Naringenin Eye Drops Inhibit Corneal Neovascularization by Anti-Inflammatory and Antioxidant Mechanisms

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Purpose. To investigate the effect of naringenin eye drops in corneal neovascularization induced by alkali (1 N NaOH) burn in mice.

Methods. Corneal neovascularization in the right eye of male Swiss mice was induced by alkali. Treatment with naringenin eye drops (0.08-80 µg; 8 µL of 0.01-10 g/L solution) or vehicle (saline) started 2 days before corneal neovascularization was induced and was performed twice a day. Mice were treated up until the time animals were euthanized and corneal tissue was collected for testing, which was 2, 4, and 6 hours after alkali stimulus for cytokine and antioxidant capacity measurements, and 3 and/or 7 days after alkali stimulus for the assessment of corneal epithelial thickness and neovascularization, neutrophil, and macrophage recruitment, and vascular endothelial growth factor (Vegf), platelet-derived growth factor (Pdgf), matrix metalloproteinase-14 (Mmp14), and pigment epithelium-derived factor (Pedef) mRNA expression.

Results. Naringenin eye drops inhibited alkali burn-induced neutrophil (myeloperoxidase activity and recruitment of Lysm-GFP cells) and macrophage (N-acetyl-B-D glucosaminidase activity) recruitment into the eye, decrease in epithelial thickness, and neovascularization in the cornea. Further, naringenin inhibited alkali-induced cytokine (IL-1β and IL-6) production, Vegf, Pdgf, and Mmp14 mRNA expression, and the reduction of ferric reducing antioxidant power and Azinobis-(3-Ethylbenzothiazoline 6-Sulfonic acid) radical scavenging capacity as well as increased the reduced glutathione and protein-bound sulfhydryl groups levels.

Conclusions. Collectively, these results indicate that naringenin eye drops are protective in alkali-induced corneal burn by inhibiting leukocyte recruitment, the proangiogenic factor expression, inflammatory cytokine production, and loss of antioxidant defenses.

Keywords: naringenin, cornea, inflammation, angiogenesis, cytokine

Neovascularization is a process that leads to formation of new capillary blood vessels as an extension of existing vasculature.1 Its presence in the eye is responsible for blindness in several eye diseases, including proliferative diabetic retinopathy, AMD (the major cause of blindness in adults), and retinopathy of prematurity (the major cause of blindness in children).2,3 The cornea is a tissue with refractive properties and is totally transparent and avascular. The presence of neovascularization in this tissue leads to loss of its transparency, and thus is considered the second most important cause of blindness and low visual acuity.4 Moreover, neovascularization represents one of the main risk factors for rejection in patients undergoing corneal transplantation.5-7 During neovascularization, several positive and negative regulators control the cascade of events that contributes to angiogenesis.8 Among them, inflammation plays an important role in pathologic neovascularization in several diseases.9

The inflammatory response occurs after tissue injury and/or infection and leads to the recruitment of neutrophils and macrophages that, in turn, promote angiogenesis by secreting proangiogenic factors, such as IL-1β and IL-6.9-10 These mediators increase vascular permeability and facilitate additional recruitment of leukocytes.11,12 Moreover, the new blood vessels generated during neovascularization exhibit increased vascular permeability, which also contributes to sustain the inflammatory response. Oxidative stress also plays an important role in angiogenesis,11,13,14 by inducing the migration of endothelial cells15,16 and production of inflammatory and proangiogenic mediators, such as IL-6.17 In this sense, compounds that present anti-inflammatory and antioxidant properties present potential to reduce corneal neovascularization.

Naringenin is 4’,5,7-trihydroxyflavanone, a flavanone found abundantly in vegetables and citrus fruits that exhibits several protective effects.18-20 It was demonstrated that naringenin inhibits myeloperoxidase (MPO) activity, an enzyme secreted by activated neutrophils,21 downregulates nuclear factor kappa B (NF-kB) activity,12 and reduces the production of NF-kB-related cytokines, including IL-1β and IL-6.20,23,24 Furthermore, naringenin reduces neutrophil-derived reactive oxygen species (ROS) and also enhances ROS detoxification by triggering the synthesis of reduced glutathione, the most abundant nonenzymatic antioxidant in cells.25-27 Importantly, naringenin possesses antiangiogenic potential and protective effect in models that angiogenesis plays a central role.25,26
Several therapeutic options are currently available to treat corneal neovascularization, such as corticosteroids and nonsteroidal anti-inflammatory eye drops, photodynamic therapy, and photocoagulation; however, all have shown limited efficiency and harmful side effects.28-32 Therefore, great effort has been devoted to finding therapeutic alternatives for this condition.30-35 Clinical studies have shown a beneficial effect of anti-VEGF treatment (Bevacizumab) for patients with corneal neovascularization.34 Interestingly, polysaccharide extract from the naturally occurring Spirulina platensis was shown to be more cost effective in the treatment of corneal neovascularization induced by alkali in rats when compared with Bevacizumab.35 Also, agarinsen, an antisense oligonucleotide preventing insulin receptor substrate-1 expression, has shown promising therapeutic potential in phase II and III studies.36,37 Expectations are that combined inhibition of different pathways may lead to better treatments for corneal neovascularization.38 Flavonoids, among them naringenin, have been considered promising candidates for a new category of anti-inflammatory drugs because they target varied inflammatory pathways without abolishing endogenous physiological process, therefore, presenting reduced side effects.39 Motivated by the pressing need for improved treatment options, and considering the emerging literature reporting the protective effects of naringenin in choroidal neovascularization,25,26 we investigated the protective effects of naringenin eye drops in alkali-induced corneal neovascularization in mice, as well as its effects on the inflammation and oxidative stress present in this model.

**METHODS**

**Animals**

Male Swiss mice (20–25 g) obtained from the Central Vivarium of Universidade Estadual de Londrina (UEL) and Lysm-eGFP mice40 (20–25 g) from Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, were kept at the Animal facilities of the Department of Health Sciences of UEL for at least 2 days before the experiments. Mice were housed in propylene cages (300 × 195 × 130 mm) with metal cover, in a temperature-controlled room (21°C) under a light/dark cycle of 12:12 hours, with free access to food and water. The maximum number of mice was of five mice per cage (area of 585 mm²). This study was approved the Research and Ethics Committee of UEL (process 70760/2012) and the animals were handled according to the guidelines of Conselho Nacional de Controle de Experimentação Animal (National Committee for the Control of Animal Experiments) and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Experimental Protocol**

Corneal neovascularization in the right eye of mice was induced by alkaline solution (1N NaOH; Vetec Quimica Fina, Duque de Caxias, Rio de Janeiro, Brazil) as previously described.41 First, mice were anesthetized by intraperitoneal injection with ketamine (80 mg/Kg) and xylazine (10 mg/kg; Ceva, Paulinia, São Paulo, Brazil) and topical ocular anesthesia was induced by hydrochloride 0.5% propacain (Anestalcon-Alcon, São Paulo, Brazil). Next, a 2-mm diameter filter paper disc uniformly soaked with alkali or vehicle was applied to the cornea of the right eye for 10 seconds and then irrigated with 20 mL of 0.9% saline solution. The right eyes of mice were treated twice a day with 0.08 to 80 µg of naringenin (8 µL of 0.01–10 g/L solution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or vehicle (saline). Daily treatment with naringenin started 2 days before the stimulus with alkali and continued up until the time animals were euthanized and cornea tissue was collected for testing. The best dose of naringenin was determined by assessing leukocyte recruitment to the cornea (MPO and N-acetyl-β-D-glucosaminidase [NAG] activity assays) 3 and 7 days after alkali stimulus.42 Alkali-induced recruitment of Lysm-eGFP cells, loss of corneal epithelium, corneal neovascularization, and Vegf, platelet-derived growth factor (Pdgf), matrix metalloproteinase-14 (Mmp14), and pigment epithelium-derived factor (Pedf) mRNA expression by quantitative (q) PCR were assessed at 3 and/or 7 days. Cytokine (IL-1β, IL-6, and IL-10) levels in the cornea tissue were determined 2, 4, and 6 hours after alkali stimulus using ELISA kits (Ready-SET-Go!; Bioscience, Carlsbad, CA, USA). Oxidative stress was assessed by determining the ferric reducing antioxidant power (FRAP), ability to scavengue 2,2′-Azinobis(3-Ethylbenzothiazoline 6-Sulfonic acid (ABTS), and the levels of reduced glutathione (GSH) and protein sulfhydryls (SH) in the cornea 2, 4, and 6 hours after alkali burn.42,43

**Confocal Microscopy**

Images of corneal tissue from Lysm-eGFP mice were captured using a Leica TCS SP8 confocal microscope (Wetzlar, Germany) for posterior quantification of Lysm-eGFP+ cells.

**Histology and Corneal Epithelial Thickness**

The ocular globes were fixed in buffered 10% formaldehyde and then paraffin embedded for conventional morphology study. Four-micrometer sections were prepared and stained with haematoxylin and eosin (H&E). Stained sections were analyzed by a pathologist at ×40 magnification using light microscopy (E-200; Nikon microscope, Tokyo, Japan) and measurements of the corneal epithelial thickness (mm) were made using a reticle ruler.

**Corneal Neovascularization Assessment**

Biomicroscopic images of the corneas were obtained and the area of neovascularization was assessed 3 and 7 days after alkali-induced corneal burn. The area of neovascularization and analysis of corneal image was performed using biomicroscopy (HS 5000 video system; Huvitz, South Windsor, CT, USA), and analyzed by program ImageJ 1.45x software (http://imagej.nih.gov/ij/) provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) 3 and 7 days after alkali burn.

**Reverse Transcription and qPCR**

Corneal tissue samples were collected 7 days after alkali stimulus and RNA extraction, reverse transcription of total RNA to cDNA, and qPCR were carried out as previously described.42 The relative gene expression was measured using the comparative 2−(ΔΔCq) method. The primers used are provided in the Supplementary Material section. The expression of β-actin mRNA was used as a reference gene to normalize data.

**Statistical Analysis**

Results are presented as means ± SEM of four to six mice per group per experiment. All experiments were performed twice. Two-way ANOVA was used to compare the groups and doses at all times when the parameters were measured at different times after the stimulus injection. The analyzed factors were treatments, time, and time versus treatment interaction. One-way ANOVA followed by Tukey’s multiple comparison tests was performed for each time. All statistical analyses were performed using Graph Pad Prism 5 (La Jolla, CA, USA). The level of significance was set at P less than 0.05.
RESULTS

MPO and NAG Activity

Several lines of evidence indicate that infiltrating leukocytes play a crucial role in the development of neovascularization in different ocular tissues.44–46 In this sense, the effect of daily treatment with naringenin eye drops (0.08–80 μg) on alkali-induced corneal burn was investigated (detailed in the Methods section). In the present study, we assessed the leukocyte recruitment by measuring the activity of MPO and NAG. Myeloperoxidase is an enzyme mainly found in cytoplasmic granules of neutrophils, but also monocytes. Nevertheless, the present MPO assay selective determines neutrophil MPO activity. NAG, on the other hand, is a lysosomal enzyme present in high levels in activated macrophages. Together, MPO and NAG serve as good markers of neutrophil and macrophage infiltration in tissues, respectively, and inflammation.42,47 Alkali significantly increased MPO and NAG activity in the cornea when compared with vehicle 3 and 7 days (Figs. 1C, 1D) after alkali-induced corneal burn. Treatment with vehicle or naringenin (80 μg)
alone did not alter the activity of the enzymes (Figs. 1A–D) in the cornea of mice without alkali corneal burn. Only the doses of 8 and 80 µg of naringenin eye drops inhibited the alkali burn–induced increase in MPO activity at 3 and 7 days (Figs. 1A, 1C). NAG activity was reduced by 0.8 to 80 µg of naringenin eye drops 3 and 7 days after alkali burn (Figs. 1B, 1D). The most effective dose of naringenin in reducing MPO and NAG activity in both time periods assessed (3 and 7 days) was 80 µg, thus this dose was chosen for the following experiments of the study. The results indicate that daily treatment with naringenin eye drops can inhibit both neutrophil and macrophage recruitment to the cornea in this model of corneal neovascularization.

Lysm-GFP<sup>+</sup> Cell Recruitment

To further confirm the inhibitory effect of naringenin eye drops (80 µg) on neutrophil recruitment to the cornea, Lysm-GFP mice were used to assess the effect of naringenin on the recruitment of Lysm-GFP<sup>+</sup> cells to the cornea 3 days after alkali burn, which was the time point most prominent alkali-induced MPO activity increase was observed (Fig. 1). Lysm-GFP<sup>+</sup> cells recruitment to the cornea was not detected (ND) in the saline and naringenin-only control groups (Figs. 2A, images a1–b3). Alkali significantly increased the number of Lysm-GFP<sup>+</sup> cells in the cornea (Figs. 2A, images c1–c3, and 2B), which was inhibited by naringenin (Figs. 2A, images d1–d3, and 2B).

Corneal Epithelial Thickness

To further investigate the therapeutic effects of daily treatment with 80 µg naringenin eye drops (8 µL of 10 g/L solution) in this model, the thickness of the epithelium was measured in H&E-stained corneal sections 3 and 7 days after alkali-induced corneal burn (Fig. 3). A decrease in corneal epithelial thickness was observed at 3 and 7 days after alkali burn (Figs. 3A, 3B), which was inhibited by naringenin eye-drops at 3, but not 7 days. These findings can be observed in the representative images of the cornea (Figs. 3C–I, black arrows). Moreover, naringenin (80 µg) alone at 3 or 7 days did not induce changes in the cornea epithelium of mice (Figs. 3A, 3B, 3D, 3G). In summary, naringenin has protective effect in alkali burn-induced loss of corneal epithelium.

Corneal Neovascularization

To investigate if daily treatment with naringenin eye drops would reduce corneal neovascularization, biomicroscopic images of the cornea were obtained (Figs. 4A, 4C) and the area of neovascularization was determined 3 and 7 days after alkali-induced corneal burn (Fig. 3). A decrease in corneal epithelial thickness was observed at 3 and 7 days after alkali burn (Figs. 3A, 3B), which was inhibited by naringenin eye-drops at 3, but not 7 days. These findings can be observed in the representative images of the cornea (Figs. 3C–I, black arrows). Moreover, naringenin (80 µg) alone at 3 or 7 days did not induce changes in the cornea epithelium of mice (Figs. 3A, 3B, 3D, 3G). In summary, naringenin has protective effect in alkali burn-induced loss of corneal epithelium.

**FIGURE 2.** Lysm-GFP<sup>+</sup> cell recruitment to the cornea. Daily treatment with 80 µg of naringenin (8 µL of 10 g/L solution) or vehicle (saline) started 2 days before alkali-induced corneal burn and was continued up to 3 days. Representative images of the cornea were obtained by confocal microscopy (A). Negative control cornea: 4′,6-diamidino-2-phenylindole (DAPI) (a1), Lysm-GFP (a2), and merge (a3); naringenin (NGN) control group: DAPI (b1), Lysm-GFP (b2), and merge (b3); alkali-induced corneal burn treated with vehicle group: DAPI (c1), Lysm-GFP (c2), and merge (c3); and alkali-induced corneal burn treated with 80 µg of naringenin group: DAPI (d1), Lysm-GFP (d2), and merge (d3). Quantification of Lysm-GFP<sup>+</sup> cells in the cornea 3 days after alkali burn (B). Note that Lysm-GFP<sup>+</sup> cells were ND in the saline and naringenin-only control groups. Results are provided as means ± SEM of four mice per group per experiment and are representative of two separate experiments (**,**<sup>*</sup><sup>p</sup> < 0.001 compared with the saline group [negative control]; ****<sup>p</sup> < 0.001 compared with vehicle-treated group with alkali-induced corneal burn. One-way ANOVA, followed by Tukey’s multiple comparison test).
neovascularization in the cornea of mice at 3 or 7 days (Figs. 4A–D). These results evidence the ability of naringenin to reduce neovascularization in the cornea.

Expression of Pro- and Antiangiogenic Factors

The effect of naringenin eye drops (80 μg) on the expression of Vegfa, Pdgf, Mmp14, and Pedf mRNA expression was assessed by qPCR 7 days after alkali burn (Fig. 5), which is the peak of corneal neovascularization. Treatment with vehicle or naringenin alone had no significant effect on the expression of any of these factors (Figs. 5A–D). Alkali increased the mRNA expression of the angiogenic related factors Vegfa, Pdgf, and Mmp14 in the cornea of mice, which was inhibited by naringenin (Figs. 5A–C). In contrast, neither alkali, nor naringenin, had any effect on the mRNA expression of the antiangiogenic factor Pedf (Fig. 5D), suggesting that naringenin eye drops inhibits alkali burn–induced increase in the expression of proangiogenic factors Vegfa, Pdgf, and Mmp14.

Cytokine Production

Treatment with vehicle or naringenin (80 μg) alone did not increase IL-1β and IL-6 or reduce IL-10 levels in the cornea of mice without alkali burn at 2, 4, and 6 hours (Fig. 6). Alkali significantly induced IL-1β (Figs. 6A, 6D, 6G) and IL-6 (Figs. 6B, 6E, 6H) production in the cornea at all time points assessed. Also, alkali reduced IL-10 levels (Fig. 6C, 6F, 6I) in the cornea 2 and 6 hours, but not 4 hours. Treatment with naringenin reduced the increase in IL-1β and IL-6 levels at all time points assessed and prevented the reduction in IL-10 levels at 6 hours (Fig. 6). Collectively, these data show that alkali induces time-
dependent cytokine production and naringenin eye drops reduces the alkali burn-induced production of proinflammatory cytokines IL-1β and IL-6, and prevents the decrease in anti-inflammatory cytokine IL-10.

**Antioxidant Capacity**

Decrease in endogenous antioxidants has been described in patients with inflammatory neovascularization cornea. The ABTS (ABTS radical cation scavenging) and FRAP (ferric reducing/antioxidant power) assays are Trolox equivalent antioxidant capacity (TEAC) assays widely used to assess antioxidant capacity in tissues. Alkali-induced burn resulted in the decrease in the ABTS radical cation scavenging and FRAP in cornea tissue samples at 2, 4, and 6 hours, which was prevented by treatment with naringenin (80 µg) (Fig. 7). Naringenin alone did not induce significant changes in these parameters compared with vehicle at any of the time points assessed (Figs. 7, 8). The effect of naringenin eye drops on antioxidant capacity following alkali burn was further explored by measuring the cellular antioxidant molecule reduced glutathione (GSH) and protein-bound groups (Fig. 8). Alkali burn induced significant reduction of GSH levels in the cornea of mice at 2, 4, and 6 hours and treatment with naringenin eye drops resulted in notable increase in GSH levels (Figs. 8A, 8C, 8E). In contrast to the effect of alkali burn on GSH levels, alkali did not decrease protein-bound SH group levels in the cornea of mice. Although naringenin alone did not increase protein-bound SH group levels, in alkali-induced corneal burn naringenin notably increased the levels of these SH groups (Figs. 8B, 8D, 8F). Together, these data show that naringenin eye drops not only prevents the depletion, but also enhances total antioxidant capacity in alkali-induced corneal burn. Nevertheless, this naringenin-induced up
regulation of protein-bound SH group occurs only in the disease context.

**DISCUSSION**

Studies have shown that naringenin possesses protective effect in models that angiogenesis plays a central role.\(^25,26,49\) Herein, we provide evidence that eye drops containing naringenin potently ameliorates corneal neovascularization and loss of corneal epithelium induced by alkali burn in mice by reducing inflammation, proangiogenic factors, and oxidative stress. The protective effects and mechanisms of treatment with naringenin in this model of neovascularization had not been reported previously.

Corneal neovascularization induced by alkali burn is closely related to inflammation.\(^50\) Herein, we show that daily treatment with naringenin eye drops inhibited both neutrophil and macrophage recruitment to the cornea. This may be an important antiangiogenic mechanism of naringenin, because infiltrating inflammatory cells possess direct angiogenic ability.\(^45\) Studies have shown that infiltrating neutrophils and macrophages can induce neovascularization in the cornea and choroid, and the inhibition of the recruitment of these cells diminishes neovascularization.\(^44,45\)

A central aspect of inflammation and neovascularization in the cornea is the disequilibrium between angiogenic and antiangiogenic stimuli.\(^51\) During and after injury to the cornea, corneal epithelial, and stromal cells and infiltrating leukocytes release a series of factors, such as cytokines (IL-1\(\beta\), TNF-\(\alpha\), TGF-\(\beta\), among others) and growth factors (VEGF, PDGF, basic fibroblast growth factor [bFGF])\(^45,51,52\) which shift the balance toward angiogenesis and neovascularization.\(^50,53\) Among these many factors, cytokines seem to play an important role in inducing both inflammation and neovascularization.\(^1,17,50,54\)

**FIGURE 5.** *Vegfa*, *Pdgf*, *Mmp14*, and *Pedf* mRNA expression in the cornea. Daily treatment with 80 \(\mu\)g of naringenin (8 \(\mu\)L of 10 g/L solution) or vehicle (saline) started 2 days before alkali-induced corneal burn and was continued up to 7 days. *Vegfa* (A), *Pdgf* (B), *Mmp14* (C), and *Pedf* (D) mRNA expression in the corneal tissue was assessed by qPCR 7 days after alkali-induced corneal burn. Results are provided as means ± SEM of six mice per group per experiment and are representative of two separate experiments (*\(P < 0.05\) compared with the saline group [negative control]; *\(P < 0.05\) compared with vehicle-treated group with alkali-induced corneal burn. One-way ANOVA, followed by Tukey’s multiple comparison test).
FIGURE 6. Cytokine production in the cornea. Daily treatment with 80 µg of naringenin (8 µL of 10 g/L solution) or vehicle (saline) started 2 days before alkali-induced corneal burn. The levels of IL-1β (A, D, G), IL-6 (B, E, H), and IL-10 (C, F, I) were determined by ELISA in the corneal tissue 2, 4, and 6 hours, respectively, after alkali-induced corneal burn. Results are provided as means ± SEM of six mice per group per experiment and are representative of two separate experiments (*P < 0.05 compared with the saline group [negative control]; #P < 0.05 compared with vehicle-treated group with alkali-induced corneal burn. One-way ANOVA, followed by Tukey’s multiple comparison test).
FIGURE 7. Total antioxidant capacity in the cornea. Daily treatment with 80 μg of naringenin (8 μL of 10 g/L solution) or vehicle (saline) started 2 days before alkali-induced corneal burn. Total antioxidant capacity was assessed by determining the ability to scavenge ABTS (A, C, E) and FRAP (B, D, F) of the corneal tissue 2, 4, and 6 hours after alkali-induced corneal burn. Results are provided as means ± SEM of six mice per group per experiment and are representative of two separate experiments (*P < 0.05 compared with the saline group [negative control]; #P < 0.05 compared with vehicle-treated group with alkali-induced corneal burn. One-way ANOVA, followed by Tukey’s multiple comparison test).
FIGURE 8. Reduced glutathione and protein-bound sulfhydryl group levels in the cornea. Daily treatment with 80 μg of naringenin (8 μL of 10 g/L solution) or vehicle (saline) started 2 days before alkali-induced corneal burn. Reduced GSH (A, C, E) and protein SH (B, D, F) levels were quantified by colorimetric assays in corneal tissue samples at 2, 4, and 6 hours after alkali-induced corneal burn. Results are provided as means ± SEM of six mice per group per experiment and are representative of two separate experiments (* P < 0.05 compared with the saline group [negative control]; # P < 0.05 compared with vehicle-treated group with alkali-induced corneal burn. One-way ANOVA, followed by Tukey’s multiple comparison test).
The reduction in proinflammatory cytokines IL-1β and IL-6 by naringenin eye drops is consistent with previous studies that have also observed that this compound can prevent cytokine production induced by inflammatory stimuli.\textsuperscript{21-23,43} Both IL-1β and IL-6 are important in the induction of neovascularization in the cornea and the inhibition of their production can reduce neovascularization in the cornea.\textsuperscript{50,55} Thus, the modulatory effect of naringenin on proinflammatory cytokine production might account for the inhibition in neovascularization in the cornea.

Interleukin-10 is an immune-modulatory cytokine that can suppress the production of proinflammatory cytokines.\textsuperscript{56} However, in the present study the reduction in IL-1β and IL-6 levels does not seem to be dependent on IL-10 modulation, at least not at 2 and 4 hours in which IL-10 levels were similar between naringenin and vehicle-treated alkali burn groups. Additionally, the data indicate that regulation of IL-10 levels is not central to the reduction in neovascularization by naringenin. Alkali induced neovascularization was present in the absence of IL-10 depletion (4 hours) and naringenin did not inhibit the depletion of IL-10 at all time points assessed. Nevertheless, a minor role of IL-10 in the protective effect of naringenin cannot be discarded, because naringenin prevented IL-10 depletion at a later time point (6 hours) and IL-10 has been shown to indirectly promote resolution of neovascularization and inflammation in the cornea by regulating lymphangiogenesis via macrophages.\textsuperscript{57}

The reduction in IL-1β and IL-6 levels by naringenin lines up well with the reduction in neutrophil and macrophage recruitment, because these cells are the main source of inflammatory cytokines in corneal and choroidal neovascularization.\textsuperscript{55,46} Also, because proinflammatory cytokines further stimulate leukocyte recruitment and activation, the reduction in cytokine production may also contribute to the reduced neutrophil and macrophage recruitment into the cornea of naringenin treated mice.\textsuperscript{12} Moreover, it is plausible that the preservation of IL-10 levels by naringenin also favors the reduction in the accumulation of these cells in the cornea. Interleukin-10 has a crucial role in lymphangiogenesis in the cornea, which is essential for the egress of cells and drainage of debris from the inflammatory site.\textsuperscript{57}

Vascular endothelial growth factor and PDGF are potent angiogenic factors and key mediators of alkali-induced neovascularization in the cornea.\textsuperscript{52,58-60} Quantitative PCR revealed that treatment with naringenin eye drops reduced alkali-induced Vegf and Pdgf mRNA expression in the cornea of mice. This is in agreement with the reduction in neovascularization by naringenin. Importantly, the cytokines IL-1β and IL-6 are known to induce the expression of Vegf and Pdgf.\textsuperscript{9,17,61} In this sense, it is possible that reduced Vegf and Pdgf mRNA expression may be secondary to the reduction in IL-1β and IL-6 levels by naringenin. However, the relation between the decreased recruitment of neutrophils and macrophages and reduction in Vegf and Pdgf mRNA expression cannot be discarded because these cells are an important source of Vegf and PDGF.\textsuperscript{46,52,62}

Matrix metalloproteinase-14 is involved in angiogenesis and extracellular matrix remodeling.\textsuperscript{63,64} and, in the cornea, this MMP plays a central role in neovascularization.\textsuperscript{65} Therefore, inhibition of Mmp14 expression is likely to be an important antiangiogenic mechanism of naringenin. Evidence also showed that naringenin inhibits the activity of other MMPs, such as MMP-9.\textsuperscript{56} In contrast, PEDF is a potent inhibitor of angiogenesis in mammalian eyes.\textsuperscript{67} however, PEDF-mediated inhibition of neovascularization does not seem to be a mechanism triggered by naringenin, because topical treatment with this flavonoid did not affect Pedf mRNA expression.

Oxidative stress plays an important role in the pathogenesis of corneal neovascularization.\textsuperscript{13} Oxidative stress can result from either increased production of reactive species or decreased antioxidant defenses.\textsuperscript{68} In the present study, daily treatment with naringenin inhibited the decrease in total antioxidant capacity and GSH levels and induced proteinbound SH groups following alkali burn. This is in agreement with previous studies showing that naringenin can induce the expression of the nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2), which is a key transcription factor that regulates the expression of enzymatic antioxidant genes,\textsuperscript{21} and increases GSH levels.\textsuperscript{20,23,27} Tissue antioxidants are essential for the protection against reactive species and oxidative stress-mediated damage and inflammation.\textsuperscript{13,68,69} Therefore, these findings are in agreement with the reduction in alkali-induced decrease in corneal epithelium and inflammation in the cornea by naringenin. Further, neutrophils depleted of ROS (nicotinamide adenine dinucleotide phosphate [NADPH] oxidase knockout) display reduced chemotaxis,\textsuperscript{70} therefore, an overall decrease in the levels of reactive species may have contributed to decreased infiltration of neutrophils and macrophages into the cornea.

Currently available therapies to treat neovascularization in the cornea, for example, bevacizumab and dexamethasone, exhibit side effects such as delay in the proliferation of corneal epithelial cells and wound healing.\textsuperscript{70,72} Herein, the thickness of the cornea epithelium was assessed and topical treatment with naringenin (80 µg) alone for up to 7 days did not affect this parameter. In fact, naringenin reduced alkali-induced decrease in epithelial thickness. However, considering that the antiangiogenic mechanisms of bevacizumab and dexamethasone (e.g., inhibition of VEGF expression and inflammatory and proangiogenic cytokine production) are shared by naringenin, further research is warranted to closely investigate the effects of topical naringenin on corneal epithelial cell proliferation, survival, and damage.

In summary, the present study showed that eye drops containing naringenin reduces alkali-induced neovascularization in mice by mechanism dependent on inhibition of neutrophil and macrophage recruitment, proinflammatory cytokine production, and proangiogenic factor expression, and the preservation of antioxidant defenses. Thus, the local treatment with naringenin and the therapeutic effects of this compound in experimental neovascularization should be considered a promising active treatment for inflammatory neovascularization. Further, it is envisaged that this novel formulation containing naringenin merits further preclinical and clinical investigation on its applicability in inflammatory neovascularization corneal disease. Conceptually, this study also raises the importance of flavonoids in pharmaceutical development.

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References
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