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Lactoferrin concentration in tears of patients with chronic conjunctivitis and effect of Lacto eyedrops in the multicomponent treatment for this disorder

G. I. Drozhzhyna, L. Iu. Riazanova, N. I. Khramenko, L. M. Velychko

SI "The Filatov Institute of Eye Diseases and Tissue Therapy of the NAMS of Ukraine";
Odesa (Ukraine)

Background: *Lactoferrin (LF) is a non-heme iron-binding glycoprotein of the transferrin family. It is a natural component of the tear film, has bacteriostatic, bactericidal, fungicidal, antiviral, and antioxidant properties, and thus plays an important role in the protection of the ocular surface from infections. A low tear LF concentration has been found in some ocular disorders, but there have been no reports on the determination of LF concentration in tears of patients with chronic conjunctivitis (CC).*

Purpose: *To determine LF concentration in tears of patients with CC, and to assess the effect of LACTO eye drops in the multicomponent treatment for the disorder.*

Material and Methods: *Eleven patients (17 eyes) with CC were included in the study. Patient age ranged from 27 to 68 years, and the duration of CC, from 3.5 to 7 months. The ophthalmological examination included biomicroscopy of the bulbar conjunctiva and cornea, fluorescein examination, determination of corneal sensation and basal tear production (Schirmer's II test), and microbiological examination of the conjunctiva. Corneal and conjunctival xerosis was assessed using the method of van Bijsterveld. Patients were administered a topical antiseptic four times daily, preservative-free hyaluronic acid artificial tears four times daily, and Lacto eye drops twice daily over a month. Concentration of LF in the tear samples was determined by a human LF enzyme-linked immunosorbent assay kit before treatment and at day 30 of treatment.*

Results: *Seven patients (11 eyes) underwent an examination after the completion of a one-month treatment with Lacto eye drops. Tear LF concentration (mean ± standard deviation (SD)) in patients with CC was 1.37 ± 0.4 mg/ml (95% confidence interval, 1.16–1.58). The examination found no microbial growth in all these 11 eyes, with 6/7 patients presenting no complaints after treatment. There was no statistically significant change in Schirmer II test values from baseline. Corneal sensation was found to be improved in 6/11 eyes. After a one-month treatment with Lacto eye drops, tear LF concentration (a) decreased from a relatively high (mean ± SD, 1.65 ± 0.45 mg/ml) value at baseline to 1.05 ± 0.33 mg/ml (p = 0.04) in four eyes (eyes nos. 1-4), (b) increased from 1.52 ± 0.21 to 2.03 ± 0.41 gm/ml in other four eyes (eyes nos. 6-8, 11), and (c) increased from a low value (0.85 ± 0.17 mg/ml) to 1.18 ± 0.27 (p = 0.07) in eyes nos. 5, 9, and 11.*

Conclusion: *Lacto eye drops were found to have an immune modulating effect, with any low tear LF at baseline increased in an amount required for normalization of the conjunctival microbiota after treatment. In addition, our microbiological study after one-month administration of Lacto eye drops as a component of therapy for CC demonstrated that the medication provided a sanitizing effect, with no conjunctival microbiota growth but subjective improvement in complaints in all patients.*

Keywords:
chronic conjunctivitis, lactoferrin, tear, antimicrobial properties

Introduction

Conjunctivitis is one of the most common eye diseases [1]. In recent years, there has been an increase in the incidence of conjunctivitis, particularly, chronic conjunctivitis (CC). It is known that, unlike acute conjunctivitis, CC commonly affects middle-age and elderly individuals. The age of onset of CC, however, has become younger recently, and the disease is often seen in young and middle-age individuals [2, 3]. Inflammation of the conjunctiva may be associated with pathogens as well as the activation of conjunctival saprophytes, given increased numbers of patients with systemic and local immune deficiency, especially after COVID-19 [4].

CC usually develops as a consequence of undertreated acute inflammatory process or mistakes in the treatment

of the latter. It may also develop as a primary disease due to negative environmental factors. The disease may be of infectious origin or non-infectious origin, with the former associated with bacterial pathogens, primarily staphylococci, streptococci and Chlamydia; viral pathogens, including adenoviruses, herpes viruses and enteroviruses; in the presence of durable uncontrolled and long-term use of antibiotics or infection with Candida, actinomyces species, etc [5]. Non-infectious CC may be caused by irritation of the conjunctiva by physical and chemical irritants like dust, smoke, other pollution; alkaline and acidic vapors; exposure to ultraviolet radiation and allergens, durable exposure to wind and dry air; low-quality make-up products; and improper soft contact lens (SCL) wear [2, 4].

Local factors like uncorrected refractive errors, impaired tear outflow, and lid disorders (such as trichiasis) also may cause CC. In addition, CC may have a systemic cause like hypovitaminosis A, B, C, E, gastrointestinal tract disorder, nasal or paranasal sinus pathology, diabetes mellitus, systemic disease of the connective tissue, etc [4, 5].

The disease usually develops gradually, has a slow course with temporal improvements in symptoms, and is commonly bilateral. Patients complain of itching, burning, foreign body sensation, eye redness, increased sensitivity to light and visual fatigue, lid heaviness, excess tearing or dryness [5, 6].

The conjunctiva of the eye provides protection and lubrication of the eye by the production of mucus and tears. If inflammation occurs, it worsens functions of the conjunctiva, which causes a loss of homeostasis of the ocular surface leading to the above complaints. Long-term ocular discomfort significantly impairs the quality of life of patients with CC.

The use of traditional medications for acute conjunctivitis (primarily antibiotics) not always leads to recovery, does not prevent recurrences, causes toxic and immunosuppressive effects, suppresses local mechanisms of non-specific defense, contributes to the development of resistant organism strains and promotes activation of fungal flora [7, 8, 9]. This warrants the development of compounds having pathogenetic effects on the conjunctival inflammation in CC. One of these compounds is lactoferrin (Lf), a non-heme cationic iron-binding glycoprotein of the transferrin family. Through its unique combination of antimicrobial action and anti-inflammatory activities Lf in the tear film plays an important role in the maintenance of ocular health. Lf has bacteriostatic, bactericidal, fungicidal, antiviral, antioxidant and transport properties, prevents the formation of free radicals, inhibits lipid peroxidation, and activates antioxidant enzymes [10, 11]. It is secreted primarily by the lacrimal gland and also by corneal, conjunctival and meibomian epithelial cells [12, 13]. Lf represents approximately 25% by weight of the total tear proteins, with an average normal tear Lf content of 1.42 g/l. Antimicrobial activity of Lf is associated with its chelating properties towards iron ions, which inhibit the formation of iron-dependent hydroxyl radicals during inflammatory reactions and microbial infections [14–16].

In recent years, there have been numerous reports on the determination of Lf levels and functions in tears. These include reports on the determination of Lf levels in normal tears and tears of patients with dry eye disease (DED) and keratoconus [17, 18, 19]. Particularly, patients with these ocular disorders were found to have lower levels of tear LF than healthy individuals. The results of recent studies suggested that Lf concentration in tears is a good candidate as a diagnostic biomarker for eye disorders, primarily DED, with significantly decreased tear Lf levels found in patients with DED [17, 23].

Lf has been shown to have antimicrobial activity against a broad range of gram-positive bacteria, gram-negative bacteria, and some Actinomyces species. One mechanism of action of LF is through the sequestration of iron, depriving the microorganisms of an essential nutrient. Another mechanism of action of LF involves Lf binding to lipopolysaccharide of bacterial walls, with the oxidized iron part of the Lf oxidizing bacteria, which results in oxidative stress and affects membrane permeability [10, 20]. Lf has an N-terminal region that is bactericidal and has lipopolysaccharide binding activities. The positively charged N-terminal region of the protein binds negatively charged lipids of the bacterial membrane, which results in depolarization of the membrane and an efflux of K ions from the bacterial cell [21].

Bacteriostatic effects of Lf are based on the capacity to bind the bacterial components which enable bacterial attachment by suppressing their interaction with cell receptors. Bactericidal effects of Lf are implemented through direct interaction of the molecule with the surface of bacteria [14, 16, 22]. These properties make Lf promising for use in the treatment of chronic conjunctivitis.

There have been reports on the determination of Lf concentration in tears of (a) patients with DED of various etiologies, Sjögren syndrome, diabetic retinopathy and keratoconus and (b) SCL wearers. However, to the best of our knowledge, there have been no reports on the determination of Lf concentration in tears of patients with chronic conjunctivitis.

The purpose of the study was two-fold: to determine Lf concentration in tears of patients with chronic conjunctivitis, and to assess the effect of LACTO eye drops in the multicomponent treatment of these patients.

Material and Methods

This study was approved by the bioethics committee of the Filatov Institute and the principles of the Declaration of Helsinki were followed throughout. Informed consent was obtained from all study participants. The study was conducted at the site of Corneal Pathology Department of the institute in January to December 2021.

Eleven patients (17 eyes) with CC participated in the study. Patient age ranged from 27 to 68 years. Chronic conjunctivitis was defined as conjunctivitis lasting at least three months with microbiological evidence of pathogenic

or potentially pathogenic organisms in the conjunctiva and/or persistent complaints despite antimicrobial therapy.

The ophthalmological examination included biomicroscopy of the bulbar conjunctiva and cornea, fluorescein examination, determination of corneal sensation and basal tear production (Schirmer's II test), and microbiological examination of the conjunctiva. Corneal and conjunctival xerosis was assessed with the scale of 0-9 using the method of van Bijsterveld (1969). The ocular surface was divided into three areas (nasal bulbar conjunctiva, cornea, and temporal nasal conjunctiva) that were assessed for fluorescein staining. Each area was given a staining score from 0 (no damage) to 3 (severe damage) points, and the total score of fluorescein staining was calculated ranging from 0 to 9 points. Conjunctival and corneal epithelium staining was defined as (a) normal if a total score was 3.5 or lower and (b) pathological if a total score was higher than 3.5.

Basal tear production was assessed using a 5-minute anesthetized Schirmer's II test. Wetting of more than 10 mm in 5 minutes was an indication of normal tear production.

A cotton wisp test was used to assess corneal sensation. A small fine-tipped cotton wisp is lightly touched first at the central cornea and then at four different quadrants. The patient feels intense irritation and tries to close the eyes if corneal sensation is normal. If this does not take place, thicker portions of the wisp are used to assess the reduction in corneal sensation. If no corneal reflex is elicited by touching the cornea, corneal sensation is believed to be absent. Corneal sensation was estimated in each of the five points using a 0 to 2 scale in which 0 was no sensation, 1 was decreased sensation, and 2 was normal sensation. The total score of corneal sensation was calculated ranging from 0 (no sensation) to 10 points (normal sensation) [24].

A smear of the conjunctival secretion for microbiological examination was obtained with a dry sterile cotton swab in the morning before administering eye drops and performing eye toilet. The lower eyelid was slightly everted, and the swab was moved over the conjunctiva of the inferior eyelid towards the interior angle of the eye. The swab was placed into a sterile tube. The sample was collected under aseptic conditions.

Patients with CC were administered topical antiseptic (miramistin or chlorhexidine 0.02%) four times daily, preservative-free hyaluronic acid artificial tears four times daily, Lacto eye drops (manufactured by NOVAX® PHARMA and containing lactoferrin) twice daily over a month. Lf concentration in tears was assessed before administration and at day 30 of administration of Lacto eye drops.

Efficacy measures were (1) the absence of conjunctival microbiota growth, (2) no worsening in Schirmer 2 test values, (3) improvement in the state of the conjunctival and corneal epithelium, (4) improvement in total score for corneal sensation, and (5) improvement in complaints at day 30.

Patients with history of surgery within prior 6 months, systemic autoimmune disease, diabetes mellitus, ocular comorbidity requiring regular administration of eye drops, or non-infections conjunctivitis of any etiology were excluded from the study.

Tear samples for the determination of tear Lf concentration were collected in the morning before diagnostic and treatment procedures were performed. For this purpose we used a sterile plastic tip attached to a pipette aid. The tear samples collected were stored at −20 °C until immunological testing. Concentration of Lf in the tear samples was determined by a human Lf enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology, Inc., Wuhan, China). The results were photometrically measured at 450 nm with an ELISA reader (Stat Fax 2100, Awareness Technology Inc, Palm City, FL).

Statistical analyses were conducted using Statistica 9.0 (StatSoft, Tulsa, OK, USA) and SPSS 11.0 (SPSS Inc., Chicago, IL) software. Normal distribution of quantitative data was assessed using the Shapiro-Wilk test. The mean, standard deviation, and 95% confidence interval (CI) of differences were calculated using the Student t test for paired and/or unpaired observations, as appropriate. Pearson's chi 2 test was used for frequency analysis. The level of significance $p \leq 0.05$ was assumed.

Results

The duration of CC ranged from 3.5 to 7 months. LF concentration in tears of patients with CC ranged from 0.65 mg/ml to 2.25 mg/ml (mean ± SD, 1.37 ± 0.4; 95% CI, 1.16–1.58).

Conjunctival and corneal epithelial staining was "pathological" (with a score of 4 conforming to punctate epitheliopathy) in 3 eyes and normal (with a score ranging from 0 to 3) in 14 eyes.

Corneal sensation was decreased (with a score ranging from 3 to 8) in 11 eyes and normal in 6 eyes. Decreased corneal sensation in patients with CC may be associated with neurotoxic effects of numerous previously used topical antibacterial medications on the corneal epithelium. In eyes with normal corneal sensation, the mean Lf concentration in tears was 1.55 ± 0.5 mg/ml, whereas in those with abnormal corneal sensation, the mean Lf concentration in tears was 1.27 ± 0.29 mg/ml ($p = 0.07$).

The mean Schirmer II test value was 17.3 ± 8.3 mm.

It is noteworthy that, despite previous antimicrobial therapy for chronic conjunctivitis, only one patient showed no microbiological evidence of microbial growth before administration of Lacto eye drops. Potentially pathogenic or pathogenic species were cultured from swabs in 16 eyes of patients with CC, indicating decreased activity of local immunological defense mechanisms. Staphylococcus haemolyticus was found most commonly (five eyes), followed by Candida albicans and Escherichia coli (four eyes each) and Staphylococcus epidermidis, Staphylococcus aureus and Enterococcus (one eye each).

Seven patients (11 eyes) underwent an examination after the completion of a one-month treatment with Lacto eye drops (Table 1).

This examination found no microbial growth in all these 11 eyes (Table 1), with six of seven patients presenting no complaints and all seven patients noting an improvement in their symptoms after treatment.

In addition, conjunctival and corneal epithelial staining was normal (with a score ranging from 0 to 3), and the mean Schirmer II test value was 16.4 ± 11.1 mm, but with no statistically significant change from baseline, in 11 eyes. Moreover, corneal sensation was normal in 5 eyes and remained decreased (with a score of 3 to 8) in 6 eyes (eyes nos. 1, 5, 6, 8, 9 and 11).

Of note that, in four eyes (eyes nos. 1-4), Lf concentration in tears was relatively high (mean ± SD, 1.65 ± 0.45 mg/ml) at baseline, and decreased, with a range of 0.76 mg/ml to 1.0 mg/ml (mean ± SD, 1.05 ± 0.33) after a one-month treatment with Lacto eye drops (p = 0.04). This decrease is likely to be explained by a decrease in the production of natural lactoferrin in the presence of instilled lactoferrin. In other four eyes (eyes nos. 6-8, 11) with high LF concentration in tears (mean ± SD, 1.52 ± 0.21; range, 1.23–1.75 mg/ml) and pathogens (E. Coli, Candida Albicans and Staph. Haemolyticus) found at baseline, Lf concentration in tears increased to a range of 1.53–2.37 mg/ml (mean ± SD, 2.03 ± 0.41) after a one-month treatment with Lacto eye drops (p = 0.04), which could be associated with the activation of the defense mechanisms of the ocular surface targeted at the elimination of invading pathogens. In eyes (eyes nos. 5, 9, 11) with low LF concentration in tears (mean ± SD, 0.85 ± 0.17 mg/ml; range, 0.65–0.94 mg/ml), LF concentration in tears increased to a range of 0.85–1.34 mg/ml (mean ± SD, 1.18 ± 0.27) after a one-month treatment with Lacto eye drops (p = 0.07).

Discussion

Although CC is highly prevalent, the pathogenesis has not been fully elucidated. Because CC causes a number of medical, social and economical problems, elucidating the causes of the conjunctivitis transition to a chronic phase is of a high medical and social value [25, 26].

It has been reported that bacterial conjunctivitis is most commonly caused by staphylococcal or streptococcal species [25, 27]. The pathogenic agents found in our patients with CC included Staphylococcus Haemoliticus, E. Coli, Candida Albicans, which is likely to be associated with the suppression of local protective mechanisms of immunological responses due to long-term use of corticosteroids and non-steroidal anti-inflammatory drugs as well as preservatives contained in these medications.

Studies vary substantially in the reported normal tear Lf content depending on numerous factors like the method used for tear sampling, method used to assess Lf concentration, sample size, geographic origin of subjects, etc. [17, 18]. In a study by Versura and colleagues (2020) [32], the mean tear Lf content was 1.3 ± 0.17 mg/ml for patients with dry eye and 2.43 ± 0.34 mg/ml for healthy individuals. Ponzini and colleagues [17] conducted a meta-analysis and found that In 58 tear samples from healthy subjects an average lactoferrin concentration of 1.42 g/l was found.

Lf is known to play a key role in the immune defense of the mucosa including the conjunctiva. With a mean concentration of approximately 2 mg/ml Lf represents approximately 25% by weight of the total tear proteins. LP expression is upregulated in response to inflammatory stimuli to inhibit the production of inflammatory cytokines and the binding ability of lipopolysaccharide endotoxin to inflammatory cells [23, 28]. It has been demonstrated that LF concentration in blood rises during inflammation [10, 22].

In the current study, the mean LF concentration in tears of patients with CC and pathogens in the conjunctival cavity was 1.37 ± 0.4 mg/ml. Our microbiological study after one-month administration of Lacto eye drops as a component of therapy for CC demonstrated that the medication provided a sanitizing effect. This may be caused by the capacity of LF to inhibit the growth of bacterial pathogens like S. mutans, S. epidermidis, E. Coli etc [14, 21]. Studies have reported potential mechanisms of bactericidal effects of LF. Particularly, it has been demonstrated that the capacity of Lf to bind iron will prevent the use of iron by bacteria for multiplication [28]. Moreover, the death of bacteria cells can be induced by the disruption of cell walls, which was caused by the interaction between the N-terminal region of Lf and related receptors, e.g., lactoferrin binding protein A and/or B on Gram-negative bacteria [20, 22] or electrostatic interactions with Gram-positive bacteria [28]. Lf was also proved to have innate antibacterial properties contributing to the inhibition of biofilm formation [29].

At the cellular level, Lf increases the number of natural killer (NK) cells, induces phagocytosis and causes activation of neutrophils. Interaction of Lf with lipopolysaccharides recruits and directs leukocytes to sites of inflammation [16, 20]. Lf plays a substantial role in the activation of immune cells: it modulates the differentiation, maturation, activation, migration, proliferation and functions of immune cells. It also promotes the cell–cell interaction and activation of polymorphonuclear leukocytes and NK cells, thus boosting the immune response. Moreover, it modulates T cell and macrophage activity to counteract bacterial and viral infections, stimulates phagocytosis [30, 31] and enhances the sensitivity of target cells to lysis by natural killer cells [31].

Limitations of the current study include a small sample size and the absence of a control group. This is due to the fact that the study was conducted during the COVID-19 pandemic and tear sampling in healthy individuals could not be performed to compare them with patients with CC in terms of tear Lf concentration.

The current study found Lacto eye drops to be well tolerable and therapeutically effective in the treatment of CC. Therefore, findings of the current study support the key

role of LF in maintaining ocular surface homeostasis, and highlight the multiple activities of this molecule, ranging from the recovery of epithelial integrity to antibacterial effects.

Conclusion

First, Lacto eye drops were found to have an immune modulating effect, with any low tear LF at baseline increased in an amount required for normalization of the conjunctival microbiota after treatment. Second, our microbiological study after one-month administration of Lacto eye drops as a component of therapy for CC demonstrated that the medication provided a sanitizing effect, with no conjunctival microbiota growth but subjective improvement in complaints in all patients. Finally, the results obtained allow us to recommend using Lacto eye drops as a component of therapy for chronic conjunctival inflammation.

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Corresponding author: *Galyna I. Drozhzhyna, serp2002@ukr.net*

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Abbreviations: *CC, chronic conjunctivitis; LF, lactoferrin; ST, Schirmer test; CS, corneal sensation; FT, fluorescein test*

Table 1. Lactoferrin concentration in tears and pathogens found in the conjunctival cavity of patients with chronic conjunctivitis before and after treatment with Lacto eyedrops

	Before/ after treatment	Tear lactoferrin concentration	Pathogens found	Schhirmer test value (mm)	Corneal sensation	Fluorescein staining score
1	Before	1.34	Escherichia coli	13	8	2
	After	0.76	No	31	9	3
2	Before	1.26	Escherichia coli	13	5	1
	After	0.94	No	11	3	3
3	Before	1.76	Staphylococcus aureus	21	10	2
	After	1.53	No	22	9	3
4	Before	2.25	Escherichia coli	10	10	2
	After	1.0	No	10	10	4
5	Before	0.96	Staphylococcus haemolyticus	12	7	1
	After	1.34	No	14	8	1
6	Before	1.54	Staphylococcus haemolyticus	30	4	4
	After	2.37	No	30	5	2
7	Before	1.23	Escherichia coli	13	10	1
	After	1.53	No	13	10	2
8	Before	1.75	Candida albicans	9	7	2
	After	1.87	No	11	10	2
9	Before	0.94	Staphylococcus haemolyticus	14	7	1
	After	1.34	No	14	8	1
10	Before	0.65	Staphylococcus epidermidis	3	10	3
	After	0.86	No	4	9	3
11	Before	1.56	Staphylococcus haemolyticus	28	4	4
	After	2.37	No	30	5	2