THE EFFECT OF LACTOFERIN ON THE FREE RADICAL AND CYTOKINE STATUS OF CORNEA IN THE EXPERIMENTAL THERMAL BURN

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The free radical and cytokine statuses of the cornea during its thermal burn and the possibility of its correction by lactoferrin have been studied in Soviet Chinchilla rabbits. The development of a corneal thermal burn was accompanied by the development of oxidative stress (increased levels of TBA-reactive substances and carbonyl derivatives of proteins, decreased activity of SOD and GPx enzymes) and a pronounced inflammatory reaction with increased levels of TNF-1α, IL-10, TGF-1β. The use of lactoferrin had a pronounced therapeutic effect, which was manifested by accelerated healing, prevention of the development of complications (corneal perforations), a decrease in the severity of oxidative stress, an increase in the concentrations of TNF-1α (in the early stages), IL-10 (in the later stages), TGF-1β (throughout the experiment). At the same time, by the end of regeneration more severe corneal opacification was recognized compared to the control group. This may be associated with an increased level of anti-inflammatory cytokines, especially TGF-1β.

Key words: corneal thermal burn; oxidative stress; cytokines; lactoferrin

DOI: 10.18097/PBMC20247003168

INTRODUCTION

The cornea is an important part of the optical system of the eye, accounting for 80% of its refractive power. Pathological changes in the cornea lead to impaired transparency and decreased visual acuity [1]. In this context improvement of approaches to the treatment of corneal injuries is an important medical and social problem.

Modern studies pay increasing attention to the analysis of the free radical and immunological statuses of the cornea as one of the key biochemical processes involved in the pathogenesis of the development of corneal damage [2].

It has been shown that in many cases the outcome of the disease depends on the severity of oxidative stress and the balance of pro-inflammatory and anti-inflammatory cytokines [3]. For example, an excessive increase in pro-inflammatory cytokines and activation of free radical processes can lead to the development of corneal perforation, and conversely, a slight increase in pro-inflammatory cytokines and reactive oxygen species (ROS) can lead to chronization of the process and excessive development of connective tissue in infectious corneal ulcers [4–6].

The free radical and immunological statuses of the cornea during thermal burns of the cornea still remain poorly investigated. The influence of correction of these disorders on the course of this pathological process has not been evaluated yet. Lactoferrin attracts attention as a substance for the correction of free radical and immunological disorders of the cornea.

Lactoferrin is produced and secreted by human mucosal epithelial cells and neutrophils. It is detected in saliva, milk, tears and is secreted by various organs, including the mammary gland, uterus, kidneys, and brain [7].

A number of studies demonstrated its antiviral and anti-inflammatory properties, as well as the ability to accelerate the skin wound healing process. At the same time, lactoferrin also exhibits antioxidant properties, which are due to its ability to bind iron, blocking the development of Fenton and Haber-Weiss reactions [8].

We have previously shown that lactoferrin is effective in treatment of purulent corneal ulcers; it reduced the severity of oxidative stress, improved the clinical course, and reduced the rate of complications [9].

The aim of this study was to investigate features of the free radical and cytokine statuses of the cornea during a thermal burn and the possibility of their correction by lactoferrin.

MATERIALS AND METHODS

The study was carried out on 45 male of the Soviet Chinchilla rabbits, weighing 3000–3500 g, aged 9–12 months. The rabbits were kept in the conventional vivarium of the Ryazan State Medical University in individual cages.

Animals were randomized into the following experimental series:

The first series, intact animals (norm), included rabbits without modeling pathology and experimental effects (3 rabbits, 6 eyes).

The second series, pathology control, included animals in which a thermal burn of the cornea was modelled and saline solution was instilled into the conjunctival cavity three times a day (3 rabbits, 6 eyes for each time point).

The third series, correction of free radical and immunological disorders by lactoferrin, included rabbits in which a thermal burn of the cornea was induced and a lactoferrin solution (0.5 mg/ml) was instilled into the conjunctival cavity (3 rabbits, 6 eyes for each time point).

Animals were withdrawn from the experiment by Zoletil overdose on day 1, day 3, day 5, day 7, day 14, day 21, and day 28 after modeling of the corneal burn. Saline solution and lactoferrin solution were used 3 times a day in the form of instillations of 1 drop into each eye every day for 28 days.

The thermal burn was modeled as follows. A stainless steel cylinder with a base diameter of 6 mm and a radius of curvature of the cylinder base of 9.0 mm was placed on an electric hotplate heated to 200°C for 2 min. The cylinder temperature was monitored using a pyrometer. Before thermal exposure, oxybuprocaine (0.4% solution, Sentiss Pharma, India) was instilled into the conjunctival cavity. The base of the cylinder, heated to 200°C, was placed on the central region of the cornea of experimental animals for 3 s. Immediately after the thermal exposure, all rabbits underwent irrigation of the cornea and conjunctival cavity with a 0.9% sodium chloride solution at room temperature. After repeated instillation of oxybuprocaine, scarification of the corneal scab was carried out within the burned tissue. All manipulations were performed under visual control using a Neitz IO-α head-mounted binocular ophthalmoscope (Neitz, Japan). To assess the area of the formed defect, the cornea was stained with FluoStrips test strips (Contacare, India).

After euthanasia of the animals, the eyeballs were enucleated. The cornea was excised with a sclera fragment (1 mm from the limbus), the mass of the cornea was measured. It was ground, and after addition of phosphate buffer (1:10 by mass), was homogenized using a DIAX 900 homogenizer (Heidolph, Germany) at 26,000 rpm in for 1 min. The resultant homogenate was centrifuged at 1800 *g* (Eppendorf, Germany) and the supernatant was used for evaluation of the oxidative stress severity. The latter was assessed by determining the level of malondialdehyde (MDA), evaluated by determining thiobarbituric acid-reactive substances (TBARS) [10], protein carbonyl derivatives [11], and activity of superoxide dismutase (SOD) [12] and glutathione peroxidase (GPx) [13].

The severity of immunological disorders was also evaluated by the levels of tumor necrosis factor (TNF-1α), interleukins (IL) 4, 10, transforming growth factor beta 1 (TGF-1β), which were analyzed by ELISA using commercially available kits from "Cloud-Clone" (China). The obtained values were normalized per mg of protein, which was determined by the Bradford method using a commercial kit (Thermo Fisher, USA) [14].

Mathematical data analysis was performed using the Statistica 10.0 program. The data distribution was assessed using the Shapiro-Wilk test. Since in all cases the data distribution was different from normal, the results were presented as median and upper and lower quartiles (ME (Q1; Q3)). The statistical significance was assessed using nonparametric tests: the Kruskal-Wallis test was used in the case of comparison of more than two groups, and further pairwise comparisons were performed using the Mann-Whitney test with Bonferroni correction; in the case of comparison of two groups, the Mann-Whitney U test was used. Differences between groups were considered statistically significant at $p<0.05$.

RESULTS

In the control group (thermal burn and saline instillation) the cornea became more swollen within the burned tissue from days 1 to 5 after the application of the corneal burn. Infiltration of the entire cornea around the burn was observed; it gradually decreased from the periphery to the center. The transparency of the cornea outside the burn was restored during the period from day 3 to day 7. From day 1 to day 3 a mixed injection of the bulbar conjunctiva developed and persisted for up to 7–10 days. Complete epithelization of the defect in the control group was observed from day 7 to day 13. In some cases, on days 3–8, areas of corneal ulceration formed, culminating in the formation of perforations on day 4 (1 eye), day 8 (3 eyes), day 10 (1 eye), day 11 (1 eye). Animals with corneal perforations were removed from the experiment and were not used in further research (Fig. 1).

Treatment of the thermal burn of the cornea with lactoferrin gave the following results. From day 1 to day 4 after corneal burns, the cornea became more swollen within the burned tissue, and the burn site was completely stained with fluorescein. Gradually decreasing infiltration of the entire cornea around the burn was noted. The transparency of the cornea outside the burn was restored from day 2 to day 4. Mixed injection of the bulbar conjunctiva remained during 5–7 days. Complete epithelization of the defect was observed from day 6 to day 8. No perforations were detected during treatment with lactoferrin.

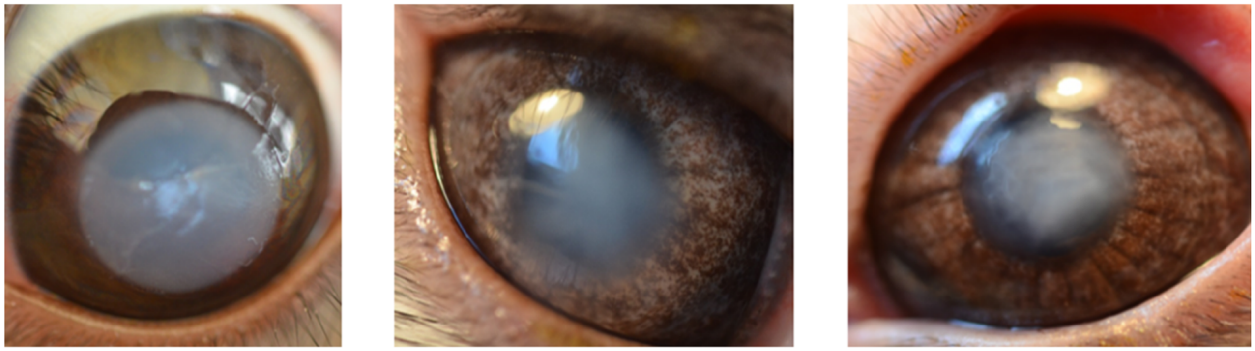

Figure 1. The effect of lactoferrin on the state of the cornea during thermal burn.

Local application of lactoferrin led to the formation of more intense corneal opacities on day 28 compared to the control group (Fig. 1).

The thermal burn modeling was accompanied by the development of oxidative stress in the cornea. The concentration of TBARS increased from day 1, reached a maximum by day 7, exceeding the norm by 120.1% ($p<0.05$), and then gradually decreased by day 28, and did not differ from the values determined in intact animals. The content of protein carbonyl derivatives also increased on days 3 and 5 of the burn modeling and exceeded normal values by 70.9% ($p<0.05$) and 47.2% ($p<0.05$), respectively (Table 1).

On the contrary, the activity of antioxidant enzymes in the cornea decreased. SOD activity was lower throughout the experiment with the minimum value observed on day 7 (by 41.3%; $p<0.05$ below normal values), and GPx activity decreased on days 7–14 by 19.7% ($p<0.05$) and 40.4% ($p<0.05$), respectively (Table 1).

The level of the pro-inflammatory cytokine TNF-1α increased on day 1, reached its maximum value on day 7 by 178.3% ($p<0.05$) exceeding the values of intact animals, and remained elevated by day 28 of the experiment (Table 2).

The content of the anti-inflammatory cytokine IL-10 also increased from day 1, reached the peak value exceeding the normal value on day 5 (93.4%; $p<0.05$), and normalized by day 28. The level of TGF-1β also increased during thermal burn throughout the experiment, reaching the maximal value exceeding norm on day 5 (77.8%, $p<0.05$). The concentration of the anti-inflammatory cytokine IL-4 did not demonstrate statistically significant changes throughout the experiment (Table 2).

The results obtained suggest the development of oxidative stress and inflammation during modeling of a corneal thermal burn, and their maximum severity coincided with the time of the development of complications, corneal perforations.

Lactoferrin applications for the correction of free radical and inflammatory disorders led to the following results. Lactoferrin had an antioxidant effect, as evidenced by a decrease in the level of TBARS compared to control levels on days 14 and 21, and carbonyl derivatives of proteins on days 14 and 28 (Table 1). Lactoferrin also accelerated the recovery of SOD activity (the activity of this enzyme decreased only from days 1 to 14, and then just insignificantly differed from normal values) and increased GPx activity, which exceeded the values of control animals on days 14 and 21 of the experiment (Table 1).

The results obtained indicate that lactoferrin has an antioxidant effect in the experimental thermal burn of the cornea.

Studying the effect of lactoferrin on the cytokine status of the cornea, the following results were obtained. The level of TNF-1α significantly exceeded normal values throughout the experiment; on day 5 it exceeded the levels of control animals by 33.6% ($p<0.05$). The concentration of IL-10 during lactoferrin application on days 1–7 was lower than in control animals, while from days 14 to 28 it exceeded control values. Also, this indicator exceeded the normal values from day 5 to day 28. The content of TGF-1β during treatment with lactoferrin exceeded both normal values and control values throughout the entire experiment (from days 1 to 28; Table 2).

Thus, the use of lactoferrin increased the level of both pro- and anti-inflammatory cytokines in the cornea during the thermal burn development.

DISCUSSION

In this study we have investigated the development of oxidative stress and the immunological status of the cornea during corneal burn modeling and the possibility of their correction with lactoferrin. It should be noted that no similar studies have

Table 1. The effect of lactoferrin on the free radical status of the cornea during the thermal burn (M(Q1-Q3))

Parameter	Experimental series	Norm	Day of experiment						
			1	3	5	7	14	21	28
TBARS, nmol/mg of protein	Control	2.29 (2.18–2.35)	4.37 (3.53–4.60)*	4.48 (4.22–4.97)*	4.97 (3.94–6.15)*	5.04 (4.64–5.45)*	3.32 (3.21–3.52)*	3.31 (3.11–3.34)*	2.31 (2.15–2.45)
	Lactoferrin		3.69 (3.29–4.28)*	3.84 (3.26–4.20)*	4.02 (3.95–4.04)*	3.28 (3.05–3.62)*	2.46 (2.43–2.49)#	2.27 (2.14–2.45)#	2.11 (2.08–2.16)
Carbonyls, nmol/mg of protein	Control	3.16 (2.93–3.20)	2.78 (2.705–2.987)	5.40 (5.310–5.680)*	4.65 (4.503–4.734)*	2.86 (2.810–2.970)	3.10 (2.970–3.230)	2.73 (2.410–2.860)	4.26 (3.990–4.390)#
	Lactoferrin		2.68 (2.150–3.160)	3.64 (3.380–4.000)	4.30 (3.940–4.590)	3.59 (3.060–3.730)	2.49 (2.430–2.550)*,#	2.61 (2.448–2.843)	2.02 (1.950–2.140)#
SOD, U/mg of protein	Control	3.12 (3.07–3.14)	2.84 (2.76–2.91)*	2.50 (2.37–2.54)*	2.05 (1.92–2.30)*	1.83 (1.70–1.92)*	2.08 (2.02–2.20)*	2.26 (2.21–2.27)*	2.81 (2.58–2.83)*
	Lactoferrin		2.86 (2.80–2.90)*	1.94 (1.76–2.83)	2.08 (1.98–2.12)*	2.66 (2.56–2.74)	2.77 (2.67–2.83)	2.90 (2.77–3.05)	3.12 (3.07–3.13)#
GPx, nmol NADPH/ min×mg of protein	Control	19.36 (17.88–23.19)	20.935 (20.210–23.290)	19.72 (18.560–21.130)	17.65 (16.700–19.840)	15.55 (15.420–16.230)*	11.53 (11.390–11.785)*	16.30 (14.420–17.330)	19.11 (18.340–20.510)
	Lactoferrin		22.83 (22.36–23.84)	22.01 (17.95–23.85)	18.82 (16.34–22.17)	17.32 (17.25–17.41)	18.44 (17.2–18.99)#	23.24 (21.92–23.76)#	23.61 (21.19–24.11)

* $p<0.05$, statistically significant changes versus norm; #, statistically significant differences versus control values. One unit of enzyme activity (U) was defined as 50% inhibition of quercetin autooxidation.

Table 2. The effect of lactoferrin on the cytokine status of the cornea during the thermal burn (M(Q1-Q3))

Parameter	Experimental series	Norm	Day of experiment						
			1	3	5	7	14	21	28
TNF-1α, pg/mg of protein	Control	120.19 (109.36–123.73)	258.61 (246.010–264.165)*	303.06 (298.840–329.560)*	221.24 (211.570–232.560)*	334.51 (330.360–345.770)*	289.04 (282.215–291.080)*	312.51 (302.590–325.360)*	258.56 (251.000–266.710)*
	Lactoferrin		255.53 (238.38–258.67)*	285.53 (271.07–299.95)*	295.63 (287.45–310.57)*,#	362.76 (345.93–368.91)*	273.32 (257.35–304.13)	270.07 (255.75–282.03)*	248.81 (233.48–250.45)*
IL-4, pg/mg of protein	Control	39.78 (36.65–41.09)	35.65 (34.40–36.69)	40.12 (37.80–41.11)	48.90 (48.40–49.35)	44.57 (43.53–45.18)	39.85 (37.64–41.90)	39.14 (37.41–42.29)	42.54 (40.32–43.73)
	Lactoferrin		48.23 (47.93–50.81)	39.23 (38.52–40.89)	43.35 (42.76–44.25)	32.96 (31.29–34.23)	40.60 (39.11–41.15)	42.37 (42.14–42.98)	41.87 (41.56–43.88)
IL-10, pg/mg of protein	Control	6.21 (5.59–6.30)	9.15 (8.60–9.45)*	11.02 (10.55–11.23)*	12.01 (11.64–13.40)*	9.12 (8.64–9.75)*	8.99 (7.84–9.37)*	8.34 (7.87–8.68)*	8.15 (6.40–9.75)
	Lactoferrin		7.34 (7.19–7.56)	6.00 (5.46–6.38)#	9.35 (8.13–10.39)*,#	8.02 (7.83–8.14)*,#	9.79 (9.10–10.30)*,#	10.94 (8.61–12.07)*,#	13.20 (12.00–13.46)*,#
TGF-1β, pg/mg of protein	Control	34.57 (34.21–36.15)	47.19 (46.38–49.21)*	53.64 (52.62–54.97)*	61.47 (60.11–62.88)*	47.80 (46.85–48.57)*	45.13 (44.77–46.26)*	43.45 (42.66–46.11)*	42.50 (41.32–44.85)*
	Lactoferrin		58.47 (57.59–59.83)*,#	79.77 (79.21–80.25)*,#	93.76 (88.31–99.03)*,#	94.87 (89.40–102.23)*,#	60.92 (52.71–63.65)*,#	61.09 (53.61–68.53)*,#	61.44 (58.98–63.64)*,#

* $p<0.05$, statistically significant changes versus norm; #, statistically significant differences versus control values.

been performed so far as and the main attention of researchers is focused on chemical burns, although the thermal factor is the cause of eye burns in 16% of cases [15].

The cornea is constantly exposed to oxygen, sunlight, ultraviolet radiation, smoke, and other toxic air pollutants. On the one hand, this predisposes the cornea to overproduction of free radicals and the development of oxidative stress [16], and on the other hand, it has evolutionarily determined a pronounced antioxidant defense system. For example, the cornea is rich in antioxidant enzymes such as SOD, catalase, GPx and glutathione reductase, as well as low molecular weight antioxidants (ascorbic acid, glutathione, vitamin E, ferritin), which are involved in protecting the cornea from free radicals [17].

This study has shown that the development of the corneal thermal burn was accompanied by the development of oxidative stress, as evidenced by an increase in the level of end products of lipid oxidation (TBARS) and products of oxidative damage of proteins, their carbonyl derivatives. At the same time, there was a decrease in the activity of the key antioxidant enzymes SOD and GPx.

The development of the corneal thermal burn was accompanied by activation of the inflammatory response as evidenced by the increased production of the pro-inflammatory cytokine TNF-1α and the anti-inflammatory cytokines IL-10 and TGF-1β.

The results obtained in our study are consistent with the results by Bazikov et al.; these authors also observed an increase in the production of TNF-1α and IL-8 within 28 days during the development of an infected alkaline burn of the rabbit cornea [18].

TNF-1α is one of the main inflammatory cytokines involved in the development of resistance to infectious agents; however, its overproduction can cause the development of complications [19]. It has been shown that ROS, through the inflammasome pathway, induce the production of TNF-1α by damaged corneal epithelium [20, 21]. TNF-1α is one of the first mediators formed after oxidative stress at the site of chemical injury of the eye.

IL-10 is an anti-inflammatory cytokine that leads to a decrease in the production of pro-inflammatory cytokines, including TNF-1α [22]; increased production of IL-10 leads to a decrease in the anti-infective protection and the development of septic complications [23].

TGF-1β is a multifunctional cytokine; its expression is associated with the physiological processes of growth, differentiation, regeneration, and stress response in many types of cells of the body. TGF-1β plays an important role in regulating the immune system. TGF-1β blocks the activation of lymphocytes and macrophages [24].

Increased levels of TGF-β family proteins are one of the main mechanisms of fibrosis development in many diseases [25].

In the present study lactoferrin has been used as the therapeutic agent because it is an endogenous component, accounting for up to 25% of the total tear proteins with an average concentration in healthy people of about 1.42 mg/ml. Most of lactoferrin is secreted by the main lacrimal gland [26]. On the other hand, lactoferrin has pronounced antioxidant, antiviral, antimicrobial and anti-inflammatory effects [7].

In this study, lactoferrin had a pronounced therapeutic effect, which was manifested in accelerating the healing of damage and preventing the development of complications — corneal perforations (in the treatment group, not a single case of perforation was recorded).

At the same time, lactoferrin exhibited a pronounced antioxidant effect: it reduced the level of TBARS and carbonyl derivatives of proteins, and increased the activity of the antioxidant enzymes SOD and GPx.

It is believed that the main antioxidant effect of lactoferrin is associated with its ability to bind iron ions and thus prevent the development of Fenton and Haber-Weiss reactions [8].

Recently, it was shown that lactoferrin could also activate the expression of catalase, GPx and SOD [27].

The study of the lactoferrin effect on the immunological status of the cornea in the early stages revealed an increase in the level of the pro-inflammatory cytokine TNF-1α and a decrease in the content of the anti-inflammatory cytokine IL-10. In the later stages the IL-10 level increased. The content of TGF-1β remained elevated over the control throughout the experiment.

Other authors have shown that that lactoferrin also influences cytokine activity, either by increasing the levels of anti-inflammatory cytokines such as IL-4 and IL-10, or by modulating pro-inflammatory cytokines such as TNF-1α, IL-1, IL-6, and granulocytes-macrophage colony-stimulating factor [28].

It is especially important to note that besides the positive effect of lactoferrin on the course of the corneal thermal burn, by the end of regeneration, more severe corneal opacification, relative to the control group, was detected. These changes may be associated with increased levels of anti-inflammatory cytokines, especially transforming growth factor. TGF-1β and other growth factors are known to overstimulate the healing process, promoting stromal fibrosis [29].

On the other hand, it has been shown that lactoferrin inhibits matrix metalloproteases MMP-9 and MMP-2 [30], which are necessary for normal corneal regeneration. Chesnokova et al. found that during the cornea thermal burn, a sharp

activation of trypsin-like proteases in the cornea required for burn wound clearance; this activation occurred from day 3 to day 14 days and then their activity decreased [31] and repair processes involving MMP-9 and MMR-2 triggered. The inhibitory effect of lactoferrin during this period could disrupt the course of the repair processes and also led to the formation of a rougher scar.

It is reasonable to suggest that to prevent excessive growth of connective tissue, the use of lactoferrin should be limited by the first 14 days.

CONCLUSIONS

In the present study, it was found that the development of the cornea thermal burn was accompanied by the development of oxidative stress (characterized by increased levels of TBARS and protein carbonyl derivatives, decreased activity of SOD and GPx), as well as the development of a pronounced inflammatory reaction (characterized by increased levels of TNF-1α, IL-10, TGF-1β). The use of lactoferrin had a pronounced therapeutic effect, which was manifested by accelerated healing, prevention of complications (corneal perforations), decreased severity of oxidative stress, increased concentrations of TNF-1α (in the early stages), IL-10 (in the later stages), and TGF-1β (throughout the experiment). At the same time, by the end of regeneration, corneal opacification was more severe compared to the control group. This may be associated with an increased level of anti-inflammatory cytokines, especially TGF-1β.

FUNDING

The work was carried out using funds from Ryazan State Medical University.

COMPLIANCE WITH ETHICAL STANDARDS

The work protocol was approved by the Bioethical Commission of Ryazan State Medical University (protocol no. 17 of November 7, 2018).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Received: 24. 03. 2024.
Revised: 29. 04. 2024.
Accepted: 02. 05. 2024.

ВЛИЯНИЕ ЛАКТОФЕРИНА НА СВОБОДНОРАДИКАЛЬНЫЙ И ЦИТОКИНОВЫЙ СТАТУС РОГОВИЦЫ ПРИ ЭКСПЕРИМЕНТАЛЬНОМ ТЕРМИЧЕСКОМ ОЖОГЕ

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На кроликах-самцах породы Советская Шиншилла изучены особенности свободнорадикального и цитокинового статуса роговицы при её термическом ожоге и возможности его коррекции с помощью лактоферрина. Развитие термического ожога роговицы сопровождается развитием окислительного стресса (повышением уровня ТБК-реактивных продуктов и карбонильных производных белков, снижением активности ферментов СОД и GPx) и выраженной воспалительной реакцией с повышением уровня TNF-1α, IL-10, TGF-1β. Применение лактоферрина оказывало выраженный терапевтический эффект, который проявлялся ускорением заживления, профилактикой развития осложнений (перфораций роговицы), снижением выраженности окислительного стресса, повышением концентраций TNF-1α (на ранних сроках), IL-10 (на поздних сроках), TGF-1β (на протяжении всего эксперимента). При этом, к концу регенерации было выявлено более грубое, относительно контрольной группы, помутнение роговицы, что может быть связано с повышенным уровнем противовоспалительных цитокинов, особенно TGF-1β.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: термический ожог роговицы; окислительный стресс; цитокины; лактоферрин

Финансирование. Работа выполнена из средств РязГМУ.

Поступила в редакцию: 24.03.2024; после доработки: 29.04.2024; принята к печати: 02.05.2024.