

## Research Article

# Current Trends in Ophthalmology

## Evaluation of Annexin A1 Protein in an Infectious Keratitis Model: Therapeutic Perspectives

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### Abstract

**Introduction:** bacterial keratitis caused by *Pseudomonas aeruginosa* is one of the causes of blindness in the world, due to pathogenicity of the bacteria that can lead to corneal perforation. Studies on the protein annexin A1 (AnxA1) point it as one of the essential mediators in homeostasis of inflammation and ocular infections. However, there are no known reports of AnxA1 in keratitis. Objective: to investigate the expression profile of AnxA1 in the *P. aeruginosa*-induced keratitis model in two strains of mice (susceptible and pathogen resistant).

**Method:** for the development of experimental keratitis, the mice had their right eyes ulcerated and immediately inoculated with 5 µl of *P. aeruginosa* in PBS. The animals were euthanized after 24 hours of the bacteria inoculation. The eyes were clinically evaluated and histopathologically processed for quantitative analyses of the inflammatory infiltrate and immunohistochemical studies of the expressions of the AnxA1 and the receptor for formylated peptides (fpr2).

**Results:** our results demonstrated a higher influx of inflammatory cells, mainly neutrophils in the susceptible animals (C57BL / 6) compared to those considered resistant (BALB / c) and controls. Immunohistochemical studies indicated weak expression of AnxA1 and fpr2 in the anterior epithelium and stroma of the cornea. However, after keratitis induction, overexpression of AnxA1 and fpr2 occurred in the corneas of both mice strains. Furthermore, greater expression of AnxA1 in the anterior corneal epithelium was observed in the C57BL / 6 animals.

**Conclusions:** AnxA1 and fpr2 may be related to infectious keratitis induced by *P.aeruginosa*, which stimulates further investigations on the use of AnxA1 as a possible adjuvant therapeutic strategy in bacterial keratitis.

**Keywords:** Keratitis, AnxA1, *Pseudomonas aeruginosa*, Ocular inflammation

**Abbreviations:** Anxa1: Protein Annexin A1; Ac2-26: Peptide of Annexin A1; BALB/c: Mice; CEUA: Ethics Committee for the Use of Animals; CFU: Colony forming unit; C57BL/6: Mice; Fpr1: Formyl Peptide Receptor-1; Fpr2: Formyl Peptide Receptor-1; IL-8: Interleukin 8; LPS: Lipopolysaccharide; MIP-2: Macrophages inflammatory protein-2; PBS: Phosphate buffered saline; Th1: Lymphocytes T helper1; Th2: Lymphocytes T helper2

### Introduction

Bacterial keratitis is an important cause of visual loss and is frequently associated with changes in corneal

defense mechanisms. Immediate diagnosis and treatment can limit tissue loss, minimize scarring, and reduce the need for future surgery [1,2]. Several mycoorganisms are known as etiological agents of keratitis [2].

Amongst bacterial organisms capable of inducing keratitis, *Pseudomonas aeruginosa* (*P. aeruginosa*) stands out for its rapid propagation that causes ulcerations [3,4]. Keratitis caused by *P. aeruginosa* is related to the continuous and prolonged use of contact lenses, that can cause micro-lesions which facilitate the installation of the bacteria [5,6].

Studies of the host's immune response against *P. aeruginosa* show that the condition and development of the disease is not only related to the production of exoenzymes and other byproducts released from the bacterial proliferation [7,8] but the production of cytokines, some eicosanoids and other molecular mediators of the host are also involved in ulceration and angiogenesis, contributing to stromal necrosis and corneal edema, during bacterial keratitis [9]. Thus, infectious ocular diseases not only involve the effects of colonization factors and bacterial virulence, but also the immune response of the host to the pathogen [10]. Immunocompromised individuals are more vulnerable to *P. aeruginosa* in ocular trauma, systemic and ocular diseases [11-13].

Due to the persistence of *P. aeruginosa* and its ability to acquire resistance [3,14,15] a wide variety of broad-spectrum antibiotic eye drops have been employed in bacterial keratitis, such as ciprofloxacin, tobramycin, gentamicin and cephalosporins [16,17]. Although topical antibiotics are the best treatment for bacterial keratitis [1], high-dose steroid treatment was significantly associated with better visual outcomes in patients with culture-positive bacterial keratitis [18]. Thus, multidimensional therapeutic strategies and adjuvant therapies related to the modulation of the immune response are interesting in the keratitis treatment [1].

In this scenario can be highlighted the protein Annexin A1 (AnxA1), an anti-inflammatory protein with calcium and membrane phospholipids binding sites that is involved in the inhibition of glucocorticoid induced eicosanoids and phospholipase A2 synthesis. Structurally, AnxA1 comprises two domains, a small N-terminal region that contains sites for post-translational processes, such as phosphorylation, glycosylation and proteolysis [19-22], and a central domain formed by four to eight replicates of a highly conserved 70 to 80 amino acid sequence [20,21,23]. Molecular findings on inflammation mechanisms [24] revealed the proactive profile of endogenous AnxA1 and its mimetic peptide, Ac2-26, derived from the N-terminal region, as strong candidates for coadjuvant treatment in

ocular inflammation [25-27].

The protein AnxA1 binds to the receptor for formylated peptides (FPR), which plays an important role in the detection of bacteria and the modulation of immune responses [28], among them Th1 versus Th2 [29]. Although AnxA1 and *fpr2* receptor have been studied in infectious uveitis [26] no reports of these proteins are known in keratitis. In view of this, it is interesting to evaluate the profile of AnxA1 and its receptor in *P. aeruginosa*-induced keratitis model in strains of animals with different immunological responses, such as the Th1 response-related C57BL / 6 mice, which are predisposed to a wide variety of pathogens and infectious antigens, and BALB / c mice, related to Th2 responses and resistant to some pathogens, among them *P. aeruginosa* [30].

The considerable medical and economic impact of infectious keratitis [4], and the increase in cases of microbial keratitis in recent years in Brazil [31,32] stimulated the development of this investigation.

## Materials and Methods

### Animals

Mice of the BALB / c and C57BL / 630 and 6 to 8 week old strains were obtained from the Didactic and Experimental Research Unit of the University Center Padre Albino of Catanduva-SP, Brazil. The animals were divided into 4 groups (n = 10 / group) and kept in cages in environment with controlled temperature (22 to 25°C), with water and food ad libitum. The experimental procedures were conducted in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and after approval of the Ethics Committee for the Use of Animals of University Center Padre Albino, Catanduva, SP, Brazil (No. 02/16). The animals were evaluated by the veterinarian of the institution.

### Preparation of bacteria for inoculation

*P. aeruginosa* bacteria (ATCC27) (NEWprov) were cultured in Nutrient Agar medium (Oxoid, CM003) for 24 hours under agitation at 160 RPM / min. After that, they were centrifuged and washed in PBS to remove bacterial metabolic residues present in the medium and that could interfere in the experiment. They were then resuspended in PBS at a concentration of approximately  $1 \times 10^6$  CFU / ml, and 5ul of the solution were used for ocular inoculation [33-37].

## Experimental model of keratitis

For the development of bacterial keratitis, the animals were anesthetized with ketamine (BioChimico, Itatiaia, Brazil) and xilasín (Ceva Santé Animale, Paulínea, Brazil) by intraperitoneal via (i.p.). The cornea of the right eye was scarified by three parallel incisions (1 mm deep) with a 21G needle. These incisions did not penetrate beyond the superficial stroma. In the right eye an aliquot of 5  $\mu$ l *P. aeruginosa*, resuspended in PBS at  $1 \times 10^6$  CFU / ml 14-30 was inoculated. To minimize discomfort, the animals received codeine, diluted in water and freely offered. Drops of lubricating eye drops were given every 12 hours. The animals induced to keratitis were euthanized after 24 hours of inoculation of the bacteria by excessive anesthetic dose. Animals without manipulation were used as controls (n = 5).

## Morphological evaluation

After 24 hours induction of bacterial keratitis, the animals were anesthetized and eyes clinically evaluated with surgical loupe aid (D.F Vasconcellos S.A Brazil), F = 160 mm (focus), an increase of 24x.

## Histopathological and immunohistochemical studies

The right eyes (n = 5 / group) were fixed in 4% formaldehyde, dehydrated in increasing order of ethanol and included in paraffin for histopathological and immunohistochemical studies.

Histopathological analyses were performed in sections of 3  $\mu$ m by staining with Hematoxylin and Eosin (HE) and 1% Toluidine Blue. Inflammatory cells quantification was performed in different random images per slide under the 40x objective of the Leica microscope (DM500) and the tissue areas were obtained in the Leica Image Analysis Software.

In the immunohistochemical studies, the expressions of the AnxA1 and fpr2 were evaluated. After antigenic recovery with citrate buffer pH 6.0 and blockade of the endogenous peroxidase activity, the sections were incubated with the following rabbit polyclonal primary antibodies: anti-AnxA1 (1: 1000) (Invitrogen, Cat No: 71-3400) and anti-FPR2 (1:2000) (Invitrogen, Cat No: 72-0293), for 12 hours. They were then incubated with the biotinylated secondary antibody (Histostain Kit, Invitrogen, Cat No: 95-9643B) and immersed in conjugated streptavidin peroxidase complex. Substrate diaminobenzidine (DAB Kit, Invitrogen, Cat No: P00-2020)

was used for development and, thereafter, the sections were stained with Hematoxylin. Proteins were quantified by densitometry using the Leica Image Analysis Software. For the densitometric analyses, three different slides of each animal were used and 20 points were analyzed in four regions of the cornea, in order to obtain a mean related to the intensity of the immunostaining. The values were obtained as arbitrary units [26].

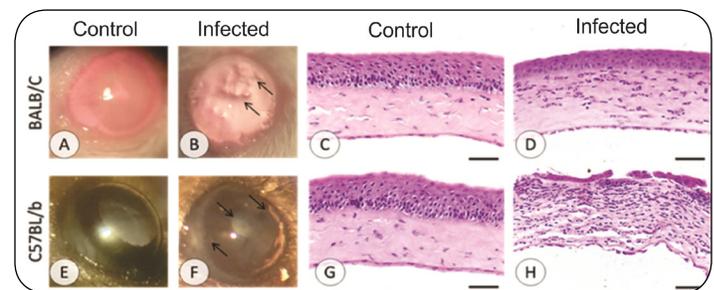
## Statistical analyses

Data from the quantitative and densitometric studies were obtained as mean  $\pm$  S.E.M. and compared by T-test. P values less than 0.05 were considered statistically significant.

## Results

### Morphological and histopathological analyses

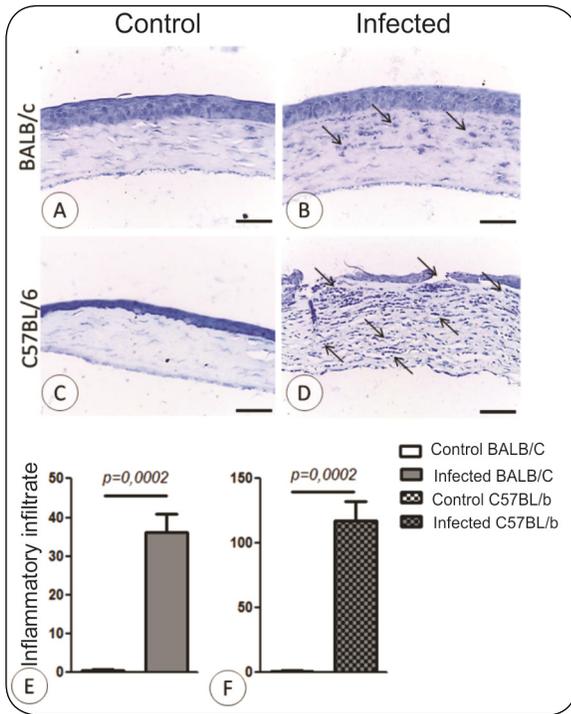
Morphological analyses of infected eyes showed opacity covering the pupil and hypopyon in the anterior chamber after 24 hours of inoculation of the *P. aeruginosa* bacterium in both strains. Histopathological studies evidenced an intense inflammatory influx, especially in the C57BL / 6 animals, which presented higher neutrophilic infiltrate (Figure 1).



**Figure 1:** Clinical and histopathological evaluation of keratitis induced by *P. aeruginosa*. Control eyes (A, E); infected eyes (B, F). Corneas of control animals, without presence of immune cells (C, G); Corneas of animals induced to keratitis, with intense inflammatory infiltrate (D, H). Sections 3  $\mu$ m. Color: Hematoxylin-Eosin Bars: 5  $\mu$ m.

### Quantification of inflammatory infiltration

Histopathological studies and the quantification of inflammatory cells after Toluidine Blue staining corroborate with the initial analyses and indicate leukocyte influx, especially neutrophils, in both strains exposed to the bacterium, with exacerbation in the C57BL / 6 lineage, compared to BALB / c (p = 0.0019) (Figure 2).



**Figure 2:** Corneal infiltrate in *P. aeruginosa*-induced keratitis. Control eye (A, C), infected eye (B, D). C57BL / 6 susceptible animals show increased neutrophilic (D) infiltration, compared to resistant BALB / c animals (B); Quantifications of infiltrated inflammatory cells (E, F). Sections 3  $\mu$ m. Color: Toluidine blue Bars: 5  $\mu$ m.

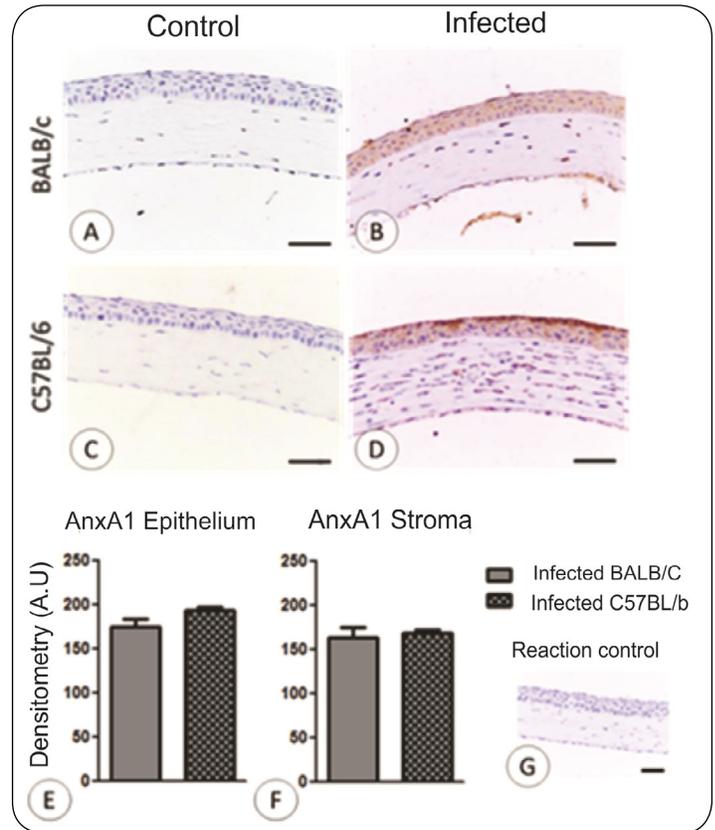
### Immunohistochemistry

Immunohistochemical studies evidenced the expression of the AnxA1 protein in the corneal epithelium and stroma in keratitis-induced animals. Mice of both strains exposed to *P. aeruginosa* overexpressed the protein compared to their respective controls. Although higher expression of AnxA1 in the epithelium of C57BL / 6 animals occurred, this difference was not statistically significant between the two mouse strains (Figure 3).

The analysis of fpr2 receptor expression showed strong staining in the corneal epithelium in keratitis-induced animals. However, no significant differences were observed in the expression profile between the animals exposed to bacteria from both strains (Figure 4).

### Discussion

Keratitis is an important ocular process that is mainly due to the action of microorganisms [1,2]. Due to the resistance to treatment and the possibility of irreversible damage to vision, studies on the expression profile of the AnxA1 protein may help in the search for new therapies to improve clinical outcomes.



**Figure 3:** Expression of AnxA1 in *P. aeruginosa*-induced keratitis. Control eye (A, C), infected eye (B, D). The densitometric analyzes indicated profile overexpression of the protein in both strains (E, F), negative control reaction (G). Counterstain: Harris hematoxylin. Bars: 5  $\mu$ m.

Our initial morphological analyses of the keratitis-induced animals revealed in both strains the common clinical characteristics of this condition, with the presence of opacity and hypopyon in the anterior chamber, more evident in the susceptible animals. Our data are consistent with the clinical characterization of *P. aeruginosa*-induced keratitis described in other investigations [1,9,14,30,38], which serves as a paradigm for human keratitis [39,40].

The bacterium's response to the host in an infectious process shows that bacteria release numerous toxins and proteases, such as exotoxin A5 [7], exoenzymes S [41] and phospholipase C [42], which cause tissue damage. Exoenzymes released from bacterial proliferation are responsible for the initial pathogenicity; however, a large part of the ocular tissue is destroyed due to the release of exoproducts from corneal cells to stimulate leukocyte migration in the fight against bacterial infection [9].

The orchestrated inflammatory process involves

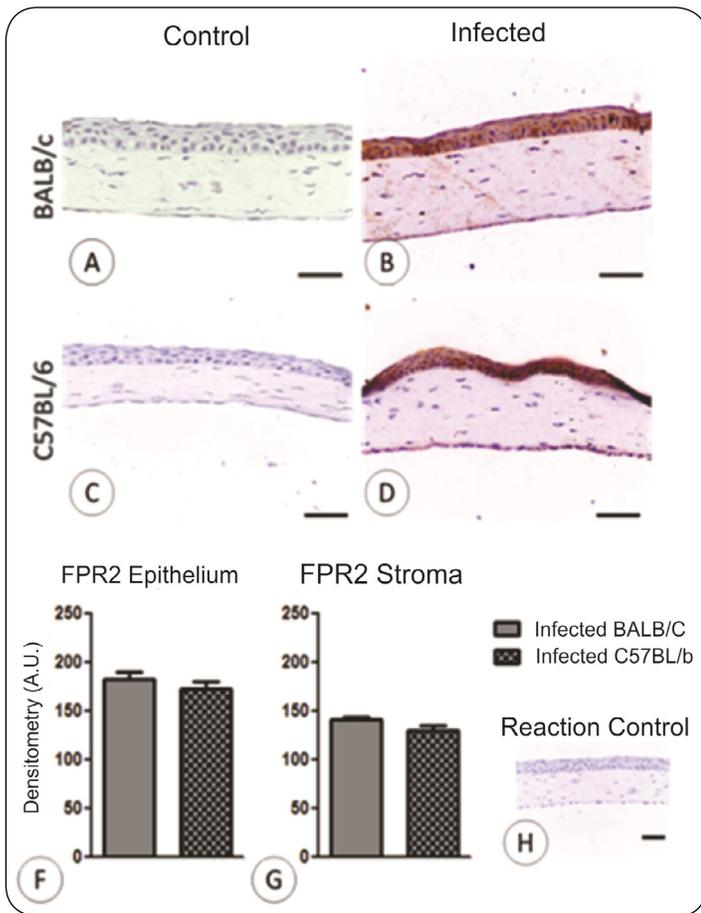
phagocytosis, lysosomal degranulation, production of toxic substances and oxygen metabolites, biosynthesis of superoxide anions and other oxidizing agents, such as: hydrogen peroxide and peroxynitrite formation, which are synthesized on the corneal surface due to or immune response [4]. In turn, these free radicals cause destruction of the stroma, degrading collagen and glycosaminoglycans, disrupting keratocyte proliferation [43]. Cytokines and chemokines also play a role in the control of pathogens, which may be beneficial or not, since they may present a role in the resolution or exacerbation of a bacterial inflammatory response induced by *P. aeruginosa* and other ocular pathogens [38].

have demonstrated direct correlation of influx of polymorphonuclear cells into tissue with the presence of the chemokine MIP-2, (functional homologue of human IL-8), capable of recruiting neutrophils [38,44]. Resistant animals BALB / c normally express less MIP-2A and the neutralization of this inflammatory mediator reflects in the decrease of the infiltrate in susceptible animals C57BL / 6 [45], which may explain the difference of the infiltrate between the strains.

After observing the validity of the model used with evidence of the most intense inflammatory process in the C57BL / 6 line and knowing the anti-inflammatory potential of the protein AnxA1, in the continuity of the investigation we observed the expression of this protein. Our studies showed overexpression of AnxA1 in animals induced-keratitis. However, there was no significant difference in protein expression between the two strains. Another research indicates that AnxA1 inhibits the production of arachidonic acid and consequently the production of inflammatory lipid mediators, such as prostaglandins and leukotriene, induced by glucocorticoids [46]. Furthermore, AnxA1 is important in the resolution of diseases caused by the pathogenicity of chronic inflammation and in the acute conditions of the innate immune system [21] and has its synthesis increased during the inflammatory process [47].

Thus, the overexpression of AnxA1 observed in this investigation suggests the compensatory action of the protein in the control of neutrophil migration. Other studies showed the relation of the AnxA1 protein and its mimetic peptide, Ac2-26, to the defense of the organism against pathogens and the resolution of inflammation by attenuating the cytokine-mediated immune responses characteristic of both Th1 and Th2 profiles [26,48]. The mechanism of action of AnxA1 in intraocular inflammation was analyzed in the ovalbumin-induced allergic conjunctivitis and indicated the importance of the Th1/Th2 balance in the development of allergic inflammatory responses, pointing a protective role for AnxA1 in ocular allergy through the negative regulation of both cytokines profiles [27].

Other researches performed at our laboratory showed increased expression of AnxA1 in the neutrophils, at 24, 48, and 72h after intravitreal *Toxoplasma gondii* infection [49] and also in leukocytes, mast cells and aqueous humor in endotoxin-induced uveitis in rats [25]. These data showed a modulation of the endogenous AnxA1 in transmigrated cells to the inflammatory sites,



**Figure 4:** Expression of the fpr2 receptor. Control eyes (A, C), infected eyes (B, D). Both lineages show similar patterns of immunostaining. Densitometry (F, G). Negative control reaction (H). Counterstain: Harris hematoxylin. Bars: 5 μm.

To evaluate the inflammatory process in this study, we performed histopathological analyses by quantification of inflammatory cells infiltrated into the cornea. These quantifications indicated exacerbated influx of neutrophils mainly in the C57BL / 6 strain. Several in vivo models of inflammation, including infectious keratitis,

indicating that the protein AnxA1 regulates the leukocyte transmigration and the production of proinflammatory mediators in neutrophils.

Finally, knowing that FPR receptors can mediate the actions of AnxA1 [26,50], we evaluated the expression of the fpr2 receptor. Our data show that the expression of fpr2 overlaps with the expression of AnxA1 and reinforces that AnxA1 / fpr2 interaction in the resolution of ocular inflammatory processes [26]. Previous studies of our research group evaluated the mechanism of action of AnxA1 and the Ac2-26 peptide in EIU and LPS-activated retinal pigment epithelial cells (ARPE-19) [26]. These results showed that AnxA1 serine-phosphorylated interact with the fpr2 and inhibits the release of inflammatory mediators. A recent investigation in experimental allergic conjunctivitis indicated increased Fpr1 and Fpr2 levels in the conjunctiva, but the lack of endogenous AnxA1 exacerbated Fpr2 expression only, what was reverted by the administration of Ac2-26 peptide. The investigators also showed the co-localization of Fpr2 and AnxA1 in the plasma membrane of mast cells, eosinophils and neutrophils, supporting that the AnxA1/Fpr system acts to modulate the anti-inflammatory and antiallergic response being an important therapeutic target in ocular inflammation.

### **Ethics approval and consent to participate**

Ethics Committee for the Use of Animals of University Center Padre Albino, Catanduva, SP, Brazil (No. 02/16)

### **Consent for publication**

Yes

### **Availability of data and material**

Yes

### **Competing interests**

There is no conflict of interest

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### **Authors' contributions**

**Experimental design:** Girol AP

**Experimental execution:** Da Silva RA, Hamade

AMA, Silva GAS, Pereira GH, Oliveira FF, Costa SS, Iyomasa-Pilon MM, Souza HR, Possebon L, Girol AP.

**Financing:** Girol AP

**Writing:** Possebon L, Souza HR, Girol AP

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University Center Padre Albino

### **Conclusion**

Our analyses point to AnxA1 as a possible mediator in infectious keratitis induced by *P.aeruginosa* in infection susceptible and resistant strains of mice, suggesting AnxA1 as a candidate for therapeutic strategy in bacterial keratitis, which deserves further studies.

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