EXPERIMENTAL ARTICLES

UDC 577.27:606.61:617.7 https://doi.org/10.15407/biotech15.05.031

PRODUCTION OF ANTI-LACTOFERRIN ANTIBODIES AND THEIR APPLICATION IN ANALYSIS OF THE TEAR FLUID IN THE HEALTHY EYE AND CORNEAL INJURIES

A. Tykhomyrov[1]
O. Yusova[1]
L. Kapustianenko[1]
V. Bilous[1]
T. Drobotko[1]
I. Gavryliak[2]
N. Greben[2]
C. A. Ağca[3]

[1]Palladin Institute of Biochemistry of NAS of Ukraine, Kyiv

[2]Bogomolets National Medical University, Kyiv, Ukraine

[3]Bingol University, Bingol, Turkiye

E-mail: artem_tykhomyrov@ukr.net

Received 08.08.2022
Revised 28.08.2022
Accepted 31.10.2022

Lactoferrin is a ubiquitous and multifunctional protein, which has antimicrobial and immunomodulatory activities. Lactoferrin plays an important role in the maintenance of ocular health.

The aim of the study was to produce polyclonal antibodies against human lactoferrin in order to apply them in evaluation of lactoferrin levels in the tear fluid collected from healthy eye and after corneal injury.

Materials and methods. Affine chromatography on Protein A-sepharose was applied in order to isolate immunoglobulin G (IgG) fraction from the blood serum of lactoferrin-immunized rabbits. Each step of protein purification was monitored by denaturing gel electrophoresis (SDS-PAGE). Target antigen recognition by produced antibodies was established by western blot analysis with the use of diluted IgG fraction. Lactoferrin levels in the tear fluids collected from healthy individuals ($n = 4$) and patients with non-penetrating corneal injures ($n = 6$) were determined immunochemically with the use of purified antibodies. The results of western blot of lactoferrin levels in the tear fluids of healthy individuals and patients with corneal wounds were analysed using Mann-Whitney U-test. The difference between group mean values was considered significant at $P < 0.05$.

Results. Using affine chromatography on Protein A-sepharose, antibodies against human lactoferrin were purified as IgG fraction from blood serum of lactoferrin-immunized rabbits. Western blot analysis showed that obtained antibodies recognize the antigen as a 75-kDa band, which corresponds to the intact human lactoferrin polypeptide. The same major polypeptide band was visualized by western blot with enhanced chemiluminescence detection in the tear fluid samples. Densitometry analysis of 75-kDa lactoferrin band showed 3.2-fold decrease in lactoferrin level in the tear fluid samples obtained from patients with non-penetrating corneal traumas as compared with samples collected from healthy persons ($P < 0.05$). Besides, tear fluid of patients with injured corneas contained large amounts of truncated lactoferrin immunoreactive polypeptides as well as high molecular weight bands, which could correspond to lactoferrin complexes with other proteins occurring during inflammation.

Conclusions. According to our data, obtained anti-lactoferrin antibodies can be used as a valuable tool for development of advanced tests and procedures for diagnostics of eye diseases associated with the corneal lesions. Reduced lactoferrin concentration might represent a potential prognostic biomarker for diagnosis of ocular diseases including non-penetrating corneal injuries in a simple and non-invasive way.

Key words: lactoferrin; antibodies; western blot analysis; corneal wounds; tear fluid.

Human lactoferrin, 80-kDa glycoprotein of the transferrin family, is a non-heme iron-binding protein, consisting of 691 amino acid residues folded into two globular lobes [1]. Lactoferrin possesses iron-binding capacities, several times greater than the affinity of transferrin [2]. Lactoferrin is a ubiquitous protein, which is synthesized in many organs of the human body and contained in exocrine fluids such as breast milk, nasal exudate, bronchial secretions, saliva, tears, sweat, sperm, and vaginal discharge [3]. The main source of lactoferrin is milk; it is contained in human colostrum in the concentration up to 7 g/l. Lactoferrin is practically absent in blood plasma. Its concentration in blood is normally about 1.0 mcg/ml, while it can increase to 200 mcg/ml during inflammatory processes. Lactoferrin represents approximately 25% of the total tear proteins by weight (1.4–2.2 mg/ml), so it represents one of the main proteins in human tears. Tear lactoferrin levels are not influenced by age or sex [4]. Lactoferrin is recently referred to as a multipotent protein, which plays several biological functions, including antibacterial, antiviral, antifungal, antiparasitic, and antiinflammatory activities to provide innate defence [2, 5]. In addition, lactoferrin is known to have antioxidant, antiangiogenic, and antitumor properties, and plays sufficient roles in neuronal differentiation, osteogenesis, and wound healing [6, 7]. Lactoferrin has been shown to hamper plasmin-mediated pericellular proteolysis through inhibiting plasminogen activation by urokinase, thus suppressing cancer cell motility [8]. Besides, inhibition of platelet formation from megakaryocytes and other antiplatelet effects underlie antithrombotic activity of lactoferrin [9].

Lactoferrin plays an important role in maintaining eye health [10]. This protein was found to be expressed in the cornea and iris, and cells of retinal pigment epithelium of human and mouse eyes. However, it is mostly secreted by the main lacrimal gland, with both epithelial cells and Meibomian acini contributing to its final tear levels [11]. Antimicrobial activity of lactoferrin was the first to be discovered, and to date is also the most widely studied. Lactoferrin has been shown to inhibit the growth of various bacterial species implicated in adverse events in tear surface including *Escherichia coli*, *Haemophilus influenzae*, *Bacillus subtilis*, *Streptococcus spp.*, *Staphylococcus spp.* and *Pseudomonas spp.* [4]. For example, Williams et al. [12] have shown that lactoferrin deposited on the contact lens surface can effectively kill *Pseudomonas aeruginosa* cells that attempt to colonize the surface. Furthermore, lactoferrin has been shown to prevent complement activation and formation of harmful hydroxyl radicals and to affect the functions of monocytes, granulocytes and lymphocytes. These findings suggest that lactoferrin, apart from its antimicrobial effects, may also be involved in the regulation of inflammatory disorders. Evidence has been presented to show that lactoferrin binds to other proteins and may be present in various forms in the composition of tear film [13]. As a multifunctional protein, lactoferrin also exhibits efficacy in the setting of viral infectious processes against human and animal pathogenic viruses. Recent studies have demonstrated the activity of lactoferrin against the most widely spread viral particles, including cytomegalovirus, herpes simplex virus, human immunodeficiency virus (HIV), hepatitis C virus, poliovirus, parainfluenza virus, human papillomavirus, and adenovirus. In particular, the antiviral activity of lactoferrin lies in the early phase of infection, when it prevents virus entry into host cells [14]. Nowadays, antiviral activity of lactoferrin appears to be of special interest, since the current pandemic coronavirus disease 2019 (Covid-19) caused by SARS-CoV-2 virus has ocular diseases, in particular, conjunctivitis, among its known clinical manifestations [15]. Several mechanisms have been proposed for SARS-CoV-2 (Covid-19) infection, for which lactoferrin has additionally shown the capability of inhibiting *in vitro* viral replication [16, 17].

Development of various pathological conditions, such as dry eye of corneal injuries, can lead to significant decrease in lactoferrin's concentration, thus providing less protection [5]. It has been also reported that lactoferrin is one of the important predictors of the stability and volume of tear film. Tear volumes from the lacrimal gland are shown to have a positive correlation with the concentration of this protein. Patients with lower tear production tend to have lower lactoferrin's concentration [18]. It has been shown that keratitis and conjunctivitis of different aetiologies result in a decrease of tear lactoferrin levels, thus exposing patients affected by these conditions to a higher risk of infection [10]. Therefore, current and future studies are warranted for clinical applications of lactoferrin for the treatment option of various ocular diseases. Topical application of lactoferrin may play a crucial role in the maintenance of a healthy ocular surface system

by compensating deficit of this protein in the tear film. Topical application of lactoferrin has been shown to reduce irradiation-induced corneal epithelial damage in mice models, as well as to promote corneal wound healing after alkali-burn injury [19].

From these circumstances, lactoferrin's concentrations can represent a potential diagnostic biomarker for diagnosis of ocular diseases in a simple and non-invasive way. Besides, determination of lactoferrin levels in the tears of patients with various eye diseases will give more insight into the physiological role of this protein in the tear film. Our study represents an example of generation and possible application of polyclonal antibody for detecting lactoferrin content and analysis of its polypeptide composition in the tear fluid by western blot. Thus, the aim of the present study was to produce polyclonal antibodies against human lactoferrin in order to apply them for evaluation of lactoferrin levels in the tear fluid in health and collected after corneal injury.

Materials and Methods

All experiments on animals were conducted in compliance with the basic rules and principles of EU Directive 2010/63 on the protection of animals, the Declaration of Helsinki (2008) and the requirements of the Law of Ukraine "On Protection of Animals from Cruelty" (№1759-VI of 15.12.2009).

Rabbit immunization. Two males of English angora rabbit (weighting 2.27 and 2.72 kg) were injected with emulsion of highly purified lactoferrin (Novax®Pharma, Monaco) with Freund's adjuvant (Sigma Aldrich, USA) subcutaneously in four sites in the back (0.25 ml per site), according to the scheme presented in Fig. 1.

Blood was taken from the ear vein, allowed to form a clot, and then centrifuged at 1,000 *g* for serum collection. Sera from two rabbits were pooled, aliquoted, and stored at −20 °C before further procedures.

Immunoglobulin G (IgG) isolation and purification. Isolation of IgG fraction was performed by two-stage fractionation of serum of lactoferrin-immunized rabbits. At the first stage, globulin fraction of serum containing antibodies to lactoferrin was obtained after half saturation with ice-cold ammonium sulphate. Proteins were allowed to precipitate at 4 °C for 12 h. Then, the pellet containing crude globulins was separated by centrifugation at 3,000 *g* for 30 min at 4 °C and dissolved in

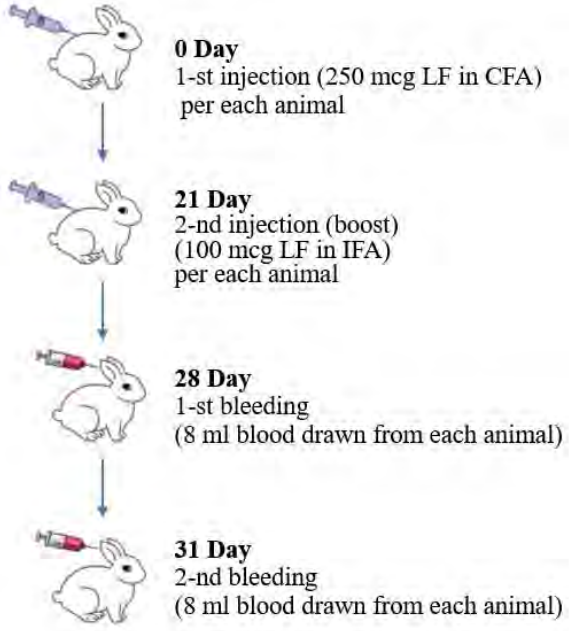

Fig. 1. **The scheme of rabbit immunization** (LF — lactoferrin, CFA — complete Freund's adjuvant, IFA — incomplete Freund's adjuvant)

0.05 M tris-HCl, pH 7.4, containing 0.15 M NaCl (TBS). Globulin solution was dialyzed against six changes of 100 volumes of the same tris-HCl buffer at 4 °C for 30 min each change till free from ammonium sulphate. After dialysis, p-nitrophenylguanidine benzoate (Sigma Aldrich, USA) was added to globulin solution to the final concentration of 1 mM.

At the second stage, IgG fraction was purified by affinity chromatography on protein A-sepharose (Sigma Aldrich, USA). The column (volume 2.0 cm^3) with protein A-sepharose was equilibrated with 10 volumes of TBS. IgG solution was loaded onto column (2:1), and unbound proteins were washed out by TBS. IgG absorbed on protein A-sepharose was eluted by glycine buffer, pH 2.2. Samples were collected, each of 1 ml, and pH was neutralized immediately by 1 M tris-HCl, pH 8.5. Concentration of IgG in eluates was monitored spectrophotometrically at the wavelength of 280 nm. Then, aliquots containing IgG were pooled and dialyzed against three changes of 100 volumes of TBS at 4 °C for 30 min each change with the use of centrifuge ultrafilters Amicon®Ultra M_m 100 kDa (Millipore, Ireland). Further, highly purified IgG fraction was used as a source of specific antibodies against lactoferrin.

Gel electrophoresis. The final purity evaluation of immunoglobulin samples was carried out by denaturing electrophoresis

in polyacrylamide gel (SDS-PAGE) in non-reducing conditions. Briefly, the samples of immune serum, globulin fraction, and isolated IgG were dissolved in Laemmli buffer (0.125 M tris-HCl, pH 6.8, containing 4% SDS, 20% glycerol, and 0.005% bromophenol blue) and loaded onto 10% gel. The samples were electrophoresed with the use of tricine-SDS cathode buffer system (0.1 M tris-HCl, 0.1 M tricine, pH 8.3) containing 192 mM glycine and 0.1% SDS, in "Mini-PROTEAN-II" vertical electrophoresis chamber ("BioRad", USA) [20]. After finishing electrophoresis, proteins in gel was fixed in 5% trichloroacetic acid and stained in 0.1% Coomassie Brilliant Blue R-250 dissolved in a mix of 40% ethanol and 10% acetic acid. After destaining, relative molecular weights of the stained protein bands were identified by comparing their migration with the location of coloured trans-blot markers Ruler™ Plus Pre-stained Protein Ladder 10–230 kDa (ThermoScientific, Lithuania). SDS-PAGE was also used for an antigen analysis before rabbit's immunization and as the first step in a western blot procedure of lactoferrin detection in a tear fluid.

Western blot analysis. After electrophoresis, proteins were transferred from the gel onto nitrocellulose membranes (GE Healthcare, Amersham Bioscience, UK, RPN 203D) with 0.45 mcm pore diameter by electroblotting using a buffer solution containing 25 mM Tris-HCl, 192 mM glycine, and 25% methanol. Membranes were blocked in a 5% solution of skimmed milk powder in phosphate buffered saline (PBS) and then probed with IgG isolated from the immune serum as a source of anti-lactoferrin antibodies (35 mcg/ml). After incubation with the primary antibodies, the membranes were washed in PBS, containing 0.1% Tween-20 (PBST), and incubated with the secondary antibodies anti-rabbit IgG (H+L)-HRP conjugate diluted 1:8,000 (Bio-Rad Laboratories, Inc., USA). Immunoreactive bands were developed by enhanced chemiluminescence (ECL) and subjected to autoradiography using Kodak X-ray films or using reaction with the chromogen substrate (0.05% diaminobenzidine and 0.03% hydrogen peroxide). Densitometric analysis of blotograms was performed with the use of densitometry software TotalLab TL120 (Nonlinear Inc, USA), signal intensities of the studied proteins were expressed as arbitrary units (a.u.).

Patients and tear sample preparation. In order to verify if antibodies isolated from immune rabbit serum are able to recognize and binds lactoferrin, we collected tear fluids from 4 healthy volunteers and 6 patients with non-penetrating corneal injures, which were observed in the clinic "Alexander Clinical Hospital" that is a clinical base of the Bogomolets National Medical University. Informed written consents were obtained from the all participants. The local ethical committee of Bogomolets National Medical University approved the study (protocol no. 138, 10 Nov. 2020) and the research is complied with the last version of Helsinki Declaration. Tear fluid was collected in a sterile plastic Eppendorf tube, mixed with Laemmli sample buffer and stored at −20 °C before laboratory examination. Total protein content in the tear fluid samples was determined spectrophotometrically by Stoscheck method [21]. Proteins of the tear fluids were separated by non-reducing 10% SDS-PAGE, loading 50 mcg total protein per track, and then lactoferrin levels were determined by western blot analysis as described above.

Statistical analysis. The results of western blot of lactoferrin levels in tear fluids of healthy individuals and patients with corneal wounds were analysed using Mann-Whitney U-test. Values are expressed as the mean ± SD. Difference between group mean values was considered significant at $P < 0.05$.

Results and Discussion

The major functions of lactoferrin related to the antioxidant, antibacterial, antiviral, antiinflammatory activities have been widely investigated. Lactoferrin is found in faecal, milk, serum, tears and other secretions from human body, and has been reported as a biomarker for several diseases, such as inflammatory bowel disease, Alzheimer's disease, and dry eye disease [22]. The present paper describes the main procedures of production design of polyclonal antibodies against human lactoferrin including immunization protocol and purification of IgG fraction from immune rabbit serum. Then, we addressed here if tear lactoferrin levels and changes in polypeptide composition of this protein could be associated with corneal injury. To do this, we measured relative content of target protein and analysed polypeptide spectrum of lactoferrin immunoreactive bands by western blot with the use of produced antibodies. Proteins of blood plasma of immunized rabbits, desolted globulin fraction and Protein A-eluted fraction containing IgG molecules were analysed by SDS-PAGE (Fig. 2).

Separated proteins of serum and globulin fraction had standard electrophoretic profile. Lanes 1 and 6 correspond to elution peak of rabbit IgG (M_m ~ 140 kDa) collected during affinity chromatography on Protein A-sepharose and concentrated. Electrophoresis showed that the eluted IgG samples contain few impurity proteins, the relative amount of which does not exceed 7-8% of total protein according to densitometry data. It was shown in further experiments that these minor impurities did not influence the immunogenicity of the produced antibodies. Then, we performed electrophoretic analysis of lactoferrin used as an antigen followed by testing produced antibodies by western blot. As shown in Fig. 3*A*, denaturing gel electrophoresis revealed intact lactoferrin polypeptide with apparent molecular weight ~75 kDa. The same major band was visualized by western blot with ECL development of immunoreactive polypeptides or their immunostaining with chromogen substrate (DAB). However, ECL-based detection as more sensitive approach was able to reveal several minor polypeptides bands with lower molecular weights (Fig. 3*B*), in comparison with blot development with the use DAB as chromogenic substrate (Fig. 3*C*).

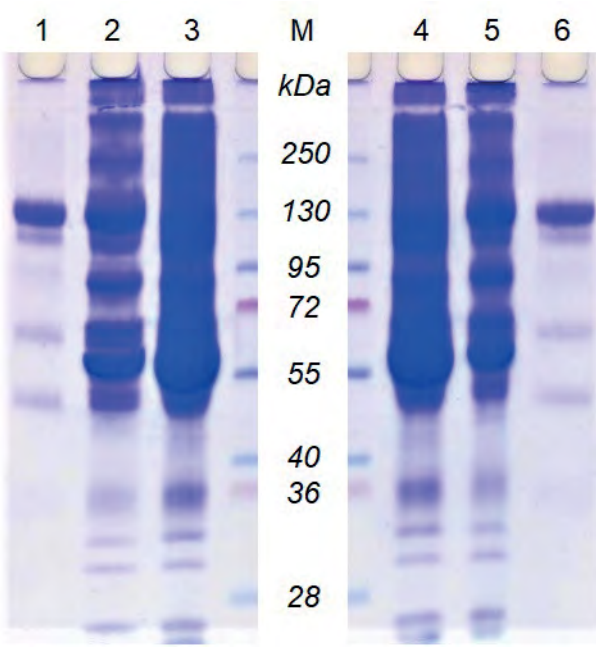

Fig. 2. **Coomassie-R250-stained electrophoregram of serum proteins (lanes 3 and 4) of rabbits immunized with lactoferrin, proteins of globulin fraction (lanes 2 and 5), and protein A-purified IgG (lanes 1 and 6) (loading 150, 50, and 10 mcg of total protein, respectively, onto 8% SDS-PAGE). Lane M — standard protein molecular weight markers**

This finding is in agreement with reported evidence that the ECL system is capable of detecting antigens even at a 20-fold increase over chromogenic western blot development [23] and thus is more appropriate technique for detection of lactoferrin in biological materials. Minor immunoreactive bands with M_m lower than 70 kDa may correspond to multiple forms of lactoferrin molecules presented in biological material or its truncated polypeptides since lactoferrin is relatively susceptible to limited proteolysis by various proteases including trypsin, chymotrypsin, pepsin, subtilisin and proteinase K [24], and even by itself owing catalytic activity [25].

Coomassie-stained electrophoregram of tear fluid proteins showed significant difference in protein profile between samples obtained from control individuals and patients with corneal injury (Fig. 4).

Comparison of the band profile of control and patient's tear samples showed a characteristic difference in intensity of two major bands, with apparent M_m 70 kDa, which corresponds to lactoferrin, and 55 kDa, which corresponds to human serum albumin. It is obviously seen that tear fluid

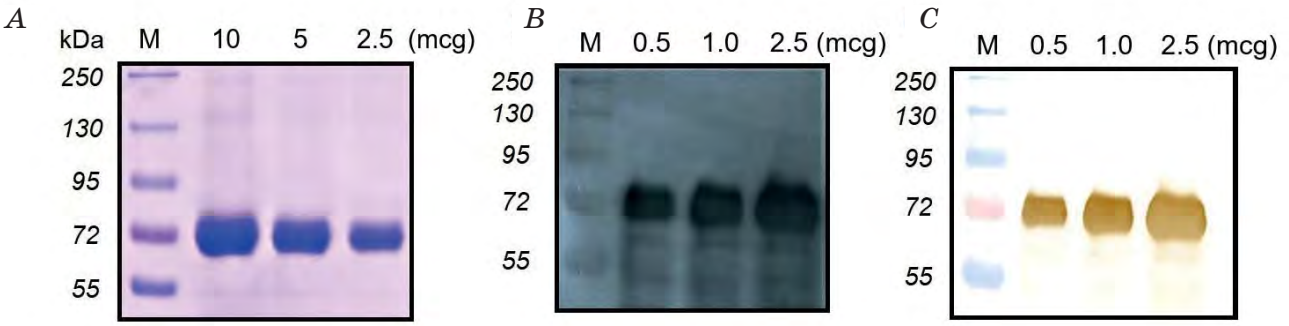

Fig. 3. **Electrophoregram of lactoferrin (*A*) and its western blot analysis with ECL detection (*B*) or staining with chromogenic substrate (*C*)**

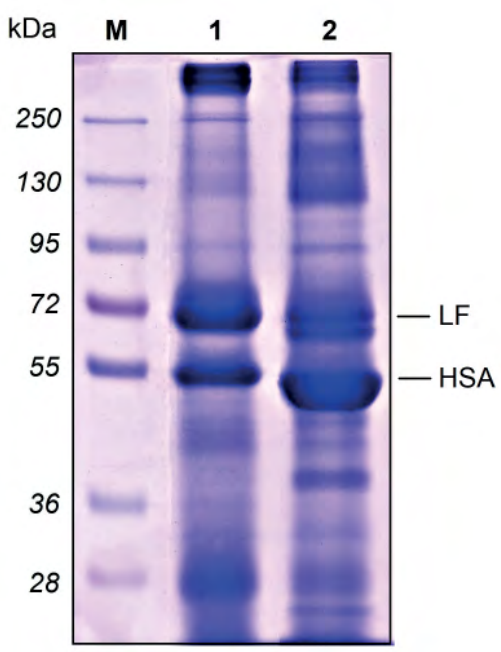

Fig. 4. **Typical example of the band profile in tear fluid samples obtained from healthy person (1) and patient with corneal non-penetrating trauma (2)** (SDS-PAGE data, loading 50 mcg of total protein per track, LF — lactoferrin, HSA — human serum albumin)

collected from intact eye contains higher levels of lactoferrin than samples obtained from traumatic eye, whereas in patients with corneal wounds, albumin level appeared to be increased in comparison with healthy persons. The high albumin level in patient's samples might be a result of a leakage of albumin from the inflamed conjunctival capillaries since albumin is a plasma-derived protein.

In order to detect lactoferrin in tear samples and evaluate differences in lactoferrin content between healthy persons and patients with corneal injuries, we used IgG fraction purified from rabbit immune serum in western blot analysis (Fig. 5). As seen in Fig. 5*A*, all tear samples of control persons contain major intact lactoferrin band with M_m ~75 kDa and minor polypeptide of ~55 kDa, while native lactoferrin polypeptide is contained in less number of patient's tear fluid.

Moreover, in addition with decreased level of lactoferrin, all tear film samples of patients with corneal injury contains well-developed band with M_m 30 kDa, which correspond of product of lactoferrin proteolytic degradation. It is also observed that several patient's tear samples contain high molecular weight immunoreactive bands, which may reflect ability of lactoferrin to form complexes with other proteins. This observation is in line with earlier published evidence that showed lactoferrin to bind other proteins including IgA, secretory components, albumin, and lysozyme, and thus it may be present in various forms in the tear film [1]. For example, human lactoferrin has been previously shown to form both *in vitro* and *in vivo* a complex with ceruloplasmin, the copper-containing protein of human plasma, which is present in the tear fluid in increased amounts during corneal inflammation [26, 27]. Densitometry analysis of 75-kDa lactoferrin band showed 3.2-fold decrease in the level of lactoferrin in the tear fluid samples obtained from patients with non-penetrating corneal trauma as compared with samples collected from healthy persons ($P < 0.05$) (Fig. 5*B*). These results of quantitative analysis of lactoferrin content may have clinical application and relevance to ophthalmic traumatology, although further testing of produced antibodies on the larger sized samples is required.

Tear fluid is a complex mixture of proteins, lipids, mucins, water and salts, and a recent study has identified more than 3,000 proteins in human tear samples by proteomic analysis [28], making them more complex (as a body fluid) than serum or plasma. Lactoferrin, also known as lactotransferrin, first isolated from milk (lacto + ferric = milk + iron) is a non-heme iron-binding protein belonging to the transferrin family. It has been estimated that a glass of cow's milk contains about 25-75 mg of this protein [2]. Lactoferrin is one of the main proteins in the tear fluid representing 25% of total tear proteins. Lactoferrin can occur as a holo-protein, which consists of a single polypeptide chain folded into two globular lobes, each with one binding site for iron and apo-protein with less than 5% iron saturation, which is more susceptible to proteolysis due to its molecular conformation that is characterized by lobes that are more 'open'. Lactoferrin performs several biological functions, including antimicrobial and immunomodulatory activities. Thanks to its multifunctional protective character, this glycoprotein is ubiquitous and it is present in different mucosal secretions such as tears, saliva, milk, and nasal secretions, among others. Lactoferrin found in most secretions is almost entirely as an apo-form and thus has the ability to tightly bind any free iron and effectively compete with bacteria for this essential cofactor [4]. Besides, lactoferrin displays broad-spectrum antiviral activity both *in vitro* and *in vivo*.

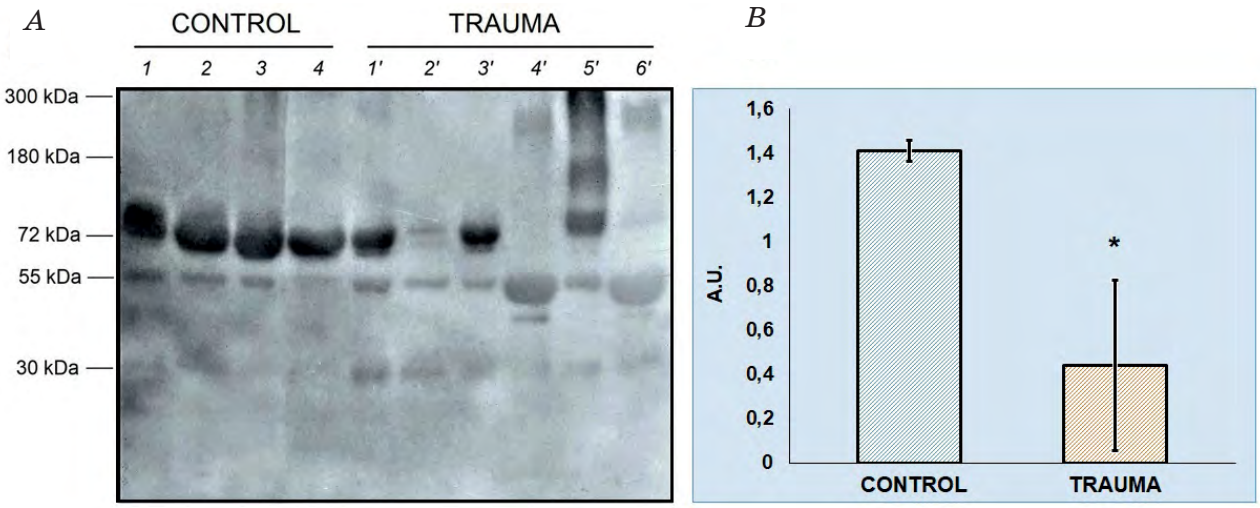

Fig. 5. **Detection of lactoferrin in samples of the tear fluid by western blot (*A*) and the results of its densitometry analysis (*B*)** (a.u. — arbitrary units)

In vitro results demonstrate that lactoferrin inhibits respiratory syncytial virus, influenza A virus (H3N2, H1N1, H3N2), as well as avian influenza A virus (H5N1), rotavirus, adenovirus, poliovirus, echovirus, herpes simplex virus (HSV-1, HSV-2), and other viruses. Orally administered bovine lactoferrin has been shown to improve the severity of viral infections including rotavirus and norovirus [14, 29]. Lactoferrin is able to bind to receptors, such as angiotensin-converting enzyme 2 (ACE2) and heparan sulfate proteoglycans (HSPGs), used by SARS-CoV-2 virus as an anchor sites in the cell membrane and thus inhibits the adsorption of the pathogen to the cell. In addition, lactoferrin is able to block the pathogen's surface receptors and prevent it from binding to the target cell [30]. It has been established that lactoferrin has not only strong *in vitro* efficacy against SARS-CoV-2, but also alleviates ocular manifestations of Covid-19 [10, 31].

Numerous earlier publications have reported a significant correlation between low levels of tear lactoferrin and the development of some ocular diseases such as dry eye disease, chronic meibomitis, and keratokonus in comparison with healthy subjects [32, 33]. Therefore, the level of lactoferrin in tears of patients with various ocular diseases has great potential to be considered as a valuable biomarker for determining, diagnosis, and prognosis of ophtalmo-pathological condition development. Other studies have also reported reduced lactoferrin tear levels with increased age and in certain diseases such as Sj gren syndrome (an autoimmune disease of the lacrimal gland), idiopathic dry eye, myotonic muscular dystrophy, vernal conjunctivitis, contact lens-induced giant papillary conjunctivitis, trachoma, herpes simplex keratitis, chronic irritative conjunctivitis keratocon-junctivitis sicca, ocular pemphigoid when it occurs concomitantly with dry eye, patients suffering cutaneous pemphigus and clinical dry eye with a marked keratopathy, post-operative cataract surgery, asymptomatic HIV-positive patients, in patients with chronic hepatitis C, and patients suffering Type 2 reactions in leprosy [reviewed in 34].

In general, patients with ocular diseases seem to have lower levels of lactoferrin compared with healthy subjects that is in complete agreement with our results, which indicate significantly decreased lactoferrin content in the tear fluid of patients with corneal injuries. Along with quantitative comparison of lactoferrin tear levels between healthy persons and patients with ocular diseases, differences in its polypeptide heterogeneity analysed by western blot may represent additional diagnostic indices. Many methods have been developed to detect lactoferrin levels during the last decades and different techniques have been used to measure concentration of this protein in tears, such as gel electrophoresis, high-performance liquid chromatography (HPLC), mass spectrometry, enzyme-linked immunosorbent assay (ELISA), or diagnostic test kits [35]. We highlight here that western blot analysis of lactoferrin in the tear fluid with the use of produced polyclonal antibodies allows performing quantitative comparison of lactoferrin levels between healthy individuals

and patients with traumatic ocular pathologies, as well as detecting differences in polypeptide composition of the studied protein in the tear samples.

Conclusion

Applicability of the produced and tested antibodies against human lactoferrin in diagnostics of ophthalmic pathology (non-penetrating corneal wound) has been clearly demonstrated. We suggest that reduced lactoferrin concentration might represent a potential diagnostic biomarker for diagnosis of ocular diseases including non-penetrating corneal injury in a simple and non-invasive way, thanks to the accessibility of tears and the convenience of tear sampling. Besides, obtained antibodies may become valuable tools for fundamental immunological research, immunohistochemistry, diagnostic testing, and drug quality control.

Conflicts of interest. Authors declare no conflict of interest.

Funding. The present research was conducted without any external financial support.

Acknowledgments. The authors gratefully thank to Andriy Wenger and Liliya Shimboretska for their kind help in animal care.

REFERENCES

1. *Kell D. B., Heyden E. L., Pretorius E.* The biology of lactoferrin, an iron-binding protein that can help defend against viruses and bacteria. *Front. Immunol.* 2020, 11, 1221. https://doi: 10.3389/fimmu.2020.01221
2. *Kowalczyk P., Kaczyńska K., Kleczkowska P., Bukowska-Ośko I., Kramkowski K., Sulejczak D.* The lactoferrin phenomenon — a miracle molecule. *Molecules*. 2022, 27 (9), 2941. https://doi:10.3390/molecules27092941
3. *Alexander D. B., Iigo M., Yamauchi K., Suzui M., Tsuda H.* Lactoferrin: an alternative view of its role in human biological fluids. *Biochem. Cell Biol*. 2012, 90 (3), 279-306. https:// doi:10.1139/o2012-013
4. *Flanagan J. L., Willcox M. D.* Role of lactoferrin in the tear film. *Biochimie*. 2009, 91 (1), 35-43. https://doi:10.1016/j.biochi.2008.07.007
5. *Rageh A. A., Ferrington D. A., Roehrich, H., Yuan, C., Terluk, M. R., Nelson, E. F., Montezuma, S. R.* Lactoferrin expression in human and murine ocular tissue. *Curr Eye Res*. 2016, 41 (7), 883-889. https://doi:10.31 09/02713683.2015.1075220
6. *Li Y.-Q. Guo C. A* Review on Lactoferrin and Central Nervous System Diseases. *Cells.* 2021, *10,* 1810. https://doi.org/10.3390/ cells10071810
7. *Bielecka M., Cichosz G., Czeczot H.* Antioxidant, antimicrobial and anticarcinogenic activities of bovine milk proteins and their hydrolysates — A review. *Int. Dairy J*. 2022, 127, 105208. https://doi.org/10.1016/j. idairyj.2021.105208.
8. *Zwirzitz A., Reiter M., Skrabana R., Ohradanova-Repic A., Majdic O., Gutekova M., Cehlar O., Petrovčíková E., Kutejova E., Stanek G., Stockinger H., Leksa, V.* Lactoferrin is a natural inhibitor of plasminogen activation. *J. Biol. Chem.* 2018, 293 (22), 8600–8613. https://doi.org/10.1074/jbc.RA118.003145
9. *Matsumura-Takeda K., Ishida T., Sogo S., Isakari Y., Taki T., Sudo T., Kiwada H.* Lactoferrin inhibits platelet production from human megakaryocytes *in vitro*. *Biol. Pharm. Bull*. 2008, 31 (4), 569–573. https://doi. org/10.1248/bpb.31.569
10. *Vagge A., Senni C., Bernabei F., Pellegrini M., Scorcia V., Traverso C. E., Giannaccare G.* Therapeutic effects of lactoferrin in ocular diseases: from dry eye disease to infections. *Int. J. Mol. Sci*. 2020, 21 (18), 6668. https://doi.org/10.3390/ ijms21186668
11. *Rusciano D., Pezzino S., Olivieri M., Cristaldi M., Gagliano C., Lupo G., Anfuso C.D.* Age-related dry eye lactoferrin and lactobionic acid. *Ophthalmic Res.* 2018, 60 (2), 94–99. https:// doi.org/10.1159/000489093
12. *Williams T. J., Schneider R. P., Willcox M. D.* The effect of protein-coated contact lenses on the adhesion and viability of gram-negative bacteria. *Curr. Eye Res*. 2003, 27 (4), 227–235. https://doi.org/10.1076/ ceyr.27.4.227.16602
13. *Hanstock H. G., Edwards J. P., Walsh, N. P.* Tear lactoferrin and lysozyme as clinically relevant biomarkers of mucosal immune competence. *Front. Immunol*. 2019, 10, 1178. https://doi.org/10.3389/ fimmu.2019.01178
14. *Wakabayashi H., Oda H., Yamauchi K., Abe F.* Lactoferrin for prevention of common viral infections. *J. Infect. Chemother.* 2014, 20 (11), 666–671. https://doi.org/10.1016/j. jiac.2014.08.003
15. *Sen M., Honavar S.G., Sharma N., Sachdev M.S.* COVID-19 and eye: a review of ophthalmic

manifestations of COVID-19. *Indian J. Ophthalmol*. 2021, 69 (3), 488-509. https://doi:10.4103/ijo.IJO_297_21
16. *Briana D. D., Papadopoulou A., Syridou G., Marchisio E., Kapsabeli E., Daskalaki A., Papaevangelou V.* Early human milk lactoferrin during SARS-CoV-2 infection. *J. Matern. Fetal. Neonatal. Med.* 2021, 1–4. https://doi.org/10.1080/14767058.2021.1920010
17. *Zimecki M., Actor J. K., Kruzel M. L.* The potential for Lactoferrin to reduce SARS-CoV-2 induced cytokine storm. *Int. Immunopharmacol*. 2021, 95, 107571. https://doi.org/10.1016/j.intimp.2021.107571
18. *Yolton D. P., Mende S., Harper A., Softing, A.* Association of dry eye signs and symptoms with tear lactoferrin concentration. *J. Am. Optom. Assoc*. 1991, 62 (3), 217–223.
19. *Pattamatta U., Willcox M., Stapelton, F., Garrett, Q.* Bovine lactoferrin promotes corneal wound healing and supresses IL-1 expression in alkali wounded mouse cornea. *Curr. Eye Res*. 2013, 38 (11), 1110–1117. https://doi.org/10.3109/02713683.2013.811259
20. *Schägger H., Von Jagow G.* Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem*. 1987, 166 (2), 368–379. https://doi.org/10.1016/0003-2697(87)90587-2
21. *Stoscheck C. M.* Quantitation of protein. *Methods Enzymol*. 1990, 182, 50–68. https://doi.org/10.1016/0076-6879(90)82008-p
22. *Zhang Y., Lu C., Zhang J.* Lactoferrin and its detection methods: a review. *Nutrients*. 2021, 13 (8), 2492. https://doi.org/10.3390/nu13082492
23. *Mruk D. D., Cheng C. Y.* Enhanced chemiluminescence (ECL) for routine immunoblotting: an inexpensive alternative to commercially available kits. *Spermatogenesis*. 2011, 1 (2), 121–122. https://doi.org/10.4161/spmg.1.2.16606
24. *Hopp T. P., Spiewak K., Matthews M. H., Athanasiou Z., Blackmore R. S., Gelbfish, G. A.* Characterization of proteolytic degradation products of vaginally administered bovine lactoferrin. *PloS One*. 2022, 17 (5), e0268537. https://doi.org/10.1371/journal.pone.0268537
25. *Massucci M. T., Giansanti F., Di Nino G., Turacchio M., Giardi M. F., Botti D., Ippoliti R., De Giulio B., Siciliano R. A., Donnarumma G., Valenti P., Bocedi A., Polticelli F., Ascenzi P., Antonini, G.* Proteolytic activity of bovine lactoferrin. *Biometals*. 2004, 17 (3), 249–255. https://doi.org/10.1023/b:biom.0000027700.90
26. *Sabatucci A., Vachette P., Vasilyev V. B., Beltramini M., Sokolov A., Pulina, M. Salvato B., Angelucci C. B., Maccarrone M., Cozzani I., Dainese, E.* Structural characterization of the ceruloplasmin: lactoferrin complex in solution. *J. Mol. Biol*. 2007, 371 (4), 1038–1046. https://doi.org/10.1016/j.jmb.2007.05.089
27. *Pulina M. O., Zakharova E. T., Sokolov A. V., Shavlovski M. M., Bass M. G., Solovyov K. V., Kokryakov V. N., Vasilyev, V. B.* Studies of the ceruloplasmin-lactoferrin complex. *Biochem. Cell. Boil*. 2002, 80 (1), 35–39. https://doi.org/10.1139/o01-206
28. *Jones G., Lee T.J., Glass J., Rountree G., Ulrich L., Estes A., Sezer M., Zhi W., Sharma S., Sharma A.* Comparison of different mass spectrometry workflows for the proteomic analysis of tear fluid. *Int. J. Mol. Sci*. 2022, 23 (4), 2307. doi: 10.3390/ijms23042307.
29. *Valenti P., Antonini G.* Lactoferrin: an important host defense against microbial and viral attack. *Cell. Mol. Life Sci.* 2005, 62 (22), 2576–2587. https://doi.org/10.1007/s00018-005-5372-0
30. *Wotring J. W., Fursmidt R., Ward L., Sexton J. Z.* Evaluating the in vitro efficacy of bovine lactoferrin products against SARS-CoV-2 variants of concern. *J. Dairy Sci*. 2022, 105 (4), 2791–2802. https://doi.org/10.3168/jds.2021-21247
31. *Alpogan O., Karakucuk S.* Lactoferrin: the natural protector of the eye against coronavirus-19. *Ocul. Immunol. Inflamm.* 2021, 29(4), 751–752. https://doi.org/10.1080/09273948.2021.1954202
32. *Kuo M. T., Fang P. C., Chao T. L., Chen A., Lai Y. H., Huang Y. T., Tseng C.Y.* Tear proteomics approach to monitoring sjögren syndrome or dry eye disease. *Int. J. Mol. Sci.* 2019, 20 (8), 1932. https://doi.org/10.3390/ijms20081932
33. *Ohashi Y., Ishida R., Kojima T., Goto E., Matsumoto Y., Watanabe K., Ishida N., Nakata K., Takeuchi T., Tsubota K.* Abnormal protein profiles in tears with dry eye syndrome. *Am. J. Ophthalmol*. 2003, 136 (2), 291–299. https://doi.org/10.1016/s0002-9394(03)00203-4
34. *Ponzini E., Scotti L., Grandori R., Tavazzi S., Zambon A.* Lactoferrin concentration in human tears and ocular diseases: a meta-analysis. *Invest. Ophthalmol. Vis. Sci*. 2020, 61 (12), 9. https://doi.org/10.1167/iovs.61.12.9
35. *Zhang Y, Lu C, Zhang J.* Lactoferrin and its detection methods: a review. *Nutrients*. 2021, 13 (8), 2492. https://doi:10.3390/nu13082492

ОТРИМАННЯ АНТИТІЛ ДО ЛАКТОФЕРИНУ ТА ЇХНЄ ВИКОРИСТАННЯ В АНАЛІЗІ СЛІЗНОЇ РІДИНИ У НОРМІ ТА ЗА ПОШКОДЖЕННЯ РОГІВКИ

А. Тихомиров[1], *О. Юсова*[1], *Л. Капустянецько*[1], *В. Білоус*[1], *Т. Дроботько*[1], *І. Гавриляк*[2], *Н. Гребень*[2], *Дж. А. Агджа*[3]

[1]Інститут біохімії ім. О. В. Палладіна НАН України, Київ, Україна
[2]Національний медичний університет ім. О. О. Богомольця, Київ, Україна
[3]Бінгольський Університет, Бінгол, Туреччина

E-mail: artem_tykhomyrov@ukr.net

Лактоферин є мультифункціональним протеїном, що синтезується різними тканинами організму та володіє антимікробною та імуномодуляторною активністю. Лактоферин відіграє провідну роль у підтриманні нормального функціонування ока.

Метою роботи було одержати поліклональні антитіла до лактоферину людини для їхнього використання у визначенні вмісту лактоферину в слізній рідині, отриманої за норми та з очей з ушкодженою рогівкою.

Матеріали та методи. Фракцію імуноглобулінів G (IgG) було виділено з сироватки крові кролів, імунізованих лактоферином, за допомогою афінної хроматографії на протеїн А-сефарозі. Ступінь чистоти протеїну на кожній стадії очищення перевіряли за допомогою денатуруючого гель-електрофорезу (SDS-PAGE). Розпізнавання цільового антигену отриманими антитілами оцінювали за допомогою вестерн-блот аналізу з використанням розчину фракції IgG. Рівень лактоферину в слізній рідині, отриманої із здорового ока ($n = 4$) та з ока пацієнтів з непроникаючим пошкодженням рогівки ($n = 6$) визначали імунохімічно з використанням отриманих антитіл. Результати визначення вмісту лактоферину в сльозі за умов норми та за травмування рогівки аналізували з використанням U-тесту Манна-Уітні. Міжгрупова різниця вважалася статистично достовірною за $P < 0{,}05$.

Результати. Антитіла до лактоферину людини у вигляді фракції IgG було виділено на протеїн А-сефарозі з сироватки крові імунізованих кролів. Вестерн-блот аналізом було показано, що отримані антитіла розпізнають відповідний антиген як зону 75 кДа, яка відповідає за молекулярною масою інтактному поліпептиду лактоферину людини. Та ж сама поліпептидна зона була визначена вестерн-блотом з підсиленою хемілюмінісцентною детекцією у зразках слізної рідини. Денситометричний аналіз поліпептиду 75 кДа уможливив встановити, що вміст лактоферину в сльозі, зібраної в пацієнтів з непроникаючими травмами рогівки є у 3,2 рази нижчим за цей показник у нормі ($P < 0{,}05$). Крім того, слізна рідина пацієнтів з пошкодженнями рогівки у значних кількостях містила також імунореактивні продукти розщеплення лактоферину, а також високомолекулярні поліпептиди, які можуть відповідати комплексам лактоферину з іншими протеїнами, що утворюються за розвитку запальних процесів.

Висновки. Згідно з наведеними даними, отримані антитіла до лактоферину можуть бути використані як корисний інструмент для створення удосконалених тестів та підходів для діагностики очних хвороб, пов'язаних з ушкодженням рогівки. Зниження вмісту лактоферину може слугувати прогностичним біомаркером перебігу ранового процесу в оці, зокрема, за непроникаючих травм рогівки, та є зручним до визначення в простий та неінвазійний спосіб.

Ключові слова: лактоферин; антитіла; вестерн блот аналіз; травми рогівки; слізна рідина.