

CONFOCAL MICROSCOPY IN OCULAR SURFACE DISEASE

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✧ Confocal microscopy is a modern clinical method, which provides in real-time mode a non-invasive possibility for *in vivo* imaging of the cornea, limbus, and conjunctiva. In several ocular surface disorders, this method could be applied for diagnostic purposes, as well as for disease monitoring and treatment efficacy evaluation. In present article, we discuss main changes observed by confocal microscopy in patients with dry eye, and propose our examination algorithm of ocular surface investigation in dry eye disease.

✧ **Keywords:** confocal microscopy; ocular surface tissues; dry eye.

ВОЗМОЖНОСТИ КОНФОКАЛЬНОЙ МИКРОСКОПИИ ПРИ ЗАБОЛЕВАНИЯХ ГЛАЗНОЙ ПОВЕРХНОСТИ

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✧ Конфокальная микроскопия — современный метод исследования, позволяющий в режиме реального времени оценить неинвазивно *in vivo* структуру роговицы, лимба и конъюнктивы. При различных заболеваниях тканей глазной поверхности метод может быть использован как с диагностической целью, так и с целью мониторинга течения заболевания и оценки эффективности лечения. В рамках данной статьи рассмотрены основные изменения, наблюдаемые при конфокальной микроскопии у пациентов с синдромом сухого глаза (ССГ), и приведён составленный авторами алгоритм исследования тканей глазной поверхности при ССГ.

✧ **Ключевые слова:** конфокальная микроскопия; ткани глазной поверхности; синдром сухого глаза.

Confocal microscopy is a modern method permitting real-time *in vivo* assessment of the cellular structure of the cornea, limbus, and conjunctiva. High-resolution confocal microscopy measures the thickness of each layer and evaluates the size, shape, and number of cells, including cells of the epithelium, stroma, and corneal endothelium [1, 2, 10, 12, 13, 17, 18, 20].

In 1955, Marvin Minsky invented the confocal microscope, which was used for imaging brain cells and *in vivo* investigation of synaptic transmission in neurons. During 1980–1990, the method of confocal microscopy was studied in detail [2, 15]. The basic principle of confocal imaging is that it uses point illumination, which allows both the illuminator and objective to focus on one point of the tissue. Thus,

we get a high-resolution image, but the field of view is limited [2, 15].

In this study, we used a confocal laser scanning microscope [Rostock Cornea Module (RCM)] of the Heidelberg Retina Tomograph (HRT3, Heidelberg Engineering GmbH, Germany). The HRT3-RCM system has a helium–neon diode laser with a wavelength of 670 nm and provides high-resolution (up to 1 $\mu\text{m}/\text{pixel}$) imaging. We performed contact scanning under epibulbar anesthesia using disposable sterile caps made of polymethyl methacrylate; the system obtains diagonal scans of all corneal layers and scans through moderately opaque tissue (corneal clouding or corneal edema).

Confocal microscopy ushered in a new era in the diagnosis of corneal disorders and other diseases of the ocular surface. The state of ocular surface tissues has attracted the attention of many physicians. The ocular surface tissues include the anterior epithelium of the cornea, limbus, and conjunctiva. Dry eye syndrome (DES) is one of the most common diseases of the ocular surface and is estimated to affect 5%–35% of the population in developed countries [5]. The Dry Eye Workshop defines DES as a disorder of the tear film due to tear deficiency or excessive tear evaporation, causing damage to the ocular surface. DES is associated with increased osmolarity of the tear film and inflammation in the ocular surface tissues [5]. Confocal microscopy can help obtaining a detailed understanding of the pathophysiological mechanisms of DES, what is important for choosing a correct treatment strategy and assessing its efficacy. Moreover, the capture of subclinical manifestations may ensure early detection of the disease.

Corneal epithelium is a non-keratinized, stratified squamous epithelium and consists of three layers: superficial, medium (wing cells), and a layer of basal cells. The thickness of corneal epithelium is approximately 50 μm . Superficial cells are polygonal with distinct borders, bright nucleus, and dark cytoplasm; their diameter is approximately 40–50 μm and thickness is approximately 4–5 μm (Figure 1a). The shape and size of wing cells varies; their diameter usually reaches 30–45 μm and thickness is approximately 10 μm . The density of these cells is approximately 5000 cells/ mm^2 (Figure 1b). Basal cells have distinct bright borders and no visible nucleus; their diameter is approximately 10–15 μm , density varies between 3600 and 8996 cells/ mm^2 (Figure 1c), and they exhibit mitotic activity. Basal cells have smaller diameter and higher density compared to those of wing cells [1, 2].

Increased reflectivity of epithelial cells indicates metabolic activity decline and the beginning of desquamation [3].

Confocal microscopy identifies abnormal morphological changes in epithelial cells and assesses their density in different layers. Some studies have reported decreased density of superficial and medium epithelial cells in patients with DES [3, 6, 21]. Erdélyi et al. revealed that the density of superficial and medium epithelial cells in patients with DES was 702–984 cells/ mm^2 and 4612–5444 cells/ mm^2 , respectively, whereas density in the control group was 1026–1398 cells/ mm^2 and 5437–6171 cells/ mm^2 , respectively [6].

Therefore, individuals with DES are characterized by a decreased density of superficial and medium

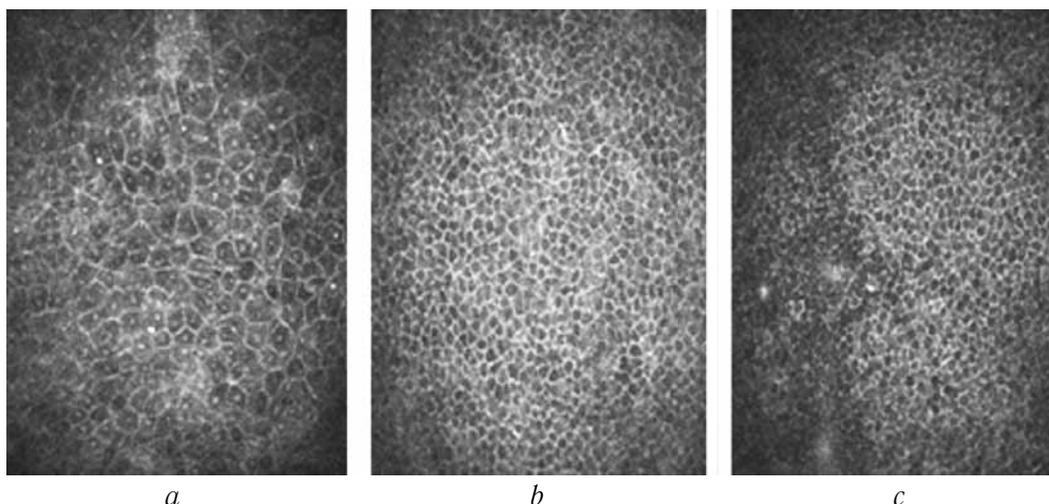


Fig. 1. Confocal microscopy of the corneal epithelium (HRT3-RCM): *a* – superficial layer, *b* – an intermediate layer, *c* – basal layer

Рис. 1. Конфокальная микроскопия эпителия роговицы (HRT3-RCM): *a* — поверхностный слой, *b* — промежуточный слой эпителия, *c* — базальный слой эпителия

epithelial cells. The data on basal cell density are controversial. Villani et al. found that DES is associated with increased basal cell density [22]. Moreover, Zhang et al. demonstrated a significant decrease in basal cell density in patients with DES compared to that in controls (9234 ± 1365 cells/mm² in mild DES and 8634 ± 998 cells/mm² in more severe DES) [28]. The Bowman and the Descemet membranes are transparent structures that do not reflect light; thus, they cannot be visualized via confocal microscopy [1].

The subepithelial nerve plexus is located under the Bowman membrane. Its nerve fibers perforate the membrane and form a sub-basal nerve plexus at the basal cell level. The fibers of this plexus are superficial and provide innervation to the basal epithelial layer [1, 2, 3, 8, 17].

The main criteria for the estimation of nerve fibers are density, width, tortuosity, reflectivity, and branching.

According to Niederer et al., the density of sub-basal nerves decreases by 0.9% each year [16]. Decreased density of sub-basal nerves is also observed in patients with diabetes mellitus, infectious keratitis, after laser-assisted in situ keratomileusis (LASIK), and after photorefractive keratectomy [4, 7, 8]. Data on the density of sub-basal nerves in DES are controversial. Hoşal et al. and Tuominen et al. observed no changes in sub-basal nerve density in patients with DES [9, 21]. Zhang et al. demonstrated increased sub-basal nerve density in patients with DES compared to that in healthy controls (1423.5 ± 609.5 $\mu\text{m}/\text{mm}^2$ and 1315.7 ± 664.7 $\mu\text{m}/\text{mm}^2$, respectively). Such controversy may be explained by the enrollment of patients with different stages and severity of DES [27]. However, several studies suggest that DES is associated with increased tortuosity and reflectivity of nerve fibers in the subepithelial nerve plexus and the sub-basal nerve plexus (Figure 3) [16, 17, 27].

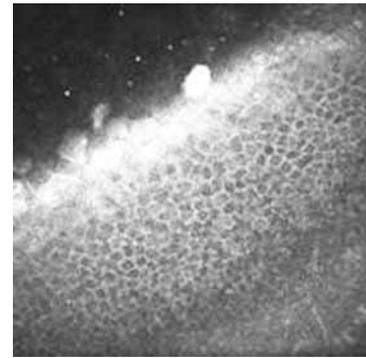


Fig. 2. Desquamation of the corneal epithelium (oblique cut, HRT 3-RCM)

Рис. 2. Десквамация эпителия роговицы (косой срез, HRT3-RCM)

DES is associated with an increased number of beaded fibers in the sub-basal nerve plexus, which indicates either damage or increased metabolic activity [3, 21, 22].

Hyperreflective dendritic cells [Langerhans cells (LCs)] can be visualized at the basal epithelial cell level and the Bowman membrane. Their density is 34 ± 3 cells/mm² at the center and 98 ± 8 cells/mm² at the periphery. LCs are antigen-presenting dendritic cells of the corneal epithelium and represent an important component of the immune system as they can limit inflammation by the activation of T-lymphocytes and other immune cells. The density of LC decreases from the periphery to the center. LCs are predominantly located near the fibers of the sub-basal nerve plexus [1, 4, 11].

According to Lin et al., compared to that of controls, patients with DES tend to have higher LC density (34.9 ± 5.7 cells/mm² and 89.8 ± 10.8 cells/mm², respectively). In addition, increase in the numbers of LC processes may indicate their activation. Normally, LCs with multiple processes are found at the periphery, whereas DES is characterized by an increased number of such cells at the center (Figure 4) [4, 11].

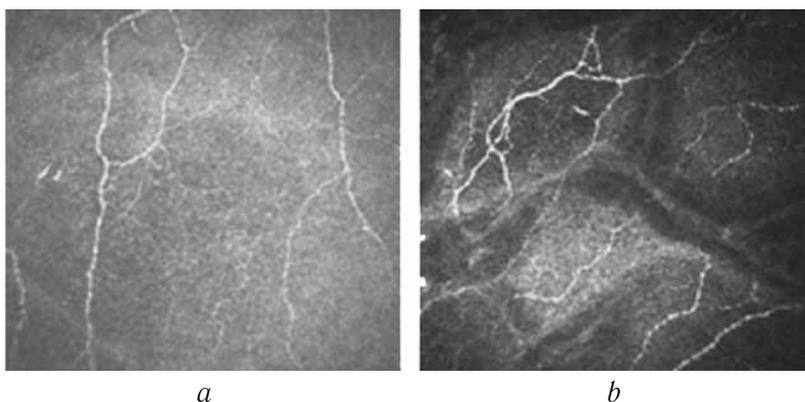


Fig. 3. Subbasal nerve plexus (HRT3-RCM): *a* – in healthy person, *b* – in dry eye disease patient

Рис. 3. Суббазальные нервные сплетения (HRT3-RCM): *a* — у здорового пациента, *b* — у пациента с синдромом сухого глаза

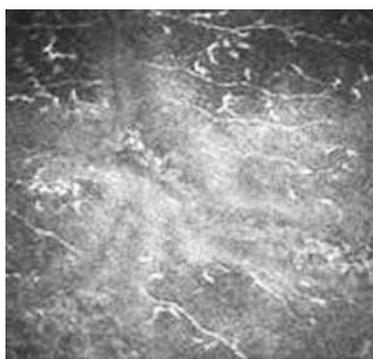


Fig. 4. Multiple dendritic cells in the optic center in a dry eye disease patient (HRT3-RCM)

Рис. 4. Множественные дендритические клетки в оптическом центре у пациента с синдромом сухого глаза (HRT3-RCM)

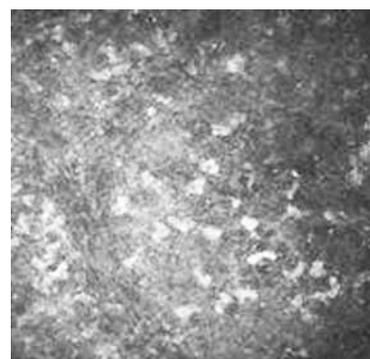


Fig. 6. Hyperreflective intercellular micro-inclusions, "activated" keratocytes (HRT3-RCM)

Рис. 6. Гиперрефлективные межклеточные микро-включения, «активированные» кератоциты (HRT3-RCM)

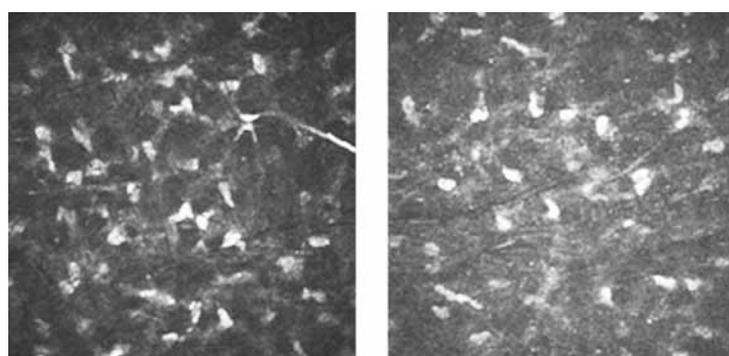


Fig. 5. Confocal microscopy of the corneal stroma (HRT3-RCM): *a* – anterior stroma, *b* – posterior stroma

Рис. 5. Конфокальная микроскопия стромы роговицы (HRT3-RCM): *a* — передняя строма роговицы, *b* — задняя строма роговицы

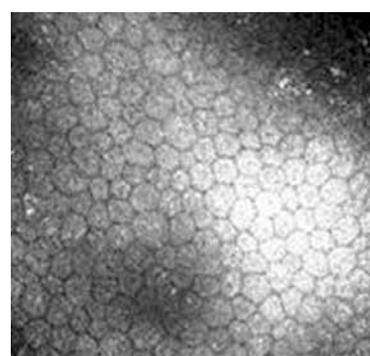


Fig. 7. Confocal microscopy of the corneal endothelium (HRT3-RCM)

Рис. 7. Конфокальная микроскопия эндотелия роговицы (HRT3-RCM)

Table 1

Ocular surface assessment algorithm with confocal microscopy *in vivo*

Таблица 1

Алгоритм оценки состояния тканей глазной поверхности при помощи конфокальной микроскопии *in vivo*

Показатель	Cell density (per mm ²)			
	Epithelial wing cells	Epithelial basal cells	Keratocytes	
			Anterior stroma	Posterior stroma
Dendritic cells (Langerhans cells)	- /+ /++/ +++			
Areas of desquamation in the superficial epithelium	- /+ /++/ +++			
Hyperreflective intercellular micro-inclusions	-/+ /++/ +++			
Thickening of the Bowman membrane	- /+ /++/ +++			
Total length of sub-basal nerve fibers in the field, mm*				
Tortuosity of sub-basal nerve fibers*	- /+ /++/ +++			
Granular structures in sub-basal nerve fibers	- /+ /++/ +++			

* These parameters can be calculated using semi-automatic analytic software CCMetrix Image Analysis Software v. 1.1

Corneal stroma comprises approximately 90% of total corneal thickness and consists of collagen fibers, intercellular substances, and keratocytes. Collagen fibers and intercellular substances are transparent and cannot be seen by confocal microscopy. The diameter of keratocyte nuclei varies between 5 and 30 μm; they are bean shaped in the anterior stroma and oval shaped in the posterior stroma (Figure 3a and 3b). Keratocyte density is observed in the anterior stroma (Figure 5) [10, 12, 14].

No DES-specific changes in the corneal stroma have been described in the literature. Several studies have demonstrated a large number of hyperreflective keratocytes and intercellular micro-inclusions (Figure 6). Some authors consider these keratocytes as activated cells or stress cells. The density of activated keratocytes is higher in patients with DES and endocrine ophthalmopathy [23]. However, there is no consensus on the underlying mechanism of this hyperreflectivity: keratocyte apoptosis, active metabolic process, or inaccuracy of the method.

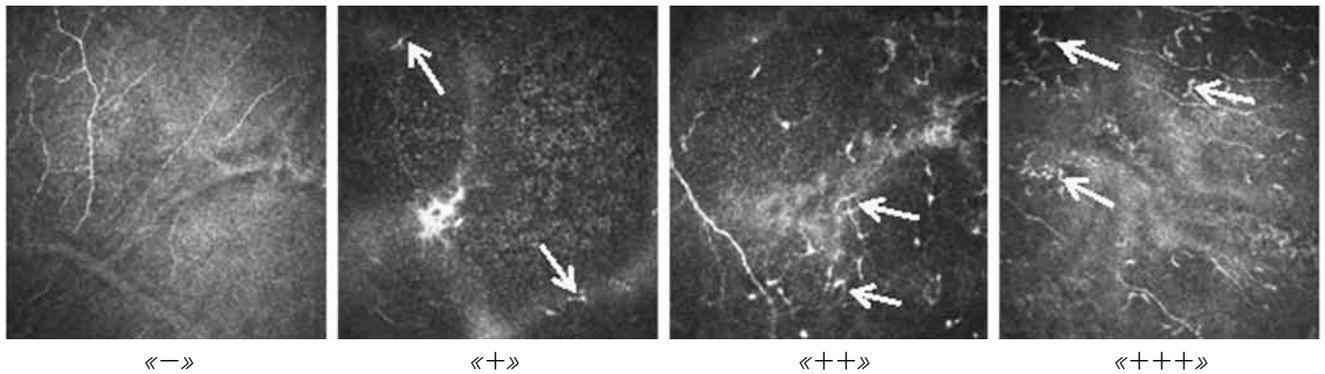


Fig. 8. Dendritic cells

Рис. 8. Дендритические клетки (Лангерганса)

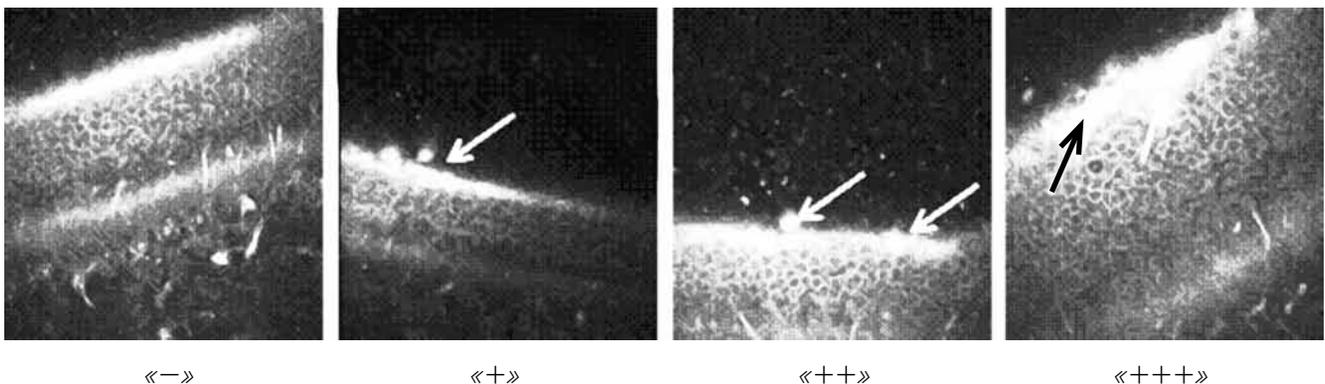


Fig. 9. Superficial epithelium desquamation focuses

Рис. 9. Очаги десквамации поверхностного эпителия

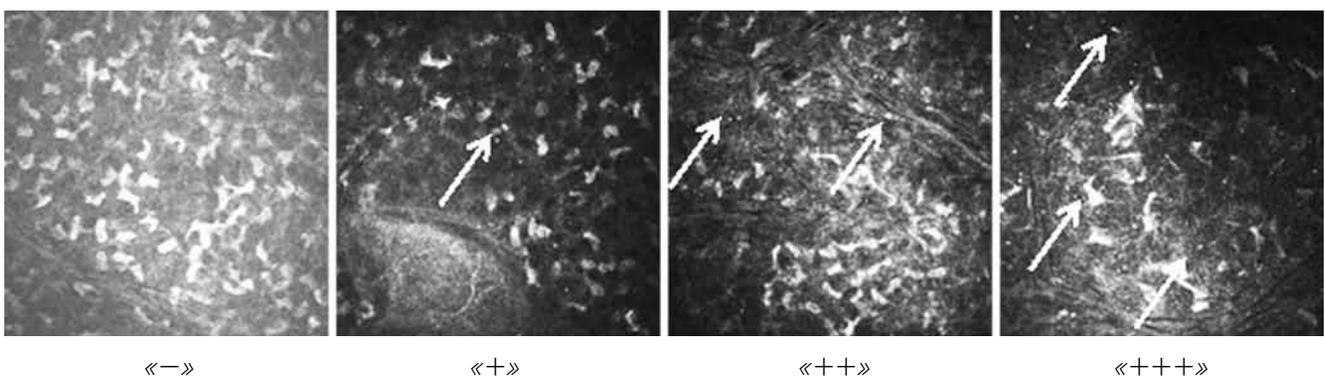


Fig. 10. Hyperreflective intercellular micro-inclusions

Рис. 10. Гиперрефлективные межклеточные микровключения

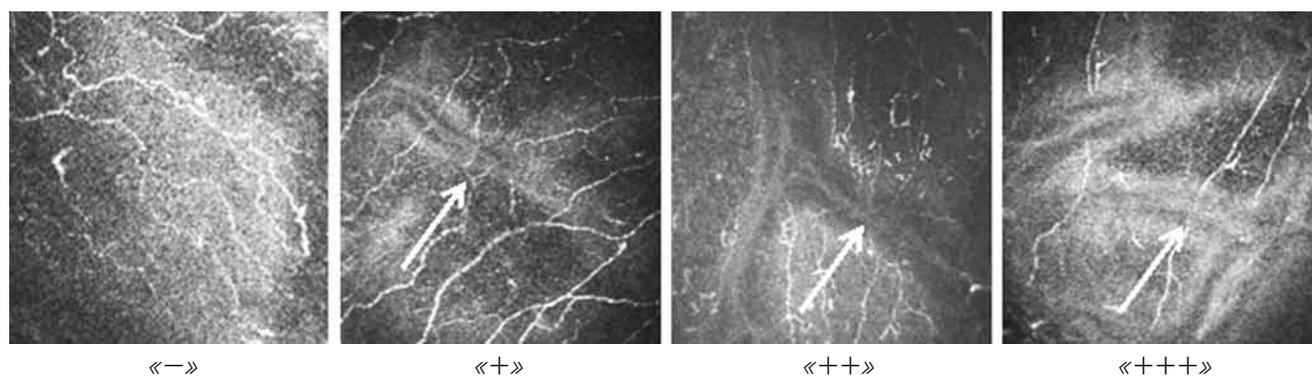


Fig. 11. Bowman's membrane stiffening

Рис. 11. Уплотнение боуеновой мембраны

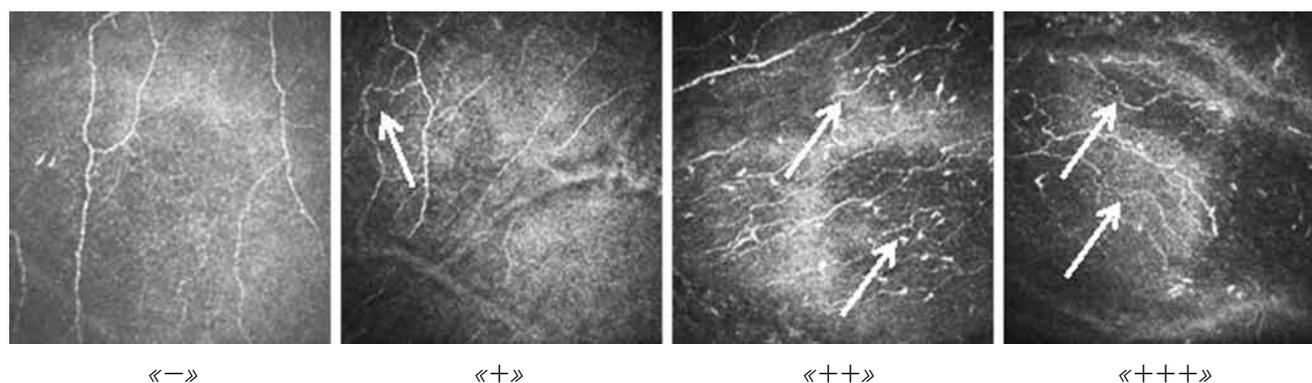


Fig. 12. Tortuosity of subbasal nerve fibers

Рис. 12. Извитость суббазальных нервных волокон

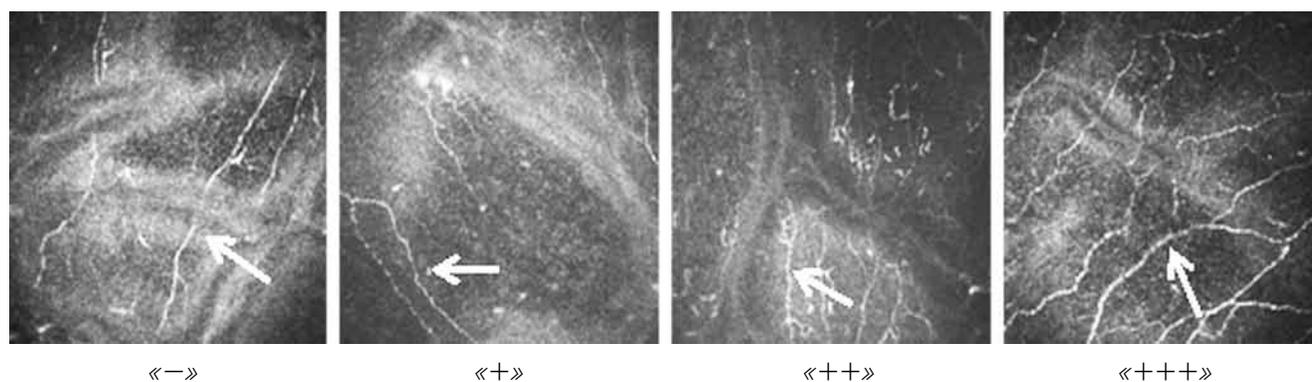


Fig. 13. Bead-like structures of subbasal nerve fibers

Рис. 13. Гранулоподобные структуры суббазальных нервных волокон

The endothelium consists of one layer of hexagonal cells with dark borders and bright cytoplasm (Figure 7). Cell nuclei are usually not visualized. On average, cell diameter is 20 μm [1, 10, 14].

Confocal microscopy allows real-time *in vivo* assessment of the cellular structure of cornea, limbus, and conjunctiva. Therefore, this method can be used for diagnosing various ocular surface disorders and monitoring treatment efficacy. Assessment of the corneal epithelium in patients with DES reveals significant lesions [9]. These

lesions are probably associated with tear film hyperosmolarity due to excessive tear evaporation, which could be caused by either morphological changes or inflammation in the corneal epithelium [19, 26]. The condition of corneal stroma requires further investigation. Particular attention should be paid to the corneal nerves. Confocal microscopy shows substantial changes in the subbasal nerve plexus of DES patients and enables noninvasive evaluation of corneal immune cells; this is especially important for patients with DES

because inflammation plays an important role in the development of this disease.

Considering all of the above mentioned data, we developed an algorithm for the evaluation of ocular surface tissues using *in vivo* confocal microscopy (Table 1).

The authors believe that this algorithm will be useful for both routine clinical practice (for patients with ocular surface disorders) and various research studies that require detailed quantitative assessment of corneal changes.

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