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THE LEVELS OF HYPOXIA- AND ANGIOGENESIS-RELATED REGULATORS AND MATRIX METALLOPROTEINASE 9 ACTIVITY IN TEAR FLUID OF PATIENTS WITH NON-PENETRATING OCULAR TRAUMAS

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Ключові слова: *слізна рідина, травма рогівки, D-димер, HIF-1α, ангіостатини, матриксна металопротеїназа 9*

Abstract. The levels of hypoxia- and angiogenesis-related regulators and matrix metalloproteinase 9 activity in tear fluid of patients with non-penetrating ocular traumas. Gavrylyak I.V., Grebin N.K., Bilous V.L., Korsa V.V., Zhaboedov D.G., Ağca C.A., Tykhomyrov A.O. *This article was focused on the evaluation of protein biomarkers related to thrombosis, hypoxia, angiogenesis, and tissue remodeling in tear fluid of patients with non-penetrating corneal trauma. 32 patients with non-penetrating corneal injures were enrolled in the study, the control group consisted of 15 healthy patients. Samples of tear fluid were collected from the patients and control volunteers with the use of a disposable end micropipette. Protein levels of D-dimer, hypoxia-inducible factor 1α (HIF-1α), angiostatins, and matrix metalloproteinase 9 (MMP-9) in tear fluids were determined by western blot analysis. Proteolytic activity values of MMP-9 were measured by gelatin zymography. Results of western blot and zymography assay were calculated by densitometry analysis and expressed as arbitrary units. Significant increase of D-dimer and HIF-1α levels in tear fluid of patients with injured cornea by 7.3 ($p<0.05$) and 56 ($p<0.001$) folds, respectively, was shown compared with control, indicating thrombotic events and hypoxia condition to be involved in pathogenesis of ocular trauma. Dramatically elevated levels/activity of MMP-9 enzyme (by 105 folds vs. control, $p<0.001$) suggest intense tissue remodeling and degradation of extracellular matrix in the damaged cornea. Up-regulation of angiostatin level, products of proteolytical cleavage of plasminogen, in tear fluid collected from patients with traumatic eye in comparison with healthy volunteers (by 7.3 folds, $p<0.05$), could represent an adaptive mechanism, which counteracts excessive hypoxia-induced neovascularization in injured cornea. It is summarized that there was a strong association between elevation of D-dimer, HIF-1α, angiostatins, and MMP-9 levels suggesting thrombosis- and hypoxia-mediated mechanisms triggering wound healing of injured cornea. The findings of this study are novel and provide a basis for further investigations of the reparation mechanisms during non-penetrating ocular trauma. Studied proteins of the tear fluid can serve as relevant biomarkers of corneal wound healing and are appropriate for diagnostic and prognostic purposes.*

Реферат. Вміст регуляторів гіпоксії та ангіогенезу та активність матриксної металопротеїнази 9 у слізній рідині пацієнтів з непроникаючими травмами ока. Гавриляк І.В., Гребінь Н.К., Білоус В.Л., Корса В.В., Жабоєдов Д.Г., Агджа Дж. А., Тихомиров А.О. *Метою представленої роботи було визначити в слізній рідині пацієнтів з непроникаючою травмою рогівки вміст білкових біомаркерів тромбозу, гіпоксії, ангіогенезу та ремоделювання тканин. До дослідження було залучено 32 пацієнти з непроникаючими ушкодженнями рогівки, контрольну групу склали 15 здорових пацієнтів. Зразки слізної рідини відбирали в пацієнтів та добровольців контрольної групи за допомогою мікропіпетки та одноразового наконечника. Рівні таких протеїнів, як D-димеру, індукованого гіпоксією фактора 1α (HIF-1α), ангіостатинів та матриксної металопротеїнази 9 (MMP-9), у слізній рідині визначали за допомогою вестерн-блот аналізу. Протеолітичну активність MMP-9 оцінювали за допомогою желатинової зимографії. Результати блотингу та зимографії обробляли за допомогою денситометричного аналізу та виражали в умовних одиницях. У слізній рідині пацієнтів з пошкодженою рогівкою виявлено значне підвищення рівнів D-димеру та HIF-1α відповідно у 7,3 ($p<0,05$) та 56 ($p<0,001$) разів порівняно з контролем, що вказує на розвиток тромботичних явищ та стану гіпоксії як провідних ланок патогенезу травми ока. Різко підвищений рівень/активність ензиму MMP-9 (у 105 разів порівняно з контролем, $p<0,001$) свідчить про інтенсивне ремоделювання тканин та деградацію позаклітинного матриксу в пошкодженій рогівці. Підвищення рівня ангіостатинів, які є продуктами протеолітичного розщеплення плазміногену, у сльозі, зібраній у пацієнтів з непроникаючими пораненнями рогівки порівняно зі здоровими добровольцями (у 7,3 раза, $p<0,05$), може становити адаптаційний механізм, необхідний для запобігання надмірній неоваскуляризації, спричиненій гіпоксією в пошкодженій рогівці. Отже, встановлено тісний зв'язок між підвищенням рівня D-димеру, HIF-1α, ангіостатинів та MMP-9, що свідчить про розвиток механізмів, опосередкованих тромбозом та гіпоксією, які є тригерами процесів загоєння пошкодженої рогівки. Уперше отримані результати представленої роботи створюють основу для подальших досліджень репаративних механізмів при непроникаючих травмах ока. Визначені протеїни слізної рідини можуть бути використані як релевантні біомаркери загоєння ран рогівки та є доцільними для використання в діагностичних та прогностичних цілях.*

The cornea is one of the main components of the refractive system of the eye, characterized by transparency and avascularity. Any damage to the corneal transparency will reduce visual acuity. Because the cornea is the most anterior part of the eye, it is extremely susceptible to injury. When any type of corneal injury occurs, a healthy cornea with enough regenerative capacity is capable to repair the structural defect and restore visual function. The type of corneal injury and the extent of damage dictate the intensity of the wound healing response [1].

Non-penetrating ocular trauma is a leading cause of visual impairment and impacts both the individual by affecting their quality of life and the community by causing loss of working capacity. There are two categories of the corneal non-penetrating injures, namely superficial (those not involving the Bowman layer) and deep (those that penetrate the Bowman layer but do not rupture the Descemet membrane). Corneal abrasions may result from foreign bodies, contact lenses, chemicals, fingernails, hair brushes, tree branches, and dust [2].

Tear fluid that washes the cornea and conjunctiva is a complex mixture of proteins, lipids, and metabolites with different structure and functionality. It has been recently reported that proteomic analysis

revealed more than 3,000 different proteins in tear fluid of healthy individuals [3]. Among them, some tear proteins have been proposed as appropriate markers of thrombosis, hypoxia-induced events, and angiogenesis, which are often developed in the damaged cornea and contribute to chronic healing of ocular superficial wounds. Chronic or prolonged hypoxia, which develops as a result of trauma, may disrupt cellular homeostasis, induce inflammation, change the composition of tear film, stimulate angiogenesis and corneal vascularization for compensating thrombosis-induced oxygen deprivation [4]. D-dimer is a final product of fibrin clot degradation by plasmin. The elevated level of D-dimer is believed to be a reliable marker of pathological coagulation that underlies the pathogenesis of most vascular pathologies [5]. Hypoxia-inducible factor 1α (HIF-1α) is a regulator of hypoxia-associated genes that act against hypoxia. For example, vascular endothelial growth factor (VEGF), which is also known to be the main pro-angiogenic factor in corneal neovascularization, is under regulation of the HIF-1α signaling pathway in the cornea [6]. It has been well-established that hypoxia induces matrix metalloproteinase-9 (MMP-9) expression via HIF-1α induction [4]. In turn, MMP-9 is responsible for degradation of extracellular matrix proteins and tissue remodeling. However, MMP-9 overexpression is positively associated with clinical levels of corneal ulceration, neovascularization and fibrosis [7]. Earlier, active forms of MMP-9 have been reported to be present in tears of severe ulcerative and ocular surface disorder patients and may serve as biomarkers of these diseases [8]. On the other hand, MMP-9 is responsible for producing angiostatins, which are known as by-products of proteolytic cleavage of precursor protein plasminogen [9]. Angiostatins are potent inhibitors of neovascularization that contribute to the restriction of reparative angiogenesis in chronic wounds [10].

Therefore, it would be reasonable to suggest that evaluation of biomarkers in tear fluid can provide valuable insights into the diagnosis, progression, or modulation of disease with or without pharmaceutical intervention, making the evaluation of ocular biomarkers a critical component of ophthalmic drug discovery and development. Thus, the aim of our study was to evaluate the levels of several protein markers of hypoxia, angiogenesis and tissue remodeling in tear fluid of patients with non-penetrating corneal trauma.

MATERIALS AND METHODS OF RESEARCH

This study was carried out in the duration for the period from December 2020 to February 2022 years. The study included 32 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. An informed written consent was obtained from the all participants. The local ethical committee of Bogomolets National Medical University approved the study (protocol No. 138, 10.11.20) and the research was conducted in accordance with the principles of bioethics set out in the WMA Declaration of Helsinki – "Ethical principles for medical research involving human subjects" and "Universal Declaration on Bioethics and Human Rights" (UNESCO).

Data collection included patient demographic data, occupation, mechanism and duration of injury, examination of patient's visual acuity (Table). Based on the examination of the patient on the slit lamp and the use of the method of corneal staining with fluorescein, the diagnosis of non-penetrating corneal damage was established. Additionally, the depth of damage was assessed using optically coherence tomography of the anterior segment of the eye. After staining with fluorescein, the corneal surface was imaged using a cobalt blue filter fitted to a slit-lamp microscope. After fluorescein staining of the cornea, an abrasion will appear yellow under normal light and green in cobalt blue light. Fluorescein staining of the cornea in case of traumatic injury allows estimating the area of damage, the depth of the injury, and the limits of abrasion (erosion). In the present study, we applied fluorescein staining and the blue light to detect damage to the cornea (Fig. 1).

Demographic and clinical characteristic of patients with corneal injuries

Characteristics	Values
Gender	
Male	**19 (59.4%)**
Female	**13 (40.6%),**
Age Mean±SD	**40.5±2.2**
Visual acuity at presentation	**From 0.06 to 0.8**
Nature of injury	
Work-related	**14(43.8%)**
Domestic	**18 (56.2%)**
Use of safety goggles (work-related injury)(n=14)	
Yes	**7(50%)**
No	**7(50%)**
Location of corneal injury	
Central (optic zone)	**11(34.4%)**
Paracentral	**21 (65.6%)**
Depth of injury	
Superficial	**18(43.8%)**
Deep	**14(43.8%)**
Corneal injury	
With foreign body	**5(15.6%)**
Without foreign body	**27(84.4%)**

According to the etiology of the injury, a mechanical factor prevailed: a blow from a metal foreign body, a child's finger, a tree branch, a leaf of a plant, cat scratch. The average duration of symptom onset to hospital presentation was 2.4±1.2 days. The commonest presenting symptoms were eye redness with pain, foreign body sensation, tearing, photophobia and visual loss in all participants. The control group consisted of 15 healthy patients.

Tear samples were obtained from the patients and control volunteers with the use of a disposable end micropipette. From inferior meniscus without instillation of anesthetic, tears were collected in sterile plastic Eppendorf tubes and stored at -20°C before laboratory examination. Total protein content in the tear fluid samples was determined spectrophotometrically by Stoscheck method [11]. Proteins of the tear fluids were separated by denaturing electrophoresis in 10% polyacrylamide gel (PAAG), loading 50 µg total protein per track. Then, the levels of HIF-1α, D-dimer, MMP-9, and angiostatins were measured by western blot analysis followed by enhanced chemiluminescence detection, processed by densitometric software and expressed as arbitrary units of the optical density of immunoreactive bands [12]. Active MMP-9 form was detected by gelatin zymography in the copolymer of 8% PAAG with gelatin (5 mg/mL) as described earlier [8]. The locations of active proteinase bands were determined by the presence of negative staining in a gel. The results of western blot were calculated by densitometric analysis and then processed statistically using Mann-Whitney U-test by "OriginPro" software (major version 9.0 SR2 Pro English) [13]. Values are represented as the mean ± SD. $P<0.05$ was considered as significant for all statistical analyses.

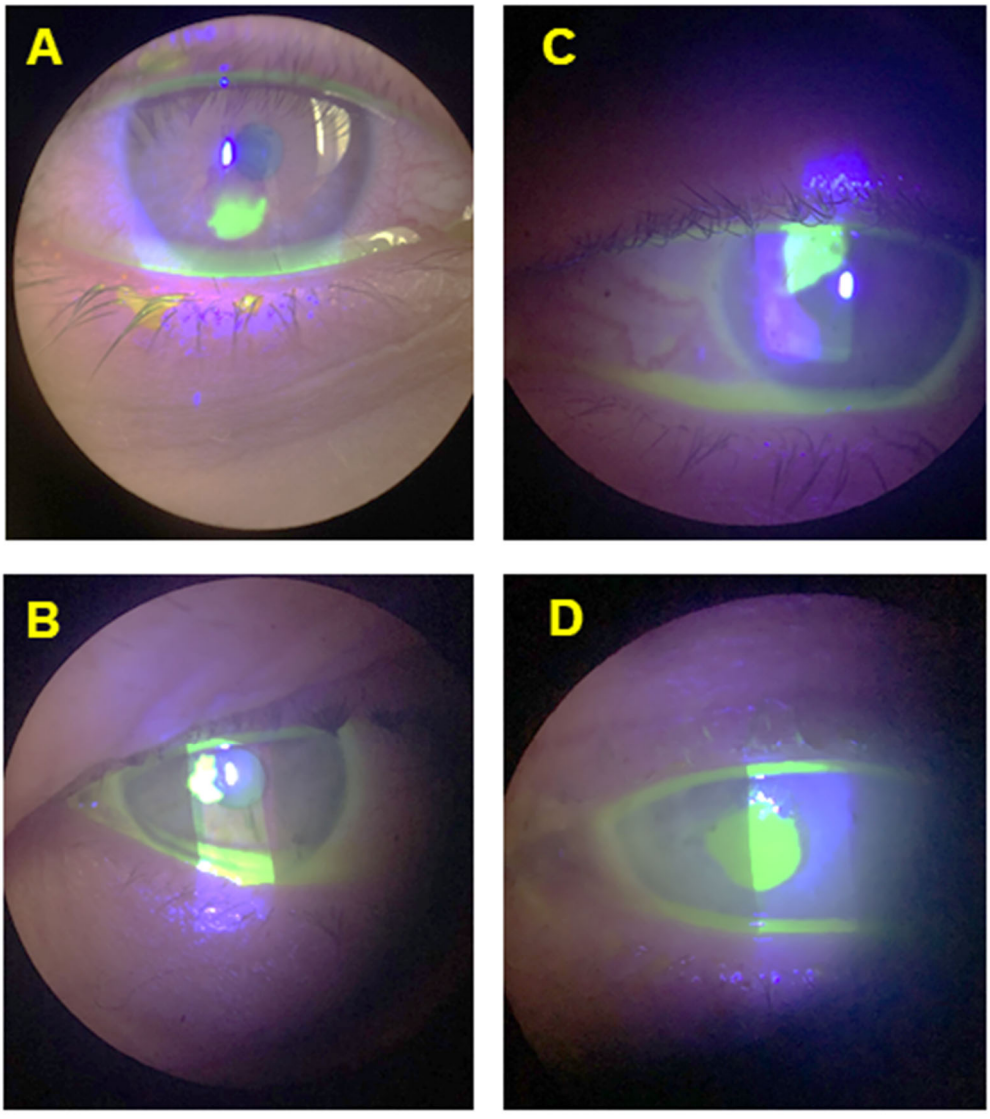
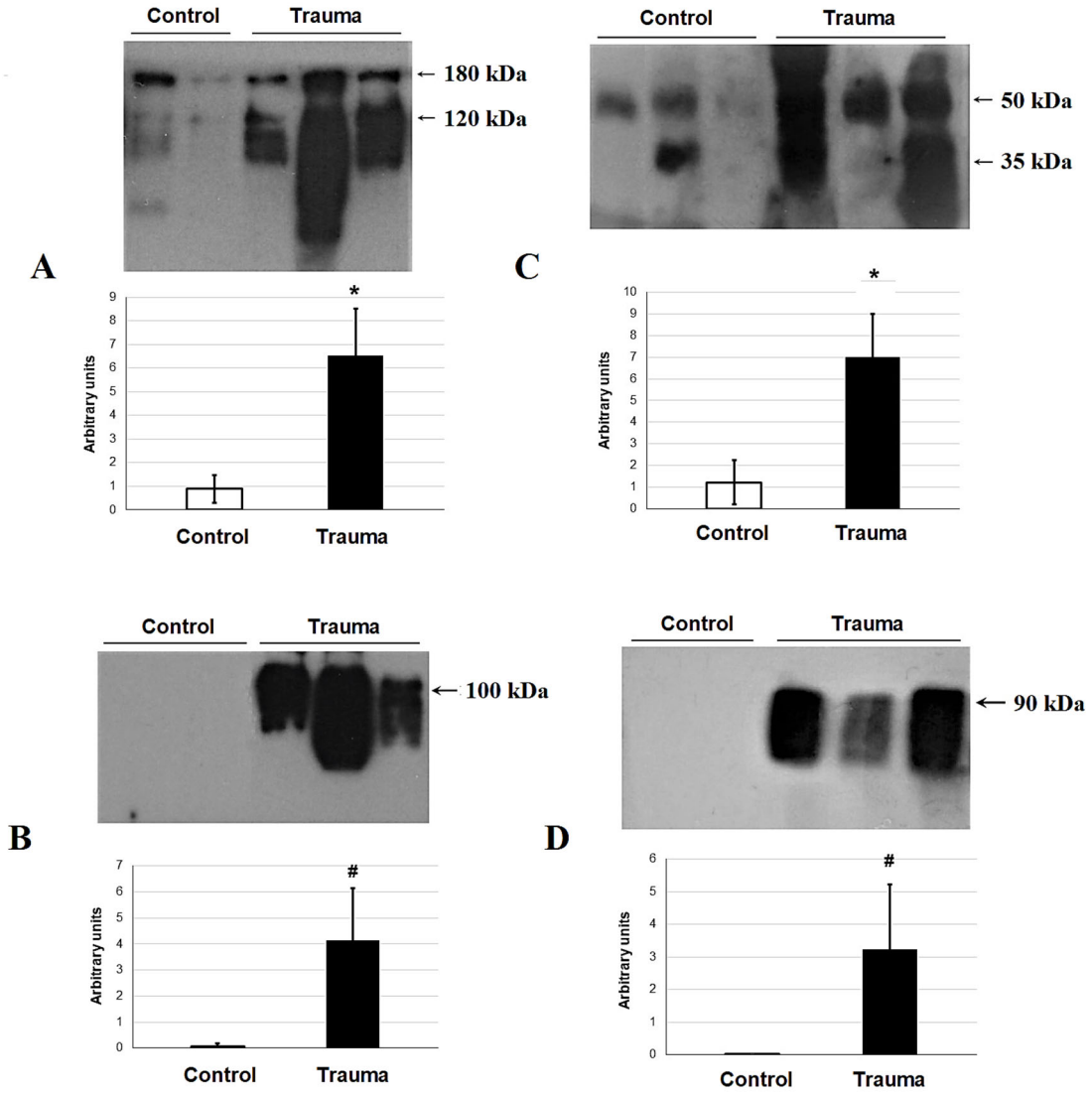


Fig. 1. Fluorescein staining of the wounded cornea: A – minor corneal abrasion, B – moderate corneal abrasion, C and D – severe corneal injury

RESULTS AND DISCUSSION

The results of western blot analysis of several hypoxia- and angiogenesis-associated regulators in lacrimal humor are presented in Fig. 2. Our findings suggested that there was significant elevation in D-dimer level in tear fluid from patients with non-penetrating ocular trauma compared with control (7.3 folds, $p<0.05$) (Fig. 2A). These data indicate that ocular trauma could be accompanied with thrombotic events in injured corneal tissue followed by development of hypoxic condition and up-regulation of transcription factor HIF-1α that activates upon sensing hypoxia. Western blot analysis demonstrated that the protein level of the key hypoxia-associated regulator HIF-1α was dramatically increased in tear fluid of patients with ocular trauma (by 56 folds, $p<0.001$) in comparison with control (Fig. 2B). HIF pathway has been observed in several pathological processes of the cornea, mainly angiogenesis, injury, and inflammation. Moreover, the HIF pathway also plays a protective role in cornea against injuries [4].

It is known that proteolytic enzymes, including components of the fibrinolytic system, are involved in regulating angiogenesis. Proteinases of plasminogen/plasmin system, besides fibrinolysis, are involved in numerous physiological and pathophysiological processes. For example, plasmin is involved in ECM remodeling through converting latent MMP pro-enzymes (pro-MMPs) into their proteolytically active forms, which are responsible for stimulating the production of cell growth factors and cleavage of proteins of ECM and basal membranes [14].



* – $p<0.05$ compared with Control; # – $p<0.001$ compared with Control.

Fig. 2. Representative blotograms of regulatory proteins in the tear fluid of healthy volunteers (Control) and patients with non-penetrating injuries of the conrea (Trauma): A – D-dimer, B – HIF-1α, C – angiostatins, D – MMP-9

On the other hand, plasminogen serves as a precursor of polypeptides, which are referred to as angiostatins due to their anti-endothelial activity and inhibition of angiogenesis [9].

As shown in Fig. 2C, two major angiostatin-like isoforms with the Mm values about 50 and 35 kDa, which are correspondent to angiostatins K1-4.5 and K1-3, were detected in the samples of tear fluid. Densitometry analysis of blotting showed 5.8-fold elevation of angiostatin level in tear fluid of studied patients compared with control volunteers ($p<0.05$).

Nevertheless, angiostatins appeared to be present in detectable quantities in tear fluids of healthy individuals. Earlier, it has been shown that angiostatins are formed constantly by corneal epithelium and involved in maintaining corneal avascularity and optical transparency through counteracting pro-angiogenic VEGF signaling and inhibiting excessive capillary growth during corneal wound and inflammation. Cysteine cathepsins, along with MMPs, have been found to cleave endogenous plasminogen and thus are responsible for angiostatin formation in the cornea [15]. In order to evaluate expression and proteolytic activity of MMP-9, we performed western blot and zymography assay, and obtained consistent results. Significant elevation of MMP-9 protein levels was revealed by immunoblot in tear fluid samples collected from patients with injured cornea as compared with the control group (by 105 folds, $p<0.001$) (Fig. 2D). In parallel, dramatically increased values of MMP-9 activity were detected in tear fluid of patients with corneal erosion (abrasion), while no detectable enzymatic MMP activity was observed in the tested samples collected from healthy eyes, as depicted in Fig. 3.

Tear fluid is a complex mixture of proteins, lipids, mucins, water and salts, and a recent study has identified >3,000 proteins with the help of proteomic analysis [3], making it less complex to perform (as a body fluid) than serum or plasma. Following this and other works [16, 17], it has been recently proposed the usefulness of tears as a source of diagnostic and predictive biomarkers. The cornea is an interesting organ for studying the mechanism of wound healing and interaction such as stromal-epithelial, stromal-endothelial due to its transparency and accessibility for manipulation and visualization [18]. Barrier protection, light refraction and ultraviolet light filtration are the main functions of the cornea to maintain normal vision. Because the cornea is the primary refractive surface of the eye, even minor changes in its structure lead to significant vision problems. The corneal epithelium is maintained in a complex balance that can be easily disturbed [l, 2].

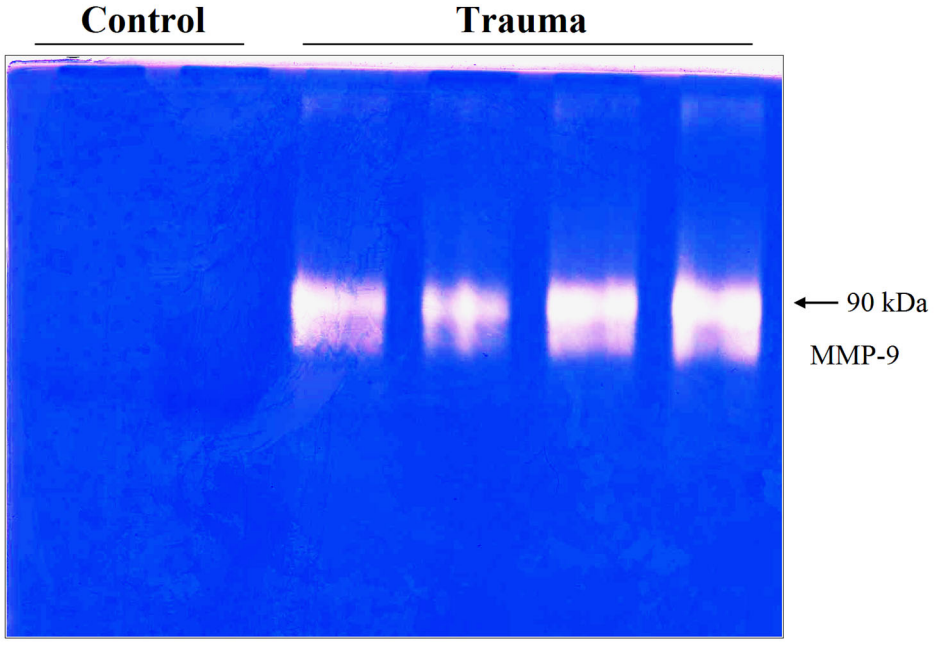


Fig. 3. Representative gelatin zymography of MMP-9 in the tear fluid of healthy volunteers (Control) and patients with non-penetrating injuries of the cornea (Trauma) (copolymer of 8% PAAG with gelatin, 5 mg/mL)

Corneal wound healing is a complex process consisting of three overlapping phases, including cell migration/inflammation, cell proliferation/differentiation, and matrix remodeling [1]. Although the cornea lacks blood vessels, which is necessary for providing its optical transparency, the surrounding

tissues including the limbus, sclera, and conjunctiva are relatively rich in vasculature, and the cornea is supplied by diffusion through the tear fluid and aqueous humor. Ulcerations and other degenerative processes in the wounded cornea may cause dramatic complications of ocular lesions, leading to difficulties in wound management and possible loss of vision. Thus, understanding pathophysiological processes that underlie corneal epithelial lesions or eye traumas is still an important unresolved task for applied ophthalmology [19].

In the case of corneal injury, there is no need to stop bleeding because the cornea is an avascular structure lacking of blood vessels. However, fibrin is deposited at the site of injury covering the surface of the exposed to provide supporting matrix together with fibronectin for the migration of epithelial cells. It has been shown that the concentration of fibrin increases in tear fluid in association with inflammation, and this fibrin may be deposited at the damaged ocular surface. Therefore, the fibrinolytic (or plasminogen/plasmin) system, which was first recognized for its role in the degradation of fibrin clots in the vasculature, has also been found to contribute to various biological processes outside of blood vessels. Fibrinolytic factors thus play an important role in biological defense of the cornea. Plasmin is a serine protease, which appears after the conversion of plasminogen by zymogen activators. It has been demonstrated that there is a direct correlation between plasmin activity in the tear fluid and corneal ulcerative processes [20]. In the present paper, evaluation of D-dimer level in the lacrimal fluid was performed in order to assess fibrinolytic activity in tears and the presence of an inflammatory reaction after injury. It is generally accepted that D-dimer is normally detected in trace amounts in the blood and some biological fluids, while is abundantly produced only after a clot has formed and is in the process of fibrinolysis. Moreover, D-dimer is responsible for the activation of inflammatory processes in injured tissues [21]. The results of our study indicate a strong association between enhanced levels of D-dimer, HIF-1α, MMP-9, and angiostatins that altogether suggests thrombosis/inflammatory- and hypoxia-associated mechanisms triggering wound healing processes in injured cornea. Hypoxia, which develops in the cornea after trauma, affects the cornea in many aspects, including impaired epithelial barrier function, corneal edema due to endothelial dysfunction and changes in stromal metabolism, and corneal stroma thinning. HIF-1α is a transcription factor that can be induced under hypoxic conditions and is thought to be the key protein regulator that contributes to adaptive changes caused by hypoxia in wounded cornea [4]. Numerous studies have demonstrated that HIF-1α participates in the regulation of angiogenesis to compensate oxygen deprivation in injured tissue through being a proximal regulator of VEGF and MMPs expression [22, 23]. Our results confirmed this paradigm, and it is obviously seen that the control group showed neither remarkable expression of MMP-9 protein nor its proteolytic activity in the tear fluid. In contrast, dramatic elevation of both protein MMP-9 levels and MMP-9-associated collagenolytic activity was shown in tear fluid of patients with ocular traumas (Fig. 3). Thus, the process of healing corneal wounds is characterized by cellular remodeling and changes in the composition of the protein tear in preparation for healing. This leads to increased production of proteolytic enzymes, including gelatinase B, or MMP-9, which destroy the damaged basement membrane of the epithelium. MMPs reduce cell adhesion and enhance cell migration. In the normal cornea, MMPs are responsible for the precise organization of collagen fibrils in the stroma of the cornea, which is essential for maintaining the purity of the cornea and the proper hydration of the stroma [24]. MMPs are also responsible for the fine regulation of angiogenesis in the healthy and injured cornea through forming angiostatins by limited proteolysis of plasminogen. In turn, angiostatins, which are constantly formed by corneal cells, play a vital role in maintaining optical transparency of cornea inhibiting corneal neovascularization and ischemia-induced vasculogenesis [25]. Sack et al. [26] have demonstrated a pivotal role of angiostatins as normal components of tear film to prevent neovascularization during overnight eye closure. Thus, angiostatins can play an essential role in preventing neovascularization in the hypoxic closed eye environment and may be up-regulated during inflammatory reactions. On the other hand, excessive increase in MMP activity can lead to abnormal degradation of the extracellular matrix, inhibition of reparative angiogenesis due to massive plasminogen degradation and the formation of abundant angiostatin amounts, which may prevent proper healing of corneal wounds [10].

Our study has some limitations. The first limitation is a relatively small sample size, however, group of patients in our study represents a relatively homogenous population. The second limitation addresses the necessity of observation of dynamic changes in the tested biomarkers during wider time frame of healing process. Finally, there is a need in elaborating optional treatments for traumatic cornea and characterizing treatment effects associated with benefits or adverse outcomes. Peculiar attention to the obtained results can be payed due to the reported data

on an increased incidence of ocular manifestation in patients with COVID-19, while patients with severe COVID-19 bronchopneumonia may develop acute corneal decompensation due to viral endotheliitis [27, 28].

Thus, our data demonstrate for the first time that quantification of protein biomarkers of hypoxia/angiogenesis together with MMP activity in the tear fluid may be useful as a basis for the non-invasive diagnosis and proper evaluation of corneal wound damage and repair.

CONCLUSIONS

1. A strong association between up-regulation of D-dimer, hypoxia-inducible factor 1α, angiostatins, and matrix metalloproteinase-9 levels in tear fluid of patients with ocular traumatic erosion suggests development of thrombosis- and hypoxia-associated mechanisms, which trigger wound healing processes in the injured cornea.
2. Elevated levels of D-dimer in patients' tear fluid indicates increased fibrinolytic activity after corneal damage, which may contribute to further inflammation and chronicity of the process.
3. Immunochemical analysis of hypoxia/angiogenesis-associated markers together with gelatin zymography of matrix metalloproteinase activity in tear fluid can be promising as a basis for the non-invasive diagnosis of corneal wound healing and choosing adequate scheme of pharmacological treatment of ocular non-penetrating traumas.

Contributors:

Gavrylyak I.V. – methodology, validation, investigation, formal analysis, writing – original draft;

Greben N.K. – methodology, resources;

Bilous V.L., Korsa V. V. – investigation, validation;

Zhaboiedov D.G. – conceptualization, writing – original draft;

Ağca C.A. – visualization, resources, writing – review & editing;

Tykhomyrov A.O. – methodology, validation, writing – original draft.

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