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PROTEIN MARKERS OF HYPOXIA AND ANGIOGENESIS IN TEAR FLUID OF PATIENTS WITH TRAUMATIC CORNEAL INJURY

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The cornea is an avascular, transparent tissue that is essential for visual function. Penetrating or perforating ocular injuries as well as infectious keratitis can cause corneal ulceration, scarring, and, in more severe cases, partial or complete vision loss. Since the vast majority of patients with traumatic corneal injuries are people of working age, this causes an important medical and social burden [1]. Numerous modulators coming from tears, inflammatory cells, extracellular matrix (ECM), neural cells, corneal epithelial cells, or stromal fibroblasts can regulate the complex wound healing process [2]. Biomarker evaluation in tear fluid may provide valuable insight into diagnosis of disease, progression or modulation of disease with and without pharmaceutical intervention, thus making ocular biomarker assessment critical component of ophthalmic drug discovery and development. It was currently reported that the tear proteome consists of about 1800–2000 proteins [3]. Among them, some specific proteins can be used as relevant markers of hypoxia and angiogenesis, which are often developed in the injured cornea and contribute to chronic corneal wound healing. Thus, the aim of our study was to evaluate tear levels of some protein endpoints that can reflect intensities of hypoxia, angiogenesis and tissue remodeling in wounded cornea.

Methods. We examined 21 patients (21 eyes) with nonpenetrating corneal injuries, which were observed in the clinic of "Alexander Clinical Hospital" that is a clinical base of the Bogomolets National Medical University in Kyiv for the period 2020–2021. The study was approved by the local ethical committee of Bogomolets National Medical University and the research is complied with Helsinki Declaration. Demographic and clinical characteristic of the patients are presented in the Table 1. Patients underwent standard ophthalmological examination including previous history and ocular symptoms, visual acuity test, complete anterior and posterior eye segments examination using slit lamp biomicroscopy, evaluation of corneal staining with fluorescein, ophthalmoscopy. Healthy volunteers ($n = 10$) served as a control.

Tear fluid was collected from patients and control volunteers with the use of a disposable tip micropipette. From the lower arch of the conjunctiva without instillation of anesthetic, tears were collected in a sterile plastic Eppendorf tube and frozen at −20 °C before laboratory examination. Proteins of tear fluids were separated by SDS-PAGE (loading 50 μg total protein per track). Then, levels of hypoxia inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF), and angiostatins were measured by western blot. Active MMP-9 levels were evaluated by gelatin zymography. The results of blot and zymography assays were processed by densitometric software and then analyzed statistically with the use of Mann-Whitney *U*-test. Values are represented as the mean ± SD. $P < 0.05$ was regarded as significant for all statistical analyses.

Results. Elevated HIF-1α ($P < 0.001$) and angiostatins ($P < 0.05$) levels were revealed by western blot in tear fluid samples collected from patients with injured cornea in comparison with the control group (Fig. 1). It is noteworthy that extremely low amounts of VEGF were detected in tear fluid from injured eyes, in spite of abundance of its transcription inducer HIF-1α.

Dramatically increased levels of active MMP-9 were found in the tear fluids of patients with corneal wounds, while no significant collagenolytic activity was observed in tears from healthy eyes (Fig. 2). There is a strong correlation between extent of corneal lesions and changes in markers expression.

Table. **Demographic and clinical characteristic of patients with corneal injuries**

Characteristics	Values
Gender Male Female	 13 (61.9%) 8 (38.1%),
Age	43.5 ± 2.2
Visual acuity	From 0.08 to 0.9
Location of corneal damage Central (optic zone) Paracentral	 9 (42.9%) 12 (57.1%)
Depth of injury Superficial Deep	 17 (81%) 4 (19%)
Pericorneal injection Mild Severe	 11 (52.4%) 10 (47.6%)

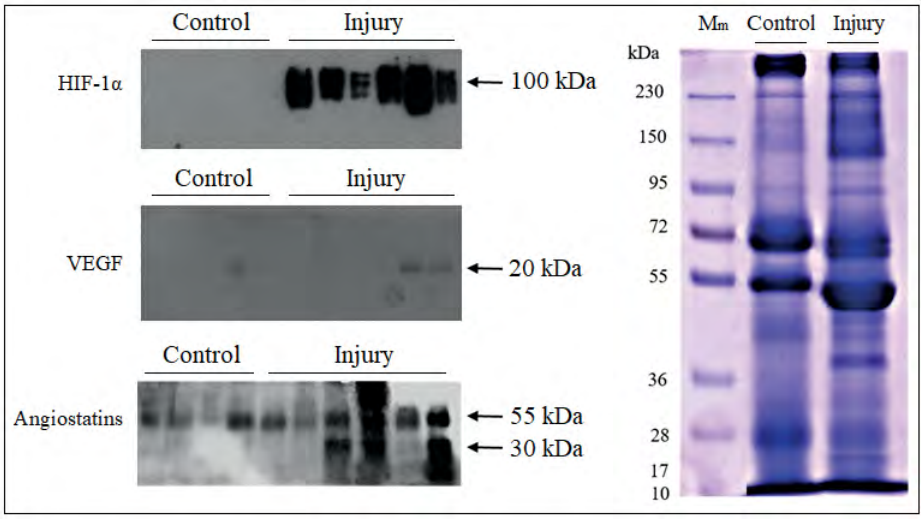

Fig. 1. **Representative blotograms of marker proteins in tear fluid of healthy volunteers and patients with ocular trauma**
right — Coomassie-stained SDS-PAGE of tear fluid proteins.

Discussion. Corneal function is vital for normal vision and includes barrier protection, light refraction, and ultraviolet light filtration. Because the cornea is the main refractive surface of the eye, even minor changes in its contour lead to significant vision problems. The corneal epithelium is maintained in a complex balance that can be easily disrupted. Hypoxia, which is developed in wounded cornea, affects the cornea in multiple aspects, including disturbance of the epithelium barrier function, corneal edema due to endothelial dysfunction and metabolism changes in the stroma, and thinning of corneal stroma. HIF-1α is the key protein facilitating hypoxia-induced changes is injured cornea [4]. VEGF is an angiogenic regulator that has been identified as an important pathophysiologic mediator in the development and maintenance of angiogenesis seen in neovascular eye disease, while angiostatins, plasminogen-derived fragments, are known to counteract VEGF-induced pro-angiogenic signaling and impede new vessel growth [5, 6]. Low VEGF levels in tear fluids from traumatic eyes can be explained by retention of VEGF in corneal tissue, where it governs pro-angiogenic signaling, or down-regulation of its expression by enhanced angiostatins [7]. Thus, corneal wound healing process is characterized by cellular remodeling and changes in protein tear composition in preparation for healing. This results in an increased production of proteolytic enzymes (including MMP-9), which degrade the damaged epithelial basement membrane. MMPs decrease cellular adhesion and help enhance cellular migration. In normal cornea, MMPs are responsible for the precise organization

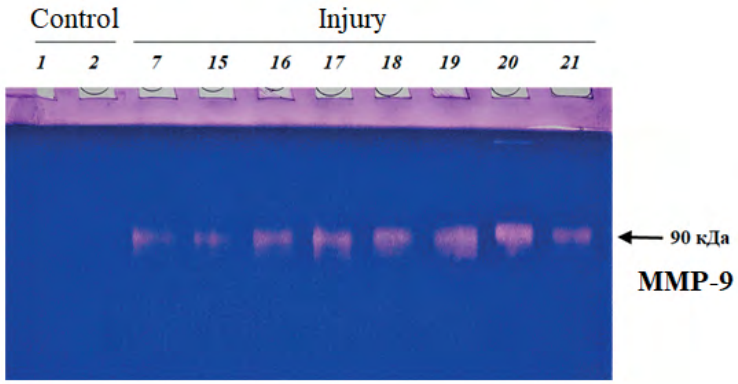

Fig. 2. **Representative gelatin zymography of matrix metalloproteinase -9 (MMP-9) in tear fluid of healthy volunteers and patients with corneal trauma**

of collagen fibrils within the corneal stroma, which is imperative to maintaining corneal clarity and appropriate stromal hydration. However, an excessive increase in MMPs activity may result in abnormal degradation of the extracellular matrix, inhibition of reparative angiogenesis through plasminogen degradation and angiostatin formation that hinder proper corneal wound healing [8]. Thus, our data demonstrate that tear levels of hypoxia/angiogenesis markers, along with MMP activity, can be helpful as ocular biomarkers to diagnose and assess corneal wound healing. In the future, the diagnostic power of these and other plausible markers in tear fluid should be verified in the different validation sets.

Conclusions. Tear levels of HIF-1α and angiostatin as well as MMP-9 activity could represent valuable biomarkers of corneal injury severity in traumatic eye.

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The authors state that they have no conflict of interest.

REFERENCES

1. *Tamhane M., Cabrera-Ghayouri S., Abelian G., Viswanath V.* Review of biomarkers in ocular matrices: challenges and opportunities. *Pharm. Res.* 2019, 36, 1–35. https://doi.org/10.1007/s11095-019-2569-8
2. *Wilson S. E.* Corneal wound healing. *Exp. Eye Res.* 2020, 197, 1–21. https://doi.org/10.1016/j.exer.2020.108089
3. *Ma J. Y. W., Sze Y.H., Bian J. F., Lam T. C.* Critical role of mass spectrometry proteomics in tear biomarker discovery for multifactorial ocular diseases (Review). *Int. J. Mol. Med.* 2021, 47(5), 1–15. https://doi.org/10.3892/ijmm.2021.4916
4. *Pang K., Lennikov A., Yang M.* Hypoxia adaptation in the cornea: current animal models and underlying mechanisms. *Animal Model Exp. Med.* 2021, 4(4), 300–310. https://doi.org/10.1002/ame2.12192
5. *Bukowiecki A., Hos D., Cursiefen C., Eming S.A.* Wound-healing studies in cornea and skin: parallels, differences and opportunities. *Int. J. Mol. Sci.* 2017, 18(6), 1–24. https://doi.org/10.3390/ijms18061257
6. *Ambati B. K., Joussen A. M., Ambati J., Moromizato Y., Guha C., Javaherian K., Gillies S., O'Reilly M. S., Adamis A. P.* Angiostatin inhibits and regresses corneal neovascularization. *Arch. Ophthalmol.* 2002, 120 (8), 1063–1068. https://doi.org/10.1001/archopht.120.8.1063
7. *Chen Y.H., Wu H.L., Chen C.K., Huang Y.H., Yang B.C., Wu L.W.* Angiostatin antagonizes the action of VEGF-A in human endothelial cells via two distinct pathways. *Biochem. Biophys. Res. Commun.* 2003, 310(3), 804–810. https://doi.org/10.1016/j.bbrc.2003.09.081
8. *Singh A., Maurya O. P., Jagannadhan M. V., Patel A.* Matrix metalloproteinases (MMP-2 and MMP-9) activity in corneal ulcer and ocular surface disorders determined by gelatin zymography. *J. Ocul. Biol. Dis. Infor.* 2012, 5(2), 31–35. https://doi.org/10.1007/s12177-012-9096-8