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Tear lactoferrin and ceruloplasmin levels in patients with traumatic and recurrent corneal erosions

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Background: Studies on the mechanisms of corneal wound healing are still important. Apart from the integrity of the corneal epithelium, tear fluid is important for maintaining homeostasis of the ocular surface; it is composed of a variety of proteins, lipids and metabolites. Studies on changes in concentrations of biochemical tear components are important for the diagnosis and treatment of corneal injuries.

Purpose: *To assess changes in tear lactoferrin (Lf) and ceruloplasmin (Cp) levels over the course of comprehensive treatment for patients with traumatic corneal erosions (TCE) and recurrent corneal erosions (RCE).*

Material and Methods: *The study sample included 62 patients (19 to 65 years of age; mean age plus or minus standard deviation, 43.5 ± 2.4 years). Group 1 included 44 patients with TCE, and group 2, 18 patients with recurrent RCE. Each patient group was divided into two subgroups on the basis of the treatment method. Subgroup 1 was administered eye broad-spectrum antibiotic (AB) eye drops and dexpantenol over a course of treatment. Subgroup 2 received AB eye drops and dexpantenol plus adjunct lactoferrin (Lf)-containing eye drops. An eye examination included visual acuity, biomicroscopy and fluorescein test. Monospecific antibodies were used to determine tear Lf and Cp levels. Tears from healthy volunteers were used as controls.*

Results: *At baseline, the tear Lf level in patients with TCR was lower than in controls (3.94 ± 0.45 arbitrary units (a.u.) versus 10.3 ± 0.4 a.u., respectively; $p < 0.05$), resulting in reduced ocular surface protection. In subgroup 1 of the TCE group, after treatment with an AB plus dexpantenol only, the tear Lf level increased to 6.38 ± 0.55 a.u. ($p < 0.05$), and the mean period of treatment was 7.6 ± 0.43 days ($p \geq 0.1$). In subgroup 2 of the TCE group, after treatment with an AB plus dexpantenol plus Lf-containing eye drops, the tear Lf level was 12.23 ± 0.6 a.u. ($p < 0.05$) and the mean period of treatment was 6.0 ± 0.23 days. The presence of Cp in the tear fluid prior to treatment for TCE or RCE indicated activation of acute inflammation; at baseline, the tear Cp level in patients with TCE was 2.37 ± 0.25 a.u. compared to controls ($p < 0.05$), and in those with RCE, 1.78 ± 0.2 a.u. On completion of treatment with Lf-containing eye drops, the tear Lf level increased and the tear Cp level decreased to the levels in controls, and there was a negative correlation between the tear Lf level and the tear Cp level ($r = -0.491$, $p < 0.001$).*

Conclusion: *The results confirmed the feasibility of utlizing Lf-containing eye drops as an adjunct in the treatment of TCE and RCE. This approach contributed to the restoration of ocular surface homeostasis, thus promoting corneal epithelialization and enabling a reduction in treatment duration.*

Keywords:

corneal trauma, lactoferrin, ceruloplasmin, recurrent corneal erosion

Introduction

The global incidence of eye injuries is estimated by the WHO to be over 55 million each year [1, 2]. Eye injury results in loss of corneal transparency and structure, leading to a significant loss of vision or blindness, which is an important social and economic problem. In developed countries, the incidence of blindness due to eye injury was 9/100,000 people, but in developing countries, that rate was 75/100,000 [1, 2, 3].

Corneal erosion is the most common type of anterior segment injury, and, among all emergency room visits with ocular complaints, about 80% present with corneal erosions. Corneal erosion is a defect in the corneal epithelium to the Bowman membrane which develops due to various mechanical, physical and chemical factors.

Superficial erosions of the corneal epithelium can re-epithelialize within 24-48 hours due to fast regeneration of multilayered non-keratinized epithelium and restoration of the morphology and function of the eye [1, 3]. Pathological epithelial regeneration occurs in injuries involving the deep corneal layers, and is manifested by the persistent changes in corneal morphology [4].

In some cases of traumatic corneal erosion, the corneal structure at the level of the basal membrane does not regenerate. In up to 65% patients with recurrent corneal erosion, a history of previous corneal trauma can be identified. Recurrent erosions occur secondary to inflammation from the inciting insult, which causes disruption of the epithelial basement membrane and proper extracellular adhesions of the hemidesmosomes [5-7]. Recurrent corneal erosion (RCE) is a multifactorial disease characterized by episodes of spontaneous corneal epithelial breakdown. Patients with RCE typically experience sudden onset of eye pain, usually on first awakening. Associated symptoms include tearing, blepharospasm, photophobia, and foreign body sensation [6, 7]. Therefore, studies on the mechanisms of corneal wound healing are still important. Apart from the integrity of the corneal epithelium, tear fluid is important for maintaining homeostasis of the ocular surface; it is composed of a variety of proteins, lipids and metabolites. Studies on changes in concentrations of biochemical tear components (e.g., such proteins as lactoferrin (Lf) and ceruloplasmin (Cp)) are important for the diagnosis and treatment of corneal injuries [8, 9].

Lactoferrin (Lf) is an iron-binding glycoprotein of the transferrin family, represents approximately 25% by weight of the total tear proteins, and is essential for ocular surface protection. Immune modulating and anti-inflammatory effects of Lf in acute and chronic inflammation of the ocular surface have been demonstrated. Studies have reported that Lf can exert effects on innate and acquired immune response, has antioxidative properties, and reduces oxidative stress effect and tissue inflammation [10, 11, 12]. Reduced lactoferrin levels were found in patients with dry eye, keratoconus and other anterior ocular surface diseases [11, 12].

Quantitative changes in tear lactoferrin during reparation processes in corneal injury have been, however, poorly investigated. Cp is an active inflammation protein, a member of the multicopper oxidase family, and its concentration is substantially increased in patients with neurodegenerative, inflammatory and metabolic conditions. The oxidation of Fe^{2+} to Fe^{3+} is a major physiological reaction catalized by ceruloplasmin and allows released iron binding to transferrin [13]. Human Lf and Cp have been shown to interact in vivo and in vitro with the formation of the complex which triggers the antioxidant defence mechanism. In addition, the Lf-Cp complex has been identified in serum and purulent exudate samples from patients with various inflammatory disorders [14].

The purpose of this study was to assess changes in tear Lf and Cp levels over the course of comprehensive treatment for patients with traumatic corneal erosions (TCE) and recurrent corneal erosions (RCE).

Material and Methods

This study included patients who presented to the Oleksandrivska Clinical Hospital (the clinical site of Bogomolets National Medical University) and were treated for a diagnosis of traumatic corneal erosion or recurrent corneal erosion in the period 2021-2023. Written informed consent was obtained from all participants. The study was approved by the local bioethics committee of Bogomolets National Medical University (committee minutes No.138 dated November 10, 2020) and adhered to the Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects and the principles of the UNESCO Declaration on Bioethics and Human Rights.

The study sample included 62 patients (19 to 65 years of age; mean age plus or minus standard deviation (SD), 43.5 ± 2.4 years). Of these, 34 (54.8%) were men, and 28 (45.2%) were women. The control group was composed of 15 practically healthy individuals (18 to 50 years of age; mean age plus or minus SD, 35.4 ± 2.1 years). Of these, 9 (60%) were men, and 6 (40%) were women. Patients were divided into two groups on the basis of clinical symptoms. Group 1 included 44 patients diagnosed for the first time with TCE of various etiologies, and group 2, 18 patients diagnosed with RCE. The diagnosis was made on the basis of eye examination and careful history taken by a specialist, taking into account any prior history of ocular surface trauma or episodes of corneal erosion.

Patient groups were matched for the major clinical and demographic characteristics. Each patient group was divided into subgroups on the basis of the treatment method. Subgroup 1 was administered eye drops with a broad-spectrum antibiotic (AB) and dexpantenol over a course of treatment. Subgroup 2 received adjunct treatment with lactoferrin-containing eye drops.

An eye examination included visual acuity, biomicroscopy and fluorescein test. Biomicroscopy was used to assess the cornea and conjunctiva and presence of infiltration and corneal stromal edema.

Grading of bulbar conjunctival hyperemia and injection severity was done on a 0–3 scale. A strip of commercially marketed sodium fluorescein containing 1 mg fluorescein (Fluostrip; Contacare Ophthalmics and Diagnostics, Gujarat, India) was used for corneal staining assessment. De-epithelialization zone was stained yellow-green and assessed using a slit-lamp microscope with a cobalt blue filter. Grading of corneal damage pattern was done on a 1-5 scale (1, central; 2, superior; 3, temporal; 4, nasal and 5, inferior). Grading of fluorescein staining for central, superior, temporal, nasal and or inferior zone was done on a 0–3 scale. A score of 0 was given for no staining; 1 was given for few punctuate staining that could be easily counted; 2 was given for moderate punctuate staining

where the individual punctuate staining was too great to count, but not yet coalesced; 3 was given for coalesced staining.

Tear samples were taken from the inferior conjunctival fornix using a sterile plastic tip attached to a pipette aid. Thereafter, they were transferred to Eppendorf tubes containing sodium phosphate buffer and stored at −20 °C until analysis. The proteins, 50 μg/lane, were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to a nitrocellulose membrane. Monospecific antibodies were used to determine tear Lf and Cp levels. Rabbit polyclonal antibodies were obtained as reported previously [15] and used for recognizing human Lf in immunoblotting. Mouse monoclonal ceruloplasmin antibodies (Santa Cruz Biotechnology; catalog number, sc-365205) were used to perform immunochemical analysis of Cp. The non-specifically bound primary antibodies were washed off, and the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (1:6000, Invitrogen Anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Catalog # G-21234; 1:8000, Invitrogen Anti-Mouse IgG (H+L) Secondary Antibody, Catalog # 31430).

Immunoblotting is a method of solid-phase immunochemical analysis. Unlike immune-enzyme assay, immunoblotting allows to establish the molecular weight of proteins under investigation, which can provide additional information about the formation of intermolecular complexes or the presence of products of proteolytic cleavage of proteins. Semi-quantitative analysis of western blots is a common method to evaluate the quantity of proteins of interest in biological samples since the intensity of immunostaining and the area of protein zone are proportional to protein content in the sample. Densitometric analysis of blot images is used for this purpose, with the protein level expressed in arbitrary units [16].

An enhanced chemiluminescence system (ECL) was used for subsequent detection of bound antibodies and the blots exposed to X-ray film (Kodak) or immunostaining bands were revealed by using the chromogenic substrate (3,3' diaminobenzidine substrate). Semi-quantitative measurements of the Western blotting results were performed using TotalLab TL120 analysis software (Nonlinear Inc., Durham NC, USA).

EZR on R Commander (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analysis. Data are presented as mean ± standard deviation (SD). The level of significance $p \leq 0.05$ was assumed. Spearman correlation coefficient was used in correlation analysis.

Results

Defects were located centrally in 24 eyes (54.5%) and paracentrally in 20 eyes (45.5%), and stromal edema and infiltration were seen in 13 eyes (29.5%) with TCE. In eyes with RCE, defects were more frequently located paracentrally (10 eyes or 55.6%) than centrally (8 eyes or 44.4%). We observed that RCE most commonly occurred at the inferior cornea. In RCE, biomicroscopy showed microform erosions, macroform erosions, microcysts, fingerprint lines and maplike patterns. In addition, stromal edema and infiltration were seen in 10 eyes (55.6%).

Clinical characteristics of patients in groups 1 and 2 are presented in Table 1.

Figs. 1 and 2 present the results of Western blot analysis for Lf and Cp in the tear fluid of patients in groups 1 and 2, before and in the course of treatment. In addition, in healthy controls, the tear Lf level as assessed by immunoblotting was 10.3 ± 0.4 a.u. Blot image of the protein Lf showed a major polypeptide of 80-kDa associated with high-molecular weight complexes of 300-kDa and minor amounts of degradation products.

At baseline, the tear Lf level in patients with TCR was rather low (3.94 ± 0.45 a.u.) and 6.36 ± 0.05 a.u. lower than in healthy controls ($p < 0.05$), resulting in reduced ocular surface protection.

In subgroup 1 of the TCE group, after treatment with an AB plus dexpantenol, the tear Lf level increased to 6.38 ± 0.55 a.u., which was 2.44 ± 0.1 greater than at baseline ($p < 0.05$), and the mean period of treatment was 7.6 ± 0.43 days. In subgroup 2 of the TCE group, after treatment with an AB plus dexpantenol plus Lf-containing eye drops, the tear Lf level was 12.23 ± 0.6 a.u., which was 5.85 a.u. greater than in subgroup 1 ($p < 0.05$), and the mean period of treatment was 6.0 ± 0.23 days, which was 1.6 ± 0.2 days shorter than in subgroup 1 ($p = 0.05$).

In the RCE group, in early disease, the tear Lf level was 4.28 ± 0.4 a.u., which was 6.02 ± 0.01 a.u. lower than in healthy controls ($p < 0.05$). In subgroup 2 of the RCE group, after treatment with an AB plus dexpantenol plus Lf-containing eye drops, the tear Lf level increased to 8.43 ± 0.8 a.u., which was 2.62 ± 0.46 a.u. greater than in subgroup 1 treated with an AB plus dexpantenol only ($p < 0.05$). The mean period of treatment was longer for subgroup 1 than for subgroup 2 of the RCE group (9.25 ± 0.94 days against 7.6 ± 0.56 days, respectively, $p < 0.05$).

Immunoblotting demonstrated (Fig. 2) that Cp was present only in trace amounts in the tear fluid of healthy controls. The presence of Cp (a major polypeptide of 150-kDa) in the tear fluid early after a traumatic event indicates activation of acute inflammation; at baseline, the tear Cp level in patients with TCE was 2.37 ± 0.25 a.u. compared to controls ($p < 0.05$). In addition, at baseline, the tear Cp level in patients with RCE was 1.78 ± 0.2 a.u. compared to controls ($p < 0.05$).

In the current study, after treatment with adjunct Lf-containing eye drops, the tear Lf level in patients with TCE or RCE increased to the level in controls and, correspondingly, the tear Cp level in these patients decreased, which was confirmed by a negative correlation between the tear Lf level and the tear Cp level ($r = -0.491$, $p < 0.001$).

Table 1. Clinical characteristics of patients with traumatic corneal erosions (group 1) and recurrent corneal erosions (group 2) before and after treatment

		Visual acuity (M±SD)		Conjunctival staining score		Corneal staining score	
		Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Group 1 n=44	Subgroup 1 (n=20)	0.57±0.2	0.88±0.12	2.13±0.83	0.6±0.19	5.68±2.15	0.6±0.68
	p	p<0.01		p<0.01		p<0.01	
	Subgroup 2 (n=24)	0.61±0.21	0.92±0.09	2.07±0.79	0.39±0.62	5.55±2.16	0.48±0.66
	p	p<0.01		p<0.01		p<0.01	
Group 2 n=18	Subgroup 1 (n=8)	0.62±0.19	0.94±0.08	1.7±1.25	0.4±0.52	5.62±2.26	0.5±0.53
	p	p=0.03		p=0.03		p<0.01	
	Subgroup 2 (n=10)	0.6±0.17	0.96±0.07	1.75±1.03	0.36±0.52	5.9±2.18	0.38±0.52
	p	p<0.01		p<0.01		p<0.01	

Note: n, number of eyes; M, mean value; SD, standard deviation; p, significance of difference between post-treatment and baseline values

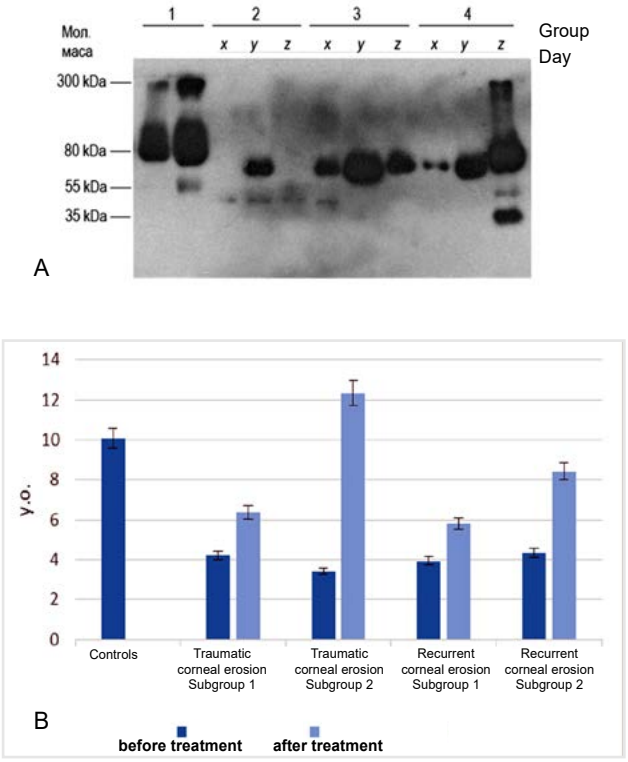

Fig. 1. Changes in tear lactoferrin levels in patients. (A) Representative blots for lactoferrin. (B) Bar graphs show results of densitometry of bands in immunoblots of tear lactoferrin in patients with traumatic corneal erosion and recurrent corneal erosion ($p < 0.05$)

Note: 1, controls (healthy volunteers); 2-4, traumatic corneal erosion group; x, before treatment; y, day 3; z, day 7.

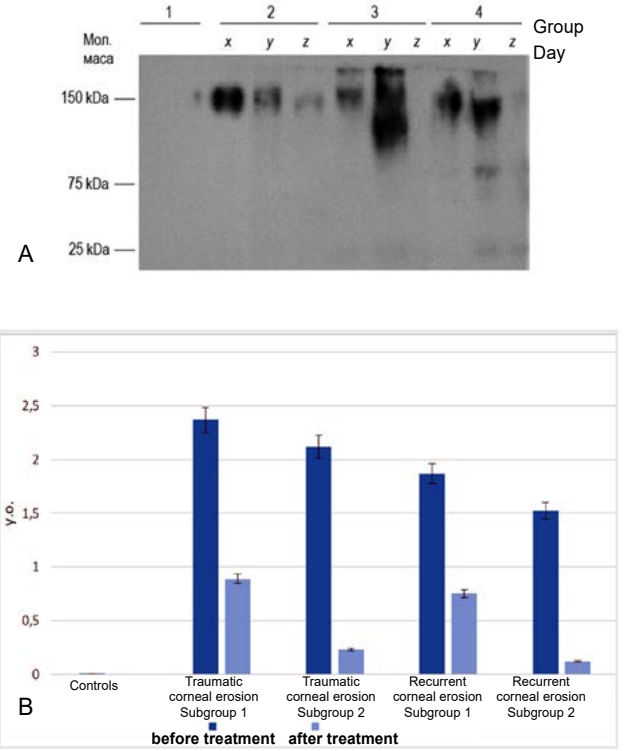

Fig. 2. Changes in tear ceruloplasmin levels in patients. (A) Representative blots for ceruloplasmin. (B) Bar graphs show results of densitometry of bands in immunoblots of tear ceruloplasmin in patients with traumatic corneal erosion and recurrent corneal erosion ($p < 0.05$)

Note: 1, controls (healthy volunteers); 2-4, traumatic corneal erosion group; x, before treatment; y, day 3; z, day 7.

Discussion

Wilson and colleagues found that corneal wound healing is a complex pathophysiological process consisting of the following phases: cell migration/inflammation, cell proliferation/differentiation and matrix remodeling [1, 3, 4, 17]. Although the inflammation phase plays an important role in successful wound healing, cheonic inflammation may result in changes in the extracellular matrix, potential leading to recurrent erosions [5, 7, 17].

Cp is an acute phase reactant in the setting of inflammation and protects tissues from free radical-induced damage [9, 13]. Human Cp is a multifunctional copper protein enabling multiple enzymatic interactions in the focus of inflammation. These features make it an effective antioxidant capable of inhibiting oxidative damage of proteins, DNA and lipids. The interaction of Cp with other proteins further expands its functions and plays a key role in most cell processes [9, 13, 14].

In the current study, an elevated baseline level of Cp in the tear fluid in eyes with TCE indicates the stimulation of inflammation after corneal injury, whereas the tear Cp level on completion of treatment with an AB plus dexpantenol plus Lf-containing eye drops was not statistically significantly higher than the tear Cp level in controls. Therefore, the results of the current study confirm that Lf contributes to the resolution of inflammation in the affected eye, and are in agreement with previous studies of others [10, 12, 13, 14]. Western blot analysis found similar changes typical for the acute trauma period (i.e., a reduction in the level of Lf and an elevation in the level of Cp compared to controls) in the tear fluid of patients with RCE. Our results confirm those by others that the induction of inflammation plays a pivotal role in the pathogenesis of RCE and in chronic setting can result in disruption of the proper extracellular adhesions of the hemidesmosomes [6, 7, 8]. Particularly, Thompson and colleagues demonstrated that Cp forms complexes with metalloproteinases, and disruption in their regulation results in a weakened adhesion of epithelial cells to the basement membrane [7, 18].

The glycoprotein Lf is known to support iron homeostasis and have antimicrobial, bacteriostatic, antiviral and immunomodulating properties [10, 11, 19].

In the current study, in healthy controls, the tear Lf level as assessed by immunoblotting was 10.3 ± 0.4 a.u., which is in agreement with the literature data [10, 11, 12, 21, 22]. Lf molecule is folded into two similarly sized homologous N- and C-lobes. Both lobes are made up of two domains, with the iron-binding sites situated within the interdomain cleft. There are two forms of Lf, namely the iron-free form (Apo-Lf) and the iron containing (holo-Lf). As a member of the transferrin family, Lf can incorporate Fe(III) ions produced during ferroxidase reaction catalyzed by Cp. Therefore, the oxidation of prooxidative Fe(II) ions catalyzed by Cp will reduce oxidative stress. The chelating agent will bind with iron ions in case of Cp oxidation [20]. The current study demonstrated a low tear Lf level and a high tear Cp level in the initial disease stage in eyes with TCE and in those with RCE, which is in agreement with findings of studies on tear Lf and Cp levels in eyes with chronic conjunctivitis or keratoconus [9, 10, 11, 23, 24].

The increase in inflammation is accompanied by necrosis of adjacent tissues, which, in turn, leads to an increase in the pool of Fe(II). Active oxygen species such as inflammation-induced hydroxyl radicals and oxygen radical superoxidase anion can react with prooxidant ions and promote oxidative damage. The formation of the Cp-Lf complex increases the ferroxidase activity of Lf, contributing to the chelation of iron ions. Effective iron chelation by the complex and further iron withdrawal from the focus of inflammation will result in a reduction in iron levels. Therefore, the Cp-Lf complex in the tear of patients with traumatic injuries neutrophil burst neutrophil burst and protects adjacent tissues from further damage [18, 19].

Of note that iron binding and chelation is associated also with antibacterial effects of Lf which competes for iron with pathogenic iron-binding systems, which in turn inhibits bacterial and microbial growth. The most obvious mechanism of antibacterial and antimicrobial action of Lf is that Lf deprives bacteria and microbes of a nutrient source via iron binding and chelation in the environment surrounding them. Another mechanism of antimicrobial activity of Lf is its direct binding to the microbial or bacterial cell, which causes cell membrane destabilization in a wide variety of microbes and bacteria [20, 21, 22]. Because Lf plays an important role in the activation of immune-protective mechanisms and modulates immune response to viral or bacterial intrusion after traumatic damage, a reduction in tear Lf level will result in reduced tear protection and abnormal homeostasis of the ocular surface.

Lf-containing eye drops, when used as an adjunct to AB and dexpantenol, resulted in a reduction in the tear Cp level and an increase in tear Lf level both in the TCE and the RCE groups. The biochemical changes in the tear fluid corresponded to the clinical picture of corneal epithelialization, and contributed to the restoration of corneal transparency and structure and reduction in subjective symptoms of corneal syndrome. The results of this study in terms of the effect of Lf on corneal epithelialization and reduction of inflammation symtoms are in agreement with findings of previous experimental studies [10, 12, 14].

Conclusion

First, in patients with TCE and in those with RCE, the tear Lf level at baseline was 6.36 ± 0.05 a.u. and 6.02 ± 0.01 a.u., respectively, lower than in healthy volunteers, which resulted in reduced tear film protection and abnormal homeostasis of the ocular surface.

Second, at baseline, patients with TCE and those with RCE showed a high tear Cp level (2.37 ± 0.25 a.u. and 1.78 ± 0.2 a.u., respectively), which indicated acute-phase inflammation in these disorders.

Third, there was a negative correlation between the tear Lf level and the tear Cp level ($r = -0.491$, $p < 0.001$) in patients treated with adjunctive Lf-containing eye drops for TCE or RCE, which indicated the interaction of these proteins during corneal inflammation and wound epithelialization.

Finally, inclusion of Lf in the treatment regimen for TCE and RCE enabled a reduction in treatment duration by 1.6 ± 0.16 days and 1.65 ± 0.65 fays, respectively.

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Disclosures

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Abbreviations: *a.u., arbitrary units; Cp, ceruloplasmin; Lf, lactoferrin; RCE, recurrent corneal erosion*