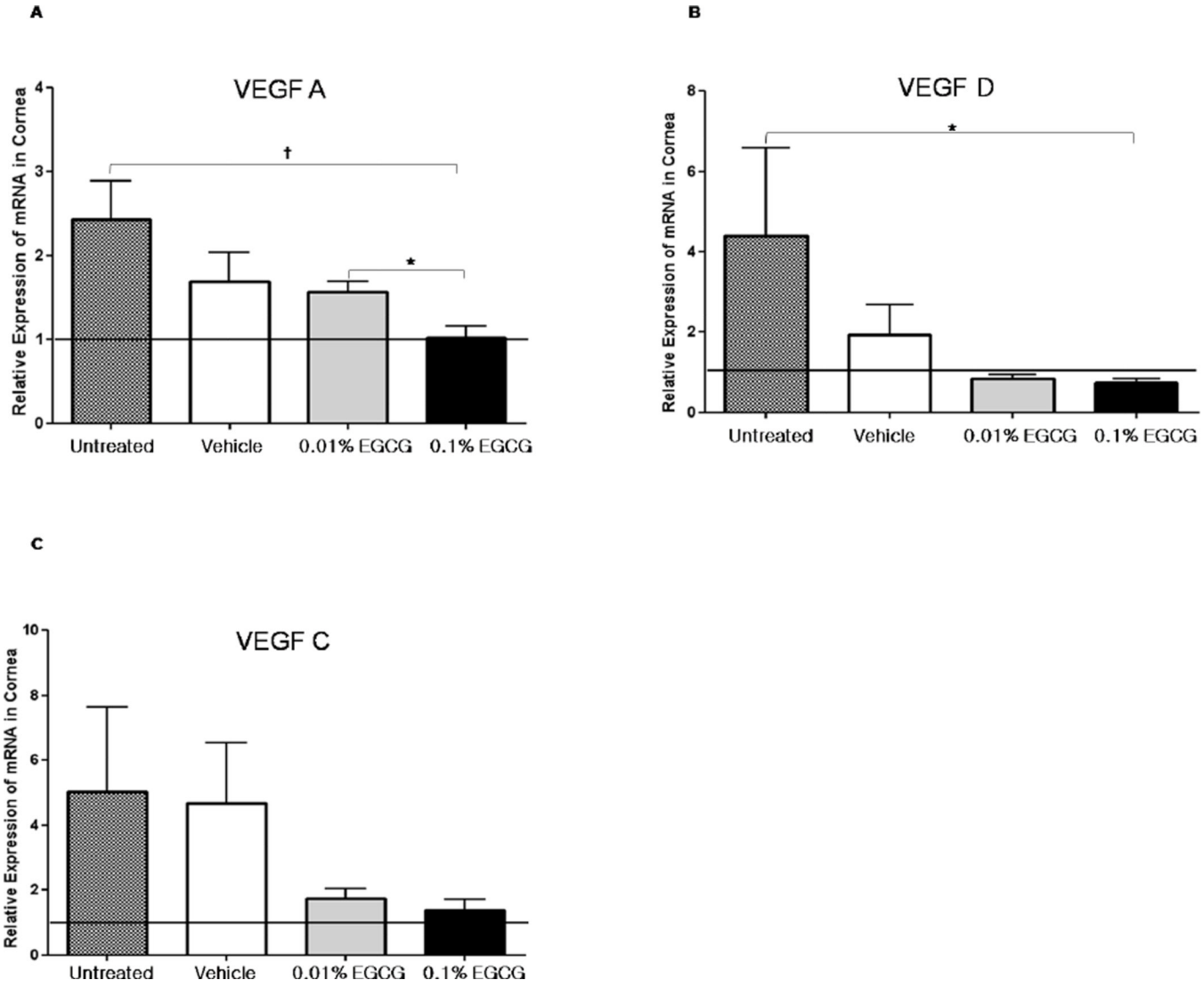

FIGURE 4.

Real-time polymerase chain reaction analysis showing that 0.1% EGCG significantly decreased relative expressions of **(A)** VEGF-A († $P<0.01$; * $P<0.05$) and **(B)** VEGF-D transcripts in dry eye corneas. EGCG at 0.01% dose did not showed the decrease of relative expressions of these VEGFs transcripts. **(C)** Both 0.01% and 0.1% EGCG showed considerable but not statictically significant decrease in VEGF-C expression. Data were normalized to GAPDH mRNA as internal control and then values were expressed as the fold change over the normal control corneas. Data are presented as mean ± SEM of three experiments. Each experiment consisted of 2 to 3 corneas per group.

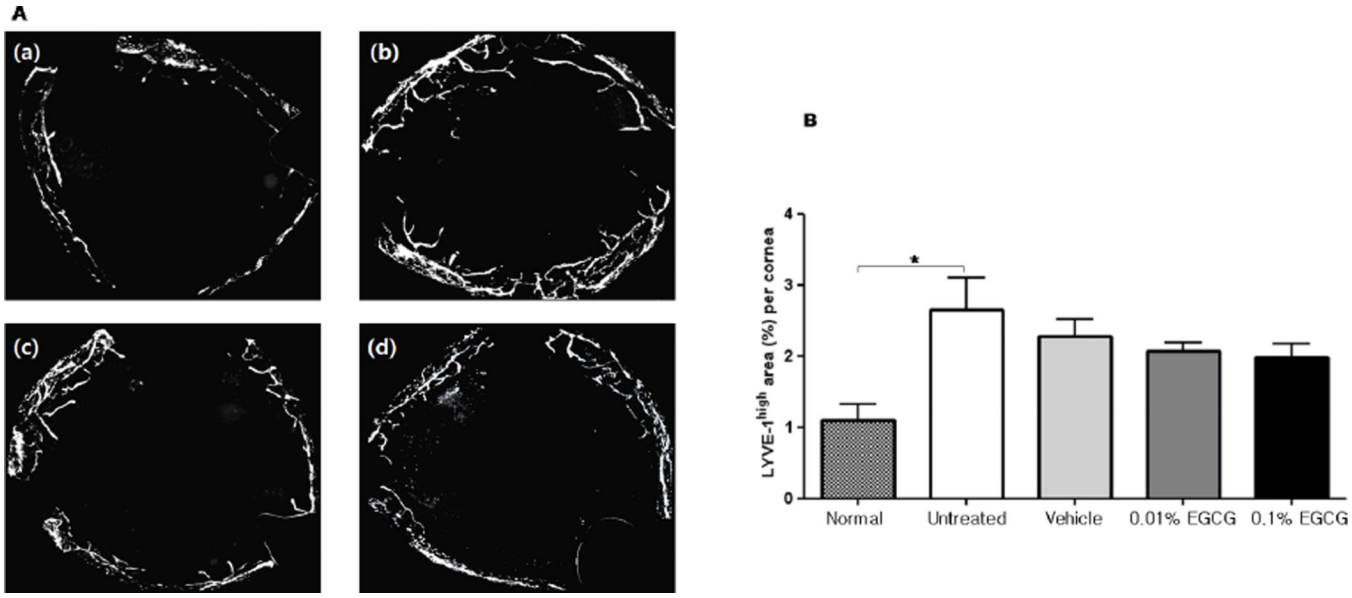

FIGURE 5.

A. Representative whole-mount corneal immunofluorescence micrographs showing lymphatic vessels (LYVE-1^{high}) in normal (a), and untreated (b) eyes, and eyes treated with vehicle (c), or 0.1% EGCG (d) at day 9 (magnification ×20).

B. The percent area of lymphangiogenesis in dry eye corneas. All treatment groups including the vehicle treated group did not show a significant regression in the area of corneal lymphangiogenesis. Asterisk indicates $P<0.05$ for normal control vs untreated group. Data are presented as mean ± SEM of three experiments. Each experiment consisted of 3 corneas per group.

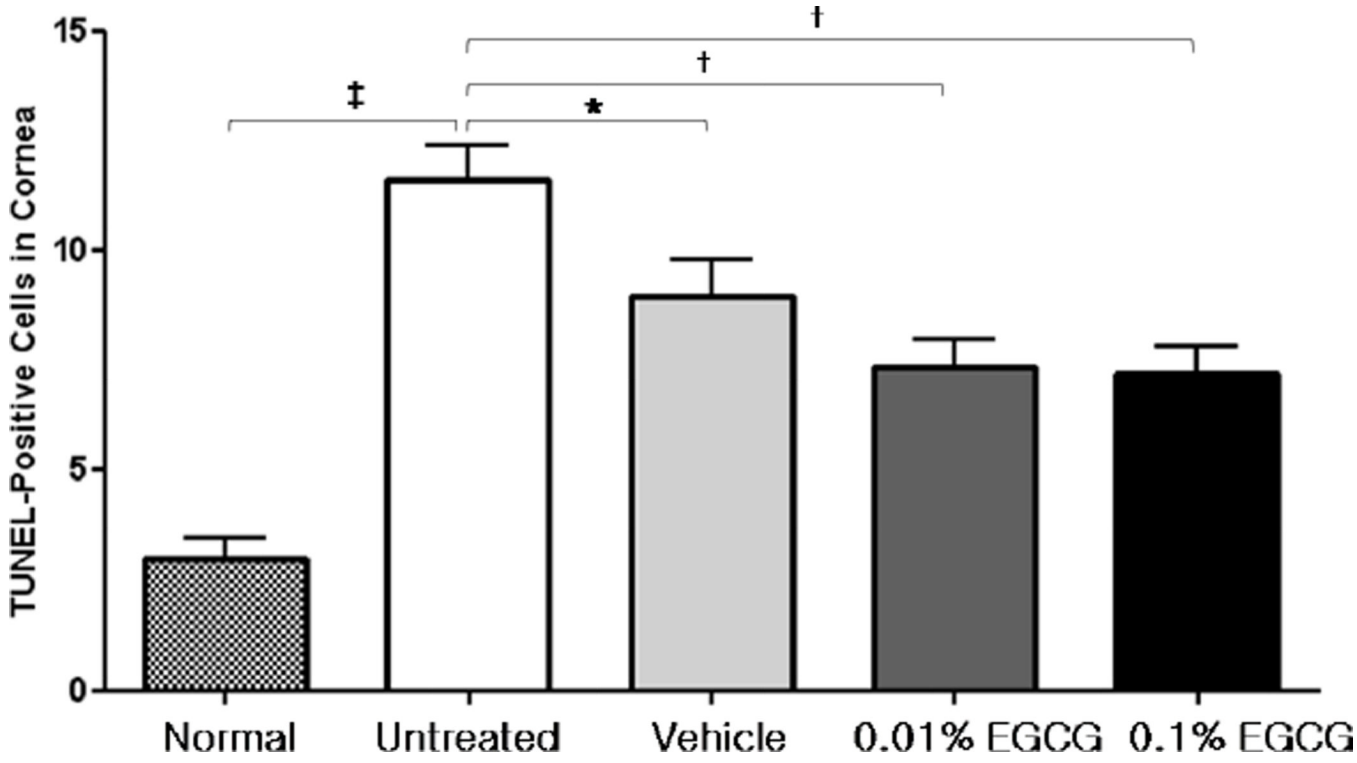

FIGURE 6.

Quantitation of TUNEL positive corneal epithelial cells. The number of apoptotic cells in dry eye corneas was significantly increased compared to normal eyes. All the treatment groups including vehicle showed significant decrease in the epithelial apoptosis. P value signs indicate: * $P<0.05$;† $P<0.01$; and‡ $P<0.001$. Data are presented as mean ± SEM of three experiments. Each experiment consisted of 3 corneas per group.