EXPERIMENTAL ARTICLES

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BENEFICIAL EFFECTS OF ANGIOSTATIN K1-3 AND LACTOFERRIN IN ALKALI-BURNED RABBIT CORNEA: A COMPARATIVE STUDY

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Corneal injury is associated with hypoxia-induced neovascularization, which interferes optical transparency of the cornea, resulting in vision loss and blindness. The treatment of corneal damage remains a significant unmet medical need. Lactoferrin (Lf) and angiostatins (AS) are naturally occurring antiinflammatory and antiangiogenic proteins, which play important roles in eye physiology and can be used as protectors against corneal diseases.

The *aim* of the study was to evaluate and compare the effects of plasminogen fragment AS K1-3 and Lf (as a referent preparation) on the levels of the principal markers of angiogenesis, apoptosis, and autophagy in the burn-injured cornea. Additionally, effects of AS on the metabolic activity of macrophages have been studied.

Materials and Methods. Experimental model of corneal burn was induced by NaOH application to rabbit's eye surface. One of the group of animals with injured cornea topically received AS, which were applied as eye drops (1 μM solution) daily for 14 days after injury, another one was treated by the equimolar solution of Lf. The levels of protein markers of angiogenesis (VEGF), apoptosis (caspase-3), and autophagy (beclin-1) were evaluated in corneal lysates by western blot. Histological analysis was performed by hematoxylin/eosin staining of corneal slices followed by light microscopy. Effects of AS in the range of concentrations 10–200 nM on the activity of phagocytic cells were assessed in murine macrophage cell line J744.2 by the test with nitroblue tetrazolium (NBT). The quantitative results were analysed with the use of Mann-Whitney U-test. The difference between group mean values was considered significant at $P < 0.05$.

Results. Dramatically increased levels of protein markers related to angiogenesis, apoptosis, and autophagy were found in the corneas with burn-induced injury. AS or Lf-based treatment reduced burn-induced overexpression of VEGF, caspase-3, and beclin-1. It is important to note that AS, along with anti-VEGF activity, appeared to more effectively suppress apoptosis and autophagy by decreasing tested markers near the control level, as compared with Lf. Histochemical examination revealed typical signs of fibrotic and necrotic changes in the injured corneas, which were associated with excessive inflammatory infiltration and intense neovascularization. Treatment of burn-induced injury with AS alleviated histopathological changes in cornea, which is evidenced by improving epithelial regeneration, reducing neovascularization, and moderating leucocyte infiltration. These observations are in agreement with the data of NBT test suggesting that AS in the concentrations >50 nM is able to moderately inhibit metabolic activity of macrophages up to 30% as compared with intact cells ($P < 0.05$).

Conclusions. Obtained results indicate that AS may serve as an effective treatment option to relief alkali-induced corneal injury with the efficacy comparable or even higher than that of firmly established eye protective protein Lf.

Key words: plasminogen kringles, angiostatin, lactoferrin, alkali burn, corneal injury, macrophages, VEGF, caspase-3, beclin-1.

The cornea is a transparent structure with significant refractive and barrier functions. The corneal avascular status is also defined as "angiogenic privilege" because the cornea is lack of both lymphatic and blood vessels, deriving nutrients and oxygen supply from the tear film, peripheral nerves, and posterior aqueous humor. The corneal angiogenic privilege is based on a subtle balance between pro-angiogenic and anti-angiogenic factors [1, 2]. Disruption in the balance of angiogenic counteracting factors can be caused by several ocular insults, including mechanical trauma, infectious keratitis, inflammatory disorders, autoimmune diseases, contact lens-related hypoxia, allergy, chemical burns, limbal stem cell deficiency and loss of the limbal stem cell barrier, corneal transplantation (keratoplasty), chronic alcohol consumption. Therefore, corneal neovascularization as a nonspecific response to different clinical insults, which occur in a wide variety of corneal pathologies, can lead to loss of transparency and an impairment of visual acuity due to the development of oedema, persistent inflammation, intrastromal protein and lipid deposition, and scarring [3].

It has been reported that 1.4 million people develop corneal neovascularization per year, 12% of whom suffer the subsequent loss of vision, and the incidence is expected to rise. Corneal neovascularization and opacity are the fourth cause of blindness globally (5.1%) after cataract, glaucoma, and age-related macular degeneration (AMD) [4]. A PubMed review was performed, analysing all publications from 1970 to 2024 concerning the topic "corneal neovascularization" and gave the result of such search as much as 3,901 articles with a tendency to constant growing.

Several treatment modalities have been derived based on the understanding of molecular mechanisms of corneal neovascularization pathogenesis. For example, corticosteroids are potent inhibitors of inflammation and remain widely accepted as first-line therapy for corneal diseases [5]. However, their antiangiogenic effects are weak and insufficient to induce long-term regression of mature corneal vessels. Agents and antibodies, which inhibit vascular endothelial growth factor (VEGF), the most influential regulator in inducing corneal angiogenesis, have provided encouragement in the management of corneal neovascularization. Nevertheless, anti-VEGF therapy produces only a partial and temporary reduction in neovascularization and is associated with serious complications, including corneal wound healing impairment, appearance of persistent epithelial defects, corneal stromal thinning, neurotrophic keratopathy, and subconjunctival hemorrhage [6]. Thus, because conventional anti-angiogenic drugs and agents have partial efficacy and may lead to a multitude of side effects, it is of great significance to develop new effective drugs to suppress the occurrence of corneal neovascularization. Several earlier studies suggest that naturally occurring plasminogen fragments, known as angiostatins (AS), are promising candidates for the suppression of angiogenesis and corneal neovascularization [7, 8]. AS comprise a group of polypeptides, which contain various number of plasminogen kringle (K) domains (K1-3, K1-4, K1-4.5, K5, etc.), which are produced by several proteases including matrix metalloproteinases, a metalloelastase, cathepsins V, and plasmin autoproteolysis [9]. AS binds to surface proteins in the activated vascular endothelial cells to hinder their migration and tubule formation [10, 11]. Although there are several studies [8, 12–15] that explored the inhibitory effects of AS on the corneal neovascularization, the objective evaluation of the injury-related molecular biomarkers in the wounded cornea is important for monitoring natural course and treatment response. It has been currently reported that proteomic profile of the human cornea is accounted 4824 proteins. Moreover, signaling pathway analysis revealed enrichment of spliceosome, phagosome, lysosome, and focal adhesion pathways, along with pro-apoptotic signaling, in the injured cornea, thereby demonstrating activated function of scavenging/autophagy corneal proteins [16]. It is also highlighted that corneal neovascularization is critically dependent on the presence of corneal macrophages, which are critical participants of injury-associated corneal angiogenesis [17]. It has been established that macrophages are able to secrete various isoforms of VEGF that promote vascular endothelial proliferation [18]. Human AS K1-3 has been earlier shown to exert anti-inflammatory effects by suppressing monocyte/macrophage migration that may help to restrict undesirable tissue angiogenesis [19].

Thus, the aim of the present study was to investigate the effects of AS K1-3 on the levels of marker proteins, which regulate key pathophysiological processes in the cornea of the eye under conditions of damage caused by alkali. The corneal levels of the following

markers of angiogenesis (VEGF), apoptosis (caspase-3), and autophagy (beclin-1) were evaluated on the model of alkaline burn of the rabbit eye. As an additional task, effects of AS on the metabolic activity of phagocytic cells were assessed. A well-known natural protein abundant in the tear fluid, lactoferrin (Lf), was used in this experiment as a reference drug. Lf is a crucial protein for the eye health due to its pleiotropic protective activities, which is widely used for the treatment of ocular disease [20, 21]. For example, bovine Lf has been generally recognized as a safe substance (GRAS) by the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) [22].

Materials and Methods

Experimental model. Chinchilla male rabbits (2-months-aged, 1.8–2.2 kg) were divided into four groups: I) intact control ($n = 3$); II) alkali burn group ($n = 4$); III) alkali burn + AS K1-3 ($n = 4$); IV) alkali burn + Lf ($n = 4$). Experimental animals received topically a drop of tetracaine as an aesthetic. Then, a 4 mm diameter Whatman no. 1 filter paper soaked in 1 N NaOH was placed on the center of the corneal surface of the eye for 30 s, and then the injured eye was thoroughly washed with 10 ml of saline [23]. Alkali burn was induced only in the right eye, while left eyes obtained equal volume of saline drops. The animals were kept and handled in compliance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental protocol has been approved by the Institutional Bioethical Committee (protocol No. 4, 8_{th} June 2021).

AS K1-3 was isolated from the products of human Glu-plasminogen elastolysis followed by affine chromatography on lysine-sepharose as described elsewhere [24]. Topical administration of 1.0 μM AS K1-3 solution dissolved in sterile buffered saline (25 μl per eye) was done daily for 14 days. The single dose of K1-3 was calculated to contain 0.75 μg of protein. The dose of AS was selected based on the data of our previous examinations [14]. Lf (Novax Pharma SARL, Monaco) was used as an equimolar solution in same regime of application. To determine if tested proteins have any side effects in the non-injured rabbit eye, K1-3 or Lf solutions were dropped to the left eye of rabbits from group III or IV, respectively, daily for the whole experimental period. After the end of the experimental period, the rabbits were sacrificed by air embolism.

Corneal sample preparations. After eye enucleation, rabbit corneas were slightly rinsed in the ice-cold phosphate-buffered saline (PBS). Each cornea was thoroughly grinded in liquid nitrogen and homogenized in lysis buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS) supplemented with the Pierce™ protease and phosphatase inhibitor cocktail (ThermoScientific, USA, cat. no. A32961) for protein extraction. Tissue to buffer ratio was 1:2 (m/v). After additional sonification with the use of ultrasound disintegrator Sartorius (Labsonic®M, Göttingen, Germany), homogenates were centrifuged at 16,000 g for 45 min at 4 °C. Concentration of total protein in supernatants were evaluated spectrophotometrically, using the measurements of absorbance at the wavelengths of 280 and 260 nm as described earlier [25]. Supernatants were then mixed with an equal volume of 2×reducing Laemmli sample buffer, boiled for 5 min, and stored at −20 °C until analysed.

Gel electrophoresis & Western blot. Sample aliquots were loaded onto 10% polyacrylamide gel (PAG) in the volume, which contains 50 μg protein per track, then corneal proteins were separated by SDS-PAGE in a vertical gel electrophoresis chamber (BioRad, USA). After electrophoresis, proteins were transferred from the PAG onto nitrocellulose membrane (GE Healthcare, Amersham Bioscience, UK, RPN 203D, 0.45 μm pore diameter) by electroblot. After 90 min blocking in a 5% solution of a skimmed milk powder in PBS, the blots were overnight incubated at 4 °C with the mouse primary antibodies to the protein markers: anti-VEGF (Invitrogen, USA, cat. no. MA5-12184, 1:3,000 diluted), mouse anti-BECN1/Beclin-1 (Santa Cruz Biotechnology, Inc, USA, clone E-8, cat. no. sc-48341, 1:1,000 diluted), anti-caspase-3 (Abcam, USA, cat. no. ab208161, 1:2,500 diluted). Beta-actin was probed as a loading control by anti-β-actin mouse antibody (Invitrogen, USA, cat. no. MA5-15739, 1:5,000 diluted). After washing each membrane six times with PBS supplemented with 0.05% Triton X-100 (PBST), the blots were incubated with HRP-conjugated goat anti-mouse IgG (1:8,000 diluted) purchased from Invitrogen, USA (cat. no. G-31430) for 2 h at 37 °C. Non-specifically bound antibodies were washed away with PBST, and thereafter specific immunoreactivity was developed by enhanced chemiluminescence (ECL) with the use of p-coumaric acid, luminol, and hydrogen peroxide as a HRP substrate. The molecular weight of each protein band was

determined by comparing their migration with the distance run by coloured markers Ruler™ Plus Prestained Protein Ladder 10-230 kDa (ThermoScientific, Lithuania, cat. no. 26619). Each band immunostaining was quantified by measuring optical density values with the use of densitometry software TotalLab TL120 (Nonlinear Inc., USA), normalized to the β-actin level, and expressed as arbitrary units (a. u.).

Histochemistry. For histological examination, enucleated rabbit corneas were taken and tissue fragments were fixed in 10% neutral buffered formalin for 16 hs. Tissue processing was performed in the LOGOS One EVO histoprocessor (Milestone, Italy). Paraffin sections of the cornea (thickness 5-6 μm) were stained with hematoxylin (Gill III Hematoxylin) and eosin according to the method described earlier [26]. Microscopic studies of sections were performed using an Olympus BX51 microscope. Ultrastructural analysis of photomicrographs of corneal tissue was performed with the use of Image J software (Wayne Rasband NIH, USA).

NBT-test. The nitro blue tetrazolium test (NBT-test) was performed according to the previously described method [27] with minor modifications. The NBT-test characterizes the extent of intracellular NADPH-N-oxidase system activation. This test is relatively simple to perform, is highly reproducible and sensitive. The principle of the method is based on the reduction of soluble colorless tetrazolium dye absorbed by phagocytic cells and its transformation into insoluble diformazan under the influence of superoxide anion (O_2^-), which is formed as a result of the NADP-H-oxidase reaction. To study the influence of AS K1-3 on the metabolic activity of macrophage cells, J744.2 cells (mouse macrophages) were used. Cells were grown to a confluent state (90–95%) in 96-well plates (cat. no. 655950, Greiner, Germany) in DMEM medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum. The number of cells was 2.5×105 per well. In 24 hs before conducting AS testing, the medium was discharged, and 90 μl of DMEM without fetal serum were added to the cells. K1-3 diluted in sterile PBS was added in the concentration range of 10–200 nM and cultured for 24 hs under standard conditions (5% CO_2, 100% humidity, 37 °C). An appropriate volume of PBS was added to the cells used as a control. Later, the culture medium was replaced with a fresh one with the addition of NBT reagent (Sigma-Aldrich, USA) in a concentration corresponding to the manufacturer's recommendations (1.0 mg/ml) and incubated for 4 hs under standard conditions. After incubation, the medium was carefully removed, 0.2 ml of dimethylsulfoxide (DMSO) solution was added for cell lysis and kept for another 5 min under intensive shaking conditions. The optical absorption of the solution was determined at a wavelength of 550 nm (test) and 620 nm (reference). The values of control cells and each concentration of angiostatin K1-3 were measured in nine replicates (n=9). To represent the results, the absorbance of cell samples that were not treated with AS (control) was taken as 100%.

Statistical analysis. The results are presented as mean ± SEM. Analysis of variances (ANOVA) followed by post-hoc Tukey's multiple comparison was used to verify significant difference between group means. *P* value of less than 0.05 was considered significant.

Results and Discussion

The present study was performed to check the relative efficacy of AS in inhibiting pro-angiogenic signaling and modulate apoptosis/autophagy pathways in the injured cornea. We chosed to use an alkali burn-induced corneal neovascularization rabbit model because it is easy-to-perform/reproduce and due to similarities in anatomical features and matrix structure between rabbit and human corneas, while rabbit eye is commonly used as an experimental model to simulate human ocular diseases [28]. In comparison with acids, which are able to bind with proteins located on the outer layer of the cornea, the hydroxyl ions, which are produced by alkaline compounds, cause saponification of fatty acids, denaturation or dissolution of stromal collagen, and can destroy the underlying ECM [29]. Corneal neovascularization is considered a sight-threatening condition that introduces vascular pathology into the normally avascular corneal tissue. Thus, regression of vessel outgrowth is thought to be emerging treatment concept in the management of neovascular ocular diseases associated with eye traumatization. The maintenance of the corneal avascular state is an active process based on continuous interactions between multiple pro- and anti-angiogenic factors. However, corneal healing response can be accompanied by loss of tissue transparency through vessel ingrowth, fluid leakage, and fibrosis [30]. These pathologies lead to a

disequilibrium between proangiogenic and antiangiogenic factors that can result in the proliferation and migration of vascular endothelial cells into the corneal stroma.

The results of western blot analysis of VEGF levels in the corneal tissue are presented in Fig. 1.

It was established that corneal damage induces an increase in VEGF level approximately by 3.3 times compared to the control ($P < 0.01$). It should be noted that the indicated changes are mostly related to the VEGF dimer, a biologically active form of this growth factor. Application of AS or Lf to the intact eye did not affect the level of VEGF in the healthy cornea. Otherwise, both tested proteins reduced the level of angiogenesis activator in the cornea of the alkali burn eye near the control value. It is interesting that AS demonstrated a more pronounced effect in comparison with Lf, which, however, does not reach the level of statistical significance. The obtained results suggest the induction of pro-angiogenic signaling in the cornea tissue during chemical burn, thus supplementing our earlier data that showed AS K1-3 to induce pronounced regression of corneal neovascularization in the model of alkali-burned injury [14].

It is known that VEGF plays a pivotal role in maintaining normal corneal function, corneal epithelium and nerve healing [31]. However, disruption of the balance between pro- and anti-angiogenic factors and overweighing of proangiogenic factors may result in pathological vessel formation [1, 3]. At present, not all mechanisms involved in the development of corneal neovascularization are completely understood. Macrophage recruitment during inflammation stage plays a key role for corneal angiogenesis. Activated macrophages are known to secrete inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and VEGF-A, -C, and -D, resulting in the induction of angiogenesis and further macrophage infiltration [17]. VEGF-A is considered to be one of the most important members of the VEGF family and a main driver for pathologic angiogenesis. Apart from macrophages, corneal fibroblasts and epithelial cells are the most important sources of VEGF-A [32]. Taking into consideration these circumstances, current anti-VEGF therapies, although efficacious, affects consequences, but not causes of pathological processes. Besides, anti-VEGF therapy requires prolonged treatment regimens, which may cause various ocular complications, such as a prolonged corneal epithelial healing period and increased the occurrence of corneal ulceration [33]. Other researcher's group has previously demonstrated that depletion of macrophages decreases angiogenesis in experimental corneal neovascularization, indicating that inactivating macrophages can be the primary target to reduce vessel in-growth in the injured

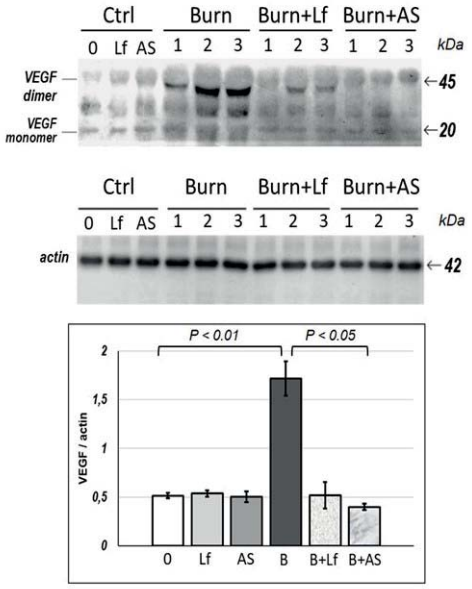

Fig. 1. **Angiostatin K1-3 and lactoferrin decrease VEGF levels in the alkali burned rabbit cornea (typical result of Western blot analysis):**
0 — intact control; B (burn); Lf (lactoferrin); AS (angiostatin); B+Lf (lactoferrin-treated burn injury); B+AS (angiostatin-treated burn injury). $P < 0.05$ is considered statistically significant difference

cornea [34]. In order to explore, if AS K1-3, which was obtained in our laboratory, may affect macrophages in terms of their metabolic activity, we organized the study on the line of phagocytic J744.2 cell assessed by NBT-test.

Fig. 2 shows that AS in the tested concentration range of 50-200 nM inhibits the activity of the NADP-H oxidoreductase system of J744.2 mouse macrophages, approximately by 30% compared to intact cells ($P < 0.05$).

Despite the possible relative species specificity of AS action, it can be assumed that the effect of K1-3 on human macrophages can be more powerful, but this issue requires further clarification. However, the data obtained in our study are generally consistent with the results of a prior research [19], which demonstrated that human AS K1-3 effectively inhibited the migration of mouse peritoneal monocytes/macrophages in a model of atherosclerosis. Therefore, the described AS effects to inhibit the activity of phagocytic cells may be useful for modulating the processes associated with hypoxia, not only during tumor growth and atherosclerosis, but also those associated with corneal damage in terms of preventing excessive neovascularization.

Therefore, the antiangiogenic activity of AS can be mediated not only by direct suppression of endothelial cells but also via inhibiting macrophage expression of VEGF in the damaged corneal tissue. It turned out that Lf is able to inhibit neovascularization by inhibiting p-p65 through binding to TNF receptor-associated factor 6 (TRAF6), thus suppressing the activation of HIF-1α and, subsequently, the expression of VEGF [35]. The effects of AS appeared to be comparable to the effectiveness of an equimolar dose of Lf, taken as a reference drug. Lf is widely used for the pharmacotherapy of eye diseases, presumably in the form eye drops, having been characterized in *in vitro* and *in vivo* studies as a biocompatible and safe substance. The choice in favor of this protein as reference was made based on the requirements of the European Medical Agency (EMA). According to the Guideline On The Investigation Of Bioequivalence (2016), the rule of pharmaceutical equivalence (or pharmaceutical alternative) is extended to the substances under investigation. The reference drug, which is used to compare the effects with the new drug, must meet a number of basic criteria: 1) contain an identical amount of the active substance and be used in the same concentration or dosage; 2) be in the same medicinal form; 3) have the same route of application; 4) affect acceptable or comparable pharmacological points (targets). Lf fully meets all the essential requirements for a reference drug to study the effects of AS in the alkali burned cornea. The comparability of the biological effects of Lf and AS is based on their antiangiogenic activity, which is well known for plasminogen fragments. However, Lf also exhibits significant antiangiogenic activity, which was recently shown in a model of tumor-induced angiogenesis [36].

Further results of our study represent important findings indicating AS to lower

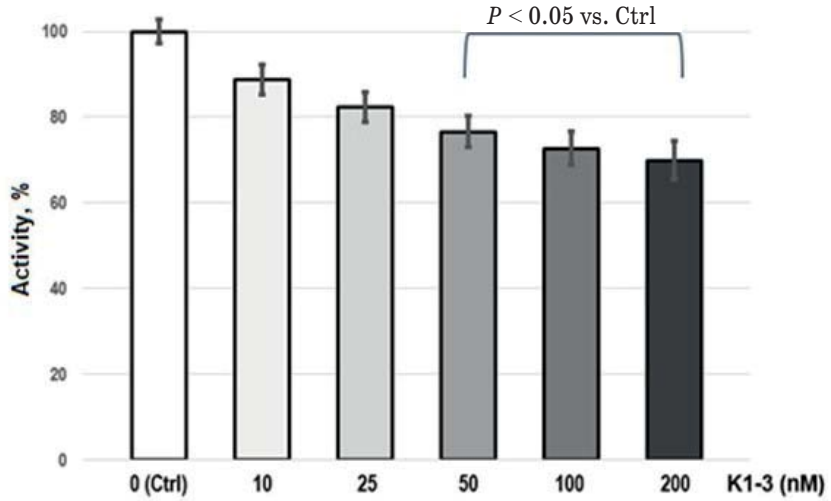

Fig. 2. **Angiostatin K1-3 affects metabolic activity of macrophage J744.2 cell line (the results of spontaneous NBT-test)**
$P < 0.05$ is considered statistically significant difference

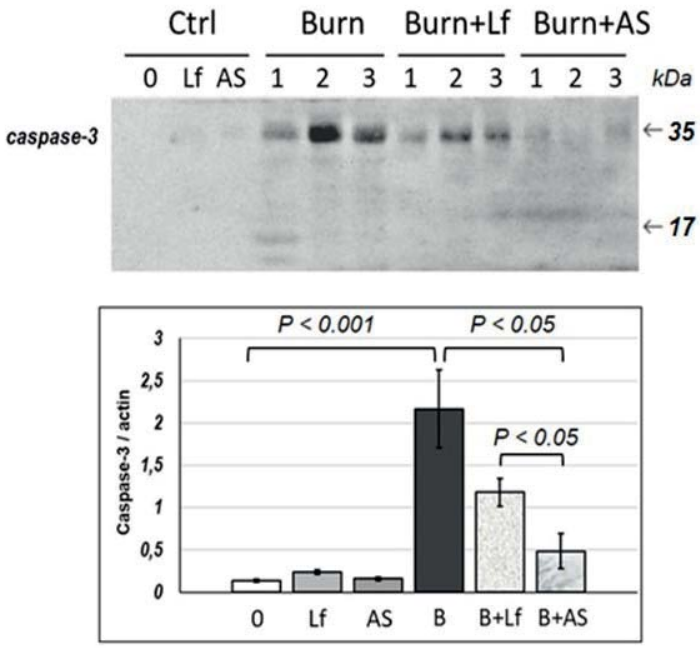

Fig. 3. **Angiostatin K1-3 and lactoferrin reduce caspase-3 levels in the alkali burned rabbit cornea (typical result of Western blot analysis):**
0 — intact control; B (burn); Lf (lactoferrin); AS (angiostatin); B+Lf (lactoferrin-treated burn injury); B+AS (angiostatin-treated burn injury). $P < 0.05$ is considered statistically significant difference

both apoptosis- and autophagy-related markers in alkali burned corneal tissue. The results of Western blot analysis of the level of the apoptotic executive protein caspase-3 in the rabbit damaged cornea are shown in Fig. 3. It is shown that this enzyme is present in trace amounts in the tissue of the intact cornea of control group animals, while the level of pro-caspase-3 in burn pathology is (zymogen) dramatically increases by 15.6 times compared to the control value ($P < 0.001$). In addition, a 17 kDa polypeptide, which corresponds to the active form of this caspase, is obviously appeared in the damaged tissue. It was established that neither AS nor Lf affect the content of this apoptosis-related protein. However, the use of Lf or AS in the form of eye drops under conditions of alkaline corneal burn contributed to a statistically significant decrease in the level of caspase-3 by 1.8 and 4.5 times, respectively ($P < 0.05$ compared to the Burn group). It is important to note that the anti-apoptotic effect of K1-3 was significantly more pronounced than that of Lf ($P < 0.05$). The obtained results indicate activation of the apoptotic pathway of corneal cell death caused by alkali burn. K1-3 used in the tested dose exerts pronounced anti-apoptotic effects under conditions of the developed experimental model of alkaline corneal burn.

Apoptosis and autophagy can be stimulated by the same stresses and may both induce cell death in some conditions. Emerging evidence indicates a crosstalk between apoptosis and autophagy [37]. The results of Western blot analysis of the level of beclin-1, an autophagy marker [38], in the rabbit cornea damaged by alkaline burn are shown in Fig. 4. In the samples of control animals, this marker protein is poorly detected that indicates the maintenance of autophagy flux at a relatively low basal level in healthy tissue. However, in chemically burned cornea, the level of beclin-1 is significantly up-regulated (31 times compared to the control, $P < 0.001$).

Both tested proteins had no effects on the basal level of the autophagy marker in the intact eye. However, the use of either AS or Lf as potential modulators of the pathophysiological processes that accompany alkaline corneal burn lead to a statistically significant decrease in beclin-1 levels by 3.2- and 12.9-folds, respectively, compared to the Burn group ($P < 0.05$). As in the case of apoptosis regulator level, AS K1-3 appeared greater compared to Lf, which was taken in equimolar amounts. Therefore, the obtained results suggest an overactivation of the autophagy pathway in the cornea caused by alkaline burn, which, together with the activation of apoptosis, may underlie the

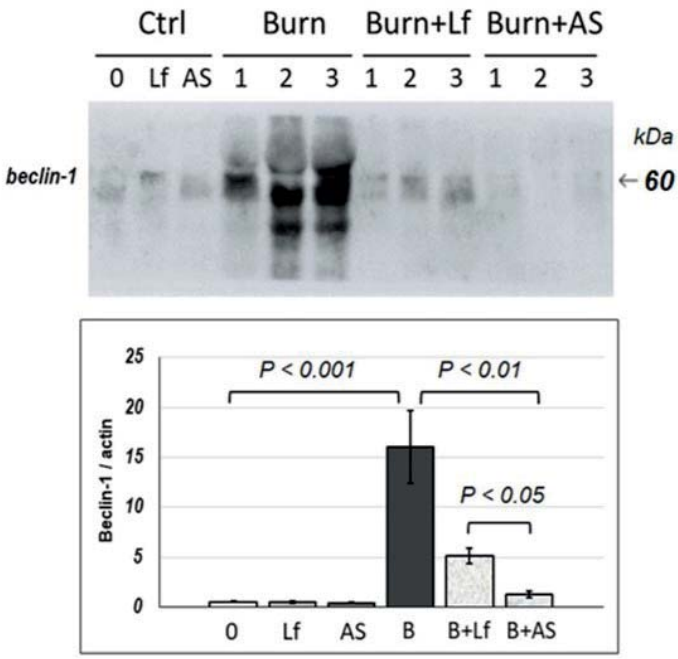

Fig. 4. **Angiostatin K1-3 and lactoferrin alleviate beclin-1 overexpression in the alkali burned rabbit cornea (typical result of Western blot analysis):**
0 — intact control; B (burn); Lf (lactoferrin); AS (angiostatin); B+Lf (lactoferrin-treated burn injury); B+AS (angiostatin-treated burn injury). $P<0.05$ is considered statistically significant difference

destructive changes in the injured eye that are corrected by AS.

The uncovered response of the cornea to alkali burn damage, which is manifested, in particular, in increased production of the pro-angiogenic factor VEGF and enhanced apoptosis/autophagy levels, can be partially explained by the activation of macrophages. Although the cornea is considered an immune-privileged structure, dendritic cells and macrophages can migrate and become activated in response to infectious agent penetration or damage [39]. These patrolling immunocompetent cells have been shown to provide a rapid but "rough" response, remaining in the tissue for a long time. At the same time, cytotoxic T-cells are transformed into long-lived memory cells present in the cornea. An important observation was made by the authors [40], who showed that tissue fragments of the cornea are capable of activating monocytes/macrophages, inducing the recruitment of other immunocompetent cells and causing the release of various cytokines (CP-1, MIP-1α and MIP-1β). It has been proven that strong overactivation of macrophages by tissue debris can be one of the causes of the development of neovascularization and allograft rejection after corneal transplantation in humans. Our study established that the effect of AS K1-3 on J744.2 macrophage cell line suppresses the metabolic activity of these cells in nearly dose-dependent manner.

Findings of histopathological examination of the corneal tissue, which are depicted in Fig. 5, mainly confirm the results of molecular/cellular analysis suggesting pronounced anti-angiogenic and pleiotropic protective effects of the tested AS polypeptide.

Fig. 5 (panel A) shows a section of a fragment of the protein shell (sclera) covered by conjunctiva. The tissue architectonic of the eye corner has a typical structure. The sclera is built of orderly arranged plates of dense fibrous connective tissue, between which single fibroblast-like cells are interposed. It should be noted that blood capillaries are found in rare cases, microvessels are most often found on the border of the conjunctiva and sclera. In the conjunctiva that covers the sclera, a multi-layered non-keratinized epithelium is clearly visible. Beneath the epithelium layer, fibrous connective tissue is loosely arranged, in which amorphous substance is dominated. There is also a small number of thin bundles of collagen fibers. In the observed area, both resident fibroblast-like cells and non-resident cells (lymphocytes and plasma cells, macrophages, and single tissue basophils) are also found in limited cases.

Histological analysis of tissue sections from chemically burned rabbit cornea

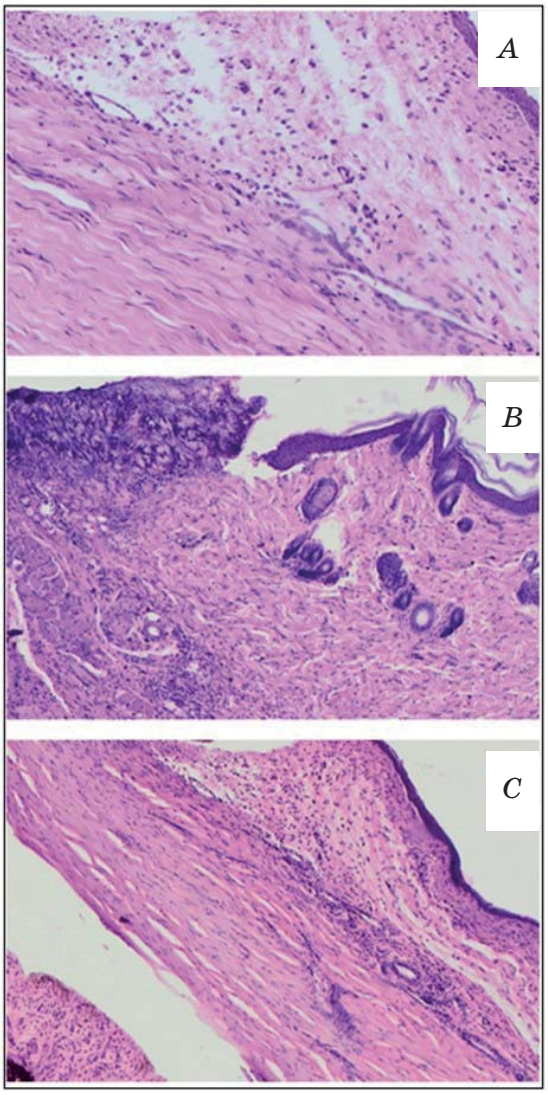

Fig. 5. **Microphotographs of rabbit cornea sections:**
A — Control group, *B* — Burn group, *C* — Burn + AS K1-3-treated group (hematoxylin/eosin staining, magnification × 200)

demonstrate the obvious signs of necrosis of the inner edge of the conjunctiva and the adjacent peripheral surface of the cornea (Fig. 5, panel B). Fibrinous-necrotic masses form a dense scar, which is located on the surface of the tissue defect. Directly under the scar, swelling and abundant inflammatory infiltration of the underlying connective tissue are observed. There is increased number of cells, mainly leukocytes, in the adjacent areas of the sclera. It has been emphasized that there are no obvious signs of regeneration of connective tissue. An abundant number of thin-walled blood microvessels are present, which are oriented towards the surface of the wound defect. The edge of the conjunctival epithelium is drastically thickened but it shows no tendency for ingrowth between the necrotic and viable tissues.

In animals with corneal burns that received treatment in the form of angiostatin-containing eye drops for 14 days, pronounced regeneration of connective tissue is observed in the area of the tissue defect (Fig. 5, panel C). It is important to note that the wound defect is already filled with new connective tissue. A significant thickening of the conjunctival lamina propria at the site of the lesion, edema, and moderate leukocyte infiltration are seen. In the deeper layers, a limited number of blood vessels with larger diameter, which develop the signs of differentiation of the wall membranes, were detected. The basal connective tissue of the cornea was practically preserved and characterized by moderate cell infiltration. Fibroblasts were present in the regenerate, as well as leukocyte cells, and tissue basophils. There were signs of epithelial regeneration that were more pronounced on the side of the conjunctiva, which grows on the newly formed connective tissue. Thus, the results of histological analysis and morphological observations confirm are in parallel with the data of immunochemical assay that demonstrate the protective effect of AS K1-3 on corneal tissue regeneration after an alkaline burn and restriction of burned-induced neovascularization. It is possible that one of the subtle mechanisms, by which tested AS is able to modulate inflammatory processes in the cornea after chemical damage, is a moderate inhibitory effect on macrophages. Reduced extent of macrophage infiltration into the damaged tissue along with moderate decrease of their metabolic activity might prevent excessive production of pro-inflammatory cytokines and extensive detrimental events in the cornea during recovery after a burn impact.

Conclusions

Two tested proteins, AS K1-3 and Lf, can be used in therapeutic regimens that diminish levels of angiogenesis-, apoptosis-, and autophagy-related markers in the rabbit cornea injured by alkali burn. The effectiveness of the AS-based topical administration appeared to be greater than that of Lf in inhibiting apoptosis and autophagy extent. Multitargeted anti-angiogenic and anti-inflammatory effects of AS may contribute to safe tissue recovery and restoration of corneal transparency.

Conflicts of interest

Authors declare no conflict of interest.

Authors' contribution

V.B. — laboratory research, data collection & analysis, writing original manuscript, funding; N.G. — histology research, conceptualization, project administration, manuscript editing; I.G. — animal model, validation, visualization, manuscript editing; C.A.A. — development of the theoretical framework, analytic calculations, manuscript review & editing.

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ЗАХИСНА ДІЯ АНГІОСТАТИНУ K1-3 ТА ЛАКТОФЕРИНУ В РОГІВЦІ КРОЛІВ ЗА УМОВ ПОШКОДЖЕННЯ ЛУЖНИМ ОПІКОМ: ПОРІВНЯЛЬНЕ ДОСЛІДЖЕННЯ

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Пошкодження рогівки асоціюється з гіпоксія-індукованою неоваскуляризацією, яка порушує оптичну прозорість рогівки, що може призводити до погіршення зору та сліпоти. Терапія пошкоджень рогівки як нагальна медична проблема потребує невідкладного вирішення. Лактоферин (Lf) та ангіостатини (AS) є природними протизапальними та антиангіогенними протеїнами, які відіграють важливу роль у нормальному функціонуванні ока і можуть використовуватися як протекторні препарати для лікування захворювань рогівки.

Мета. Оцінити та порівняти вплив фрагмента плазміногену AS K1-3 та Lf (як референтного препарату) на рівень основних маркерів ангіогенезу, апоптозу та аутофагії в рогівці за умов лужного опіку. Як окреме завдання було поставлено вивчити вплив AS на метаболічну активність макрофагів.

Матеріали та методи. Експериментальна модель опіку рогівки була створена нанесенням 1 н. розчину NaOH на поверхню ока кролика. Одна група тварин з пошкодженою рогівкою ока отримувала AS, який застосовували у вигляді очних крапель (1 мкМ розчин) щоденно протягом 14 днів після травми, інша група отримувала еквімолярний розчин Lf. Рівень протеїнів-маркерів ангіогенезу (VEGF), апоптозу (каспаза-3) і аутофагії (беклін-1) в лізатах рогівки визначали вестерн-блотом. Гістологічний аналіз проводили фарбуванням зрізів рогівки гематоксиліном/еозином з наступною візуалізацією за допомогою світлової мікроскопії. Вплив AS в діапазоні концентрацій 10 200 нМ на активність фагоцитарних клітин оцінювали з використанням макрофагів миші лінії клітин J744.2 за допомогою тесту з нітросинім тетразолієм (НСТ). Результати кількісних вимірювань аналізували за допомогою *U*-критерію Манна-Уітні. Різниця між середніми значеннями групи вважалася статистично достовірною при $P < 0{,}05$.

Результати. У тканині рогівки, пошкодженою лужним опіком, спостерігалося різке підвищення рівнів маркерних протеїнів, асоційованих з ангіогенезом, апоптозом і аутофагією. Застосування AS або Lf зменшило спричинену опіком надмірну експресію VEGF, каспази-3 та беклін-1. Важливо зазначити, що AS, крім анти-VEGF активності, більш ефективно пригнічує апоптоз і аутофагію через зниження рівня відповідних маркерів практично до контрольного рівня, у порівнянні з Lf. Гістохімічні дослідження виявили характерні ознаки фіброзно-некротичних змін у пошкодженій рогівці, які супроводжувалися надлишковою запальною інфільтрацією та інтенсивною неоваскуляризацією. Внесення AS до ока з опіковою травмою рогівки значно покращувало гістопатологічні зміни, про що свідчить підсилена регенерація епітелію, зменшення надмірної неоваскуляризації та уповільнення інфільтрації лейкоцитів. Ці спостереження узгоджуються з даними НСТ-тесту, які свідчать про те, що AS у концентраціях >50 нМ помірно пригнічує метаболічну активність макрофагів (до 30% у порівнянні з інтактними клітинами, $P < 0{,}05$).

Висновки. Отримані результати вказують на те, що AS може слугувати ефективним лікувальним засобом для корекції патологічного стану рогівки, спричиненого лужним опіком. Ефективність коригувальної дії AS є порівняно вищою за ефекти відомого протекторного протеїну Lf.

Ключові слова: кринглі плазміногену, ангіостатин, лактоферин, лужний опік, пошкодження рогівки, макрофаги, VEGF, каспаза-3, беклін-1.

активність *L. officinalis* вивчали за допомогою когерентного монохроматичного лазерного світла низької інтенсивності із заданими спектральними та інтенсивними характеристиками. В експерименті використовувалися водні колоїдні розчини наночастинок біогенних металів, таких як FeNPs, MgNPs і AgNPs, отримані методом об'ємного електроіскрового диспергування металів у рідині.

Результати. Оброблення інокулюму *L. officinalis* колоїдними розчинами наночастинок усіх використаних металів посилює ріст на 31–54%, а опромінення інокулюму гриба лазерним світлом у середовищі з наночастинками знижує ростову активність міцелію на 14,4–22,6%. Усі наночастинки металів пригнічували біосинтез позаклітинних полісахаридів, тоді як оброблення посіву колоїдними розчинами FeNPs та MgNPs стимулювало синтез ендополісахаридів. Водночас опромінення лазерним світлом у присутності AgNPs збільшувало кількість ендополісахаридів, тоді як FeNPs та MgNPs дещо пригнічувало їх синтез. Оброблення посівного матеріалу колоїдними розчинами металів і лазерним випромінюванням впливало на кількість загальних фенольних сполук (ТРС) у міцеліальній масі. Найвищі значення ТРС зафіксовано у етанольних екстрактах міцеліальної маси з AgNPs та опромінених лазерним світлом становлять 97,31±3,7 мг еквівалента галової кислоти на 1 г сухої маси (мг ЕГК/г). Найнижчі значення у розчинах метанолу з MgNPs без опромінення становили 58,12±3,2 мг ГКЕ/г сухої маси.

Висновки. Результати досліджень дають підстави розглядати наночастинки біогенних металів (AgNPs, FeNPs, MgNPs) та низькоінтенсивне лазерне світло як перспективний регулятор біосинтетичної активності *L. officinalis* у біотехнології його культивування.

Ключові слова: лазер, міцелійна маса, полісахариди, загальні фенольні сполуки, антиоксидантна активність.