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Cytologic features of the bulbar conjunctiva in patients with primary open-angle glaucoma-associated dry eye disease

T.M. Zhmud,[1] V.Iu. Tetarchuk,[1] O.O. Andrushkova,[1] A.V. Demchuk,[2] K.Iu. Hrzhymalska,[1] S.P. Veretelnyk[1]

[1] National Pirogov Memorial Medical University, Vinnytsya;

[2] Vinnytsya Regional Anatomic Pathology Office

Vinnytsya (Ukraine)

Purpose: *To examine the features of the bulbar conjunctiva in patients who developed dry-eye disease (DED) after drug treatment for primary open-angle glaucoma (POAG).*
Methods: *Impression cytology was performed by applying twice a strip of cellulose acetate filter to the ocular surface to remove the superficial epithelial layers of the temporal bulbar conjunctiva. The strips were removed with a peeling motion in a few seconds, and the samples were immediately fixed in 95% ethyl alcohol, stained with hematoxylin and eosin, mounted on glass slides and coverslipped for light microscopy. Squamous metaplasia was graded according to Nelson's grading system on the basis of cell morphology, staining and integrity as well as the nucleus-to-cytoplasm ratio. This study included a case group of 80 patients (mean age, 63.8 ± 6.7 years) with POAG-associated DED, with the group being divided into four subgroups. Subgroups 1 and 2 were composed of 40 patients each, with glaucoma duration of less or more than 5 years, respectively. Subgroups a and b were composed of 40 patients each, with a number of topical ocular hypotensive drugs used equal to one or at least two, respectively. The control group was composed of 20 apparently healthy volunteers (mean age, 67.9 ± 8.9 years). All patients underwent a routine eye examination.*
Results: *All patients with glaucoma had symptoms of DED with Ocular Surface Disease Index (OSDI) scores of at least 15. In subgroup 1, 60% had Nelson's grade 1 and 40%, Nelson's grade 2 squamous metaplasia. In subgroup 2, 10% had Nelson's grade 1; 60%, Nelson's grade 2 and 30%, Nelson's grade 3 squamous metaplasia. In subgroup a, 20% had Nelson's grade 1; 60%, Nelson's grade 2 and 30%, Nelson's grade 3 squamous metaplasia. In subgroup b, 10% had Nelson's grade 1; 60%, Nelson's grade 2 and 30%, Nelson's grade 3 squamous metaplasia.*
Conclusion: *Changes in the bulbar conjunctival epithelium corresponded to Nelson's grade 2 or 3 squamous metaplasia in 80% of patients who developed DED after drug treatment for POAG. The severity of squamous metaplasia correlated with the duration of glaucoma and, consequently, longer use of hypotensive eye drops (r1 = 0.15, p1 = 0.02, p2 = 0.01). Findings of the current study and international guidelines argue for the use of the medications containing no preservatives or potentially toxic components in long-term therapy against glaucoma.*

Keywords:

glaucoma, dry eye disease, impression cytology, bulbar conjunctiva, hypotensive eye drops, preservatives

Introduction

Most patients with primary glaucoma should have medical treatment throughout most of their lives. Chronic side effects of anti-glaucoma medications are of major concern. Of these side effects, ocular surface abnormalities are relatively common.

Hypotensive eye drops containing preservatives may cause ocular surface disorders like squamous metaplasia, subconjunctival fibrosis and loss of goblet cells [1]. Ocular surface health depends on the normal function of goblet and epithelial cells and adequate quantitative and qualitative composition of the tear film. The tear film mucin is produced mostly by conjunctival goblet cells which are particularly sensitive to toxic agents and inflammatory reactions.

Primary open-angle glaucoma (POAG) is a chronic progressive sight-threatening optical neuropathy causing loss of the optic nerve rim and retinal nerve fiber layer (RNFL) with associated visual field defects [2]. Drug therapy is the first-line treatment for POAG, and patients would normally need to be on life-long treatment with hypotensive medications which can cause degenerative changes in the eye, particularly, the ocular surface. These changes are mostly caused by toxicity of active substances and preservatives [3, 4]. Studies have assessed the toxic effect of long-term anti-glaucoma medications on the corneal epithelium [5, 6] and reported that corneal epithelial cell damage was caused directly, whereas abnormal tear film function, indirectly by long-term treatment with anti-glaucoma medications. Toxic conjunctival changes induced by topical anti-glaucoma medications have been widely reported [4-8]. Nonetheless, to date, no study has been conducted to analyze possible conjunctival modifications directly induced by the physiopathologic mechanisms related to the onset and/or progression of POAG and ocular hypertension (OH) [9].

The prevalence of dry eye disease (DED) in people older than 50 years is estimated at 5–30% [10]. The wide prevalence range is due to different definitions of dry eye and the profile of population surveyed [10]. Glaucoma is a common pathology, whose prevalence expands with age from 1% in people aged 40-49 up to 8% in people aged over 80 [11].

Both glaucoma and dry eye disease are chronic conditions and it is important to treat both diseases. Because the treatment of both often includes using eye drops, keeping up with the regimen can be challenging for patients. Treating the DED is very important both for the patient's comfort and for the long-term health of the surface of the eye. Addressing the glaucoma treatment almost always takes precedence over treating the DED—even though the dry eye disease bothers the patient more.

DED and glaucoma commonly occur together. Long-term use of anti-glaucoma eye drops can cause significant changes in the ocular surface due to altered tear secretion, which plays a special role in DED [12]. The resulting ocular surface disease in patients with glaucoma can lead to poor medication compliance from the associated symptoms and, therefore, to low efficacy of drug treatment (due to issues associated with tolerability of ocular hypotensive agents) as well as surgical treatment (due to conjunctival fibrosis as a complication of filtration surgery) for glaucoma [7, 8]. Low compliance is a major problem and a cause of the development of optic neuropathy in patients with glaucoma. This explains the need for early detection of DED and initiation of specific DED treatment in patients with glaucoma [12].

Impression cytology is a technique used in ophthalmology to verify morphological changes in the ocular surface. The technique refers to the application of cellulose acetate Millipore filter to the ocular surface to collect conjunctival specimens on the slide, with these specimens sequentially stained and examined by light microscopy [3, 4]. Impression cytology is low invasive, easy and fast to use, and enables repeat sample taking. Impression cytology represents a non- or minimally invasive biopsy of the ocular surface epithelium with no side effects or contraindications. It has demonstrated to be a useful diagnostic aid for a wide variety of processes involving the ocular surface. In addition, and mainly during the last decade, its use as a research tool has experienced an enormous growth and has greatly contributed to the understanding of ocular surface pathology [13]. Thus, impression cytology found changes in epithelial and goblet cells of patients with DED. Thus, in a study by Nelson and Wright [14], compared with normal eyes, eyes with DED demonstrated a 17% greater goblet cell loss on the interpalpebral bulbar compared with the inferior palpebral ocular surface. In an impression cytology study of the bulbar conjunctival epithelium by Zhmud and colleagues [15], most patients (94.1%) with type 2 diabetes mellitus (T2DM) had squamous metaplasia grade (Nelson) of 2 or 3, and 80% of those with squamous metaplasia grade (Nelson) of 3 had T2DM duration of more than 5 years.

Conjunctival impression cytology is an essential diagnostic tool in ocular surface disorders. The cell density and characteristics of the conjunctival surface may differ according to localization, and the changes in ocular surface disorders are first observed in bulbar than in palpebral conjunctiva [16]. The technique is interesting due to its capacity to detect changes in the conjunctival mucosa of the globe and the palpebral mucosa for the diagnosis of disorders associated with ocular surface disease.

The purpose of the study was to examine the features of the bulbar conjunctiva in patients who developed DED after drug treatment for POAG.

Material and Methods

This study included a case group of 80 patients (mean age, 63.8 ± 6.7 years) with glaucoma-associated dry eye and a control group of 20 apparently healthy volunteers (mean age, 67.9 ± 8.9 years).

Exclusion criteria for both groups were a systemic disease presenting a risk of DED (Sjogren's syndrome, ankylosing spondylitis, etc.) or a history of eye surgery. The case group was divided into two subgroups based on the duration of glaucoma. The case group was divided into two equal subgroups of 40 patients each, subgroup 1 (glaucoma duration of less than 5 years) and subgroup 2 (glaucoma duration of more than 5 years). In addition, the case group was divided into two subgroups based on the number of topical ocular hypotensive drugs containing preservatives. Subgroups a and b were composed of 40 patients each, with a number of topical ocular hypotensive drugs equal to one or at least two, respectively.

In addition to a routine examination of the eye, Schirmer I test and tear film break-up time (TBUT) test, patients had their corneal fluorescein staining (CFS) scored using the Oxford schema, severities of dry eye graded according to

the DEWS II classification and Ocular Surface Disease Index (OSDI) obtained [17, 18].

The study followed the ethical standards stated in the Declaration of Helsinki, European Convention on Human Rights and Biomedicine, relevant provisions of the WHO, and Order of the Ministry of Health of Ukraine No.281 of November 1, 2000.

Impression cytology was performed by applying a strip of cellulose acetate Millipore filter to the temporal bulbar conjunctiva. The filter strip was gently pressed against the conjunctiva and removed with a peeling motion in a few seconds. Thereafter, another conjunctival impression was taken from the same site as previously. Samples were immediately fixed in 95% ethyl alcohol, stained with hematoxylin and eosin, mounted on glass slides and coverslipped [19]. The slides were examined by light microscopy (Leica DM500; Leica Microsystems, Wetzlar, Germany) at magnification of x100, x200, and x400 to assess epithelial cell shape, arrangement, nuclei, cytoplasm, and nucleus-to-cytoplasm ratio. In addition, we assessed whether the intercellular spaces were well preserved, and whether and how many goblet cells and inflammatory cells were present in the field of vision.

The cytologic changes in the bulbar conjunctival epithelium were graded according to Nelson's grading system (1983) [14] as follows:

Grade 0: Small and round epithelial cells with a nucleocytoplasmic ratio of 1:2, large nuclei and eosinophilic cytoplasm; abundant, plump, oval goblet cells with well preserved intercellular spaces;

Grade 1: Slightly larger and more polygonal epithelial cells with nucleo-cytoplasmic ratio 1:3 and eosinophilic cytoplasm. The nuclei are smaller. The goblet cells are decreased in number; however, they still maintain their plump and oval shape. The intercellular spaces are somewhat widened at some sites;

Grade 2: Larger and polygonal, occasionally multinucleated epithelial cells with variable staining cytoplasm and a nucleo-cytoplasmic ratio 1:4-1:5. Smaller and less intensely PAS-positive goblet cells with poorly defined cellular borders and markedly decreased in number. The intercellular spaces are widened and there is loss of intercellular bonds at some sites;

Grade 3: Large polygonal epithelial cells with basophilic cytoplasm. Small, pyknotic and in many cells completely absent nuclei. There is loss of intercellular bonds and keratinization. Goblet cells absent.

Grade 0 and 1 were regarded as normal whereas grade 2 and 3 as abnormal cytology of the bulbar conjunctiva. Irregular cell shape and nucleus size and positive cytoplasmic staining of epithelial cells, reduced number of absence of goblet cells, and widened intercellular spaces are signs of conjunctival squamous metaplasia.

Statistical analyses were conducted using Statistica 10.0 (StatSoft, Tulsa, OK, USA) software. Spearman's correlation was used to assess if there was an association of the duration of glaucoma or the number of ocular hypotensive medications used with Nelson's squamous metaplasia grade. The level of significance P value was set at $P < 0.05$.

Results

An OSDI score > 15 was present in all patients, indicating the presence of dry eye symptoms. Our morphological and morphometric studies of the bulbar conjunctiva demonstrated substantial differences between the control group and subgroups of patients.

In subgroup 1, most patients (60%) had grade 2 conjunctival squamous metaplasia, and the rest had grade 1 squamous metaplasia (and the difference between subgroup 1 and the control group was statistically significant, $p1 = 0.02$).

Grade 1 squamous metaplasia appeared as mild conjunctival epithelial changes, with small polygonal epithelial cells with a nucleo-cytoplasmic ratio of ≤ 1:3 among sheets of round epithelial cells. In addition, there were one to five plump goblet cells in the field of vision and insubstantially widened intercellular spaces. This contributed to some preservation of mucosal functions (Fig. 1).

Grade 2 squamous metaplasia appeared as a number of goblet cells with poorly defined cellular borders in the field of vision decreased to 0-3; epithelial cells with an abnormal polygonal shape and a nucleo-cytoplasmic ratio of ≤ 1:5, loss of intercellular bonds, widened intercellular spaces, and, consequently, a decreased arrangement of cells in sheets. These changes decrease the density of the superficial conjunctival layers, resulting in the loss of protective capacity of the epithelium (Fig. 2). In these patients, the mean Schirmer test score was 8.6 ± 0.2 mm/min, and mean TBUT value, 10.5 ± 0.1 s. In subgroup 2, 60% had grade 2 and 30% had grade 3 squamous metaplasia ($p2 = 0.02$) (Table 1).

The most significant pathological processes in the conjunctival epithelium were seen in grade 3 squamous metaplasia, and were characterized by the presence of large polygonal epithelial cells with basophilic cytoplasm and two or three small sometimes pyknotic nuclei, loss of intercellular bonds, keratinization, complete loss of goblet cells, and degenerative changes (Fig. 3 a,b).

Degenerative epithelial processes (Fig. 4) appeared as cellular edema, larger cells, karyolysis, cariorexis, and vacuolized protoplasm, and were parallel with lymphocytic infiltration. This degeneration was especially severe in patients treated with topical anti-glaucoma medications for more than 5 years.

Of note, there was a mild positive correlation of glaucoma duration with cytological conjunctival changes ($r = 0.15$, $p = 0.01$) in patients treated with topical anti-glaucoma medications for more than 5 years.

In subgroup 2, the mean Schirmer test score was 7.6 ± 0.2 mm/min, and mean TBUT value, 8.5 ± 0.1 s. Grade 2 squamous metaplasia and grade 3 squamous metaplasia were seen in 60% and 20%, respectively, of patients in subgroup a, and 60% and 30%, respectively, of patients in

Table 1. Numbers and percentages of patients with Nelson's grades 1, 2 and 3 squamous meraplasia in subgroups of patients with (1) glaucoma duration less than 5 years and (2) more than 5 years, as assessed by impression cytology of the bulbar conjunctiva

Nelson's squamous metaplasia grade	Patients (n = 40) with glaucoma duration less than 5 years, n (%)	Patients (n = 40) with glaucoma duration more than 5 years, n (%)
Grade 1	32 (40%)	8 (10%)
Grade 2	48 (60%)	48 (60%)
Grade 3	0 (0%)	24 (30%)
	p_1=0.02	p_2=0.01
		r_1=0.15

Note: p1, significance of difference between subgroup 1 and controls; p1, significance of difference between subgroup 2 and controls; r1, quotient of correlation between cytological changes in the conjunctiva and the duration of glaucoma; n, number of patients

subgroup b (pa = 0.01)(Table 2). In addition, no significant difference was found between patients treated by a single topical hypotensive medication and those treated with at least two topical hypotensive medications (i.e., subgroups a and b). This may indicate that even a single topical hypotensive medication is capable of causing substantial changes in the bulbar conjunctiva (r2=-0.35). It was, however, noteworthy that not only signs of squamous metaplasia, but also a non-uniform arrangement of basal epithelial cells were seen in 23/57 patients (43%) patients treated with a single topical anti-glaucoma medication for less than 5 years.

Piles of nuclei of these cells with poorly visualized cytoplasm margins and the formation of multilayer sheets were noted (Fig. 5). This process may characterize reactive hyperplasia of deep epithelial layers as a manifestation of compensatory and adaptive mechanisms with the potential recovery of the epithelium with treatment with a single topical anti-glaucoma medication in glaucoma treated for less than 5 years.

Conjunctival impression samples taken from glaucoma patients treated with topical medications for more than 5 years exhibited a thinner epithelium with degenerative changes (Fig. 6). Morphologically, these changes confirm a decreased mucosal protective capacity leading to the development of DED.

Squamous metaplasia of the bulbar conjunctival epithelium of grades 0, 1 and 2 was seen in 2 subjects (10%), 16 subjects (80%) and 2 subjects (10%), respectively, of the control group. Therefore, most conjunctival impression samples taken from controls reflected a normal state of the conjunctiva. Grade 0 squamous metaplasia was characterized by sheets of small and round cells with a nucleocytoplasmic ratio of 1:2, large and even nuclei and eosinophilic cytoplasm; and abundant, plump, oval goblet cells with well preserved intercellular spaces (Fig. 7). Other control subjects exhibited mild (grade 1 or 2) squamous metaplasia which could be a normal compensatory response to stimuli or age-related processes.

Discussion

In a study by Richhariya and colleagues [20], the mean conjunctival impression cytology grades were higher in patients on the long-term use of anti-glaucoma agents than

Table 2. Numbers and percentages of patients with Nelson's grades 1, 2 and 3 squamous meraplasia in subgroups of patients using (a) one ocular hypotensive medication and (b) at least two ocular hypotensive medications, as assessed by impression cytology of the bulbar conjunctiva

Nelson's squamous metaplasia grade	Patients using one ocular hypotensive medication, n (%)	Patients using at least two ocular hypotensive medications, n (%)
Grade 1	16 (20%)	8 (10%)
Grade 2	48 (60%)	48 (60%)
Grade 3	16 (20%)	24 (30%)
	$p_{a=}$0.01	$p_{6=}$0.01
	r_2=-0.35	

Note: p1, significance of difference between subgroup a and controls; p1, significance of difference between subgroup b and controls; r2, quotient of correlation between cytological changes in the conjunctiva and the number of ocular hypotensive medications used; n, number of patients

in the control group. The medication group had higher impression cytology grade than the control group (median [range]: 1.0 [1:2 to 1:6] vs 0.6 [1:2 to 1:4]; $P < 0.001$). In addition, the monotherapy group reported lower cytology grades than the fixed combination therapy group [16].

Ciancaglini and colleagues [9] analyzed the mean density and the mean area of conjunctival epithelium microcysts in eyes on unfixed combination therapy (beta-blocker plus prostaglandin) versus those on monotherapy with either beta-blocker or prostaglandin. Patients on unfixed combination therapy (beta-blocker plus prostaglandin) showed a significantly larger microcyst area than did eyes on monotherapy with either beta-blocker or prostaglandin. In addition, the potential toxic effect of the preservative contained in the ophthalmic solutions (mainly benzalkonium chloride; BAC) has to be carefully considered as a potential factor involved in structural tissue modifications. Of interest, the presence of conjunctival microcysts in untreated hypertensive eyes did not support the hypothesis that microcyst formation could be exclusively related to an unpredictable effect of BAC. On the other hand, we cannot completely exclude that a higher dosage of BAC, as in patients receiving combined therapy, is an additional factor involved in the development of a greater microcyst area, as observed in this group of patients in [9].

Based on this hypothesis, the topical preservative toxicity may act as an accessory mechanism that contributed to and perhaps enhanced microcyst formation independent of the main mechanism of induction [9]. However, the contribution of the active ingredients in the medication or the interaction with pathological ocular surface tissues at the initiation of therapy cannot be ruled out [21].

As an example of the direct effects of an active compound, topical application of preservative-free latanoprost was shown to decrease tear production, induce conjunctival goblet cell loss, disrupt corneal epithelial barrier, and promote cell apoptosis and inflammation of the ocular surface [21]. Beta-blockers, with or without preservatives, have also been shown to induce ocular surface damage, including corneal staining, conjunctival goblet cell loss and squamous metaplasia [22].

Conclusion

Changes in the bulbar conjunctival epithelium corresponded to grade 2 or 3 (Nelson) squamous metaplasia in 80% of patients who developed DED after drug treatment for POAG. In addition, the severity of squamous metaplasia correlated with the duration of glaucoma and, consequently, longer use of topical ocular hypotensive medications ($r1=0.15$, $p1 = 0.02$, $p2 = 0.01$). This may be associated with the glaucomatous process as well as the presence of a preservative in topical ocular hypotensive medications. Findings of the current study and international guidelines argue for the use of medications containing no preservatives or potentially toxic components in long-term therapy against glaucoma.

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Disclosures

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Corresponding author: *Nataliya Khramenko, Email: khramenkon@gmail.com*

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Disclaimer. *We declare that the views expressed in this article are our own and not an official position of the institution or funder.*

Ethical approval: *All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee. The study followed the ethical standards stated in the Declaration of Helsinki, European Convention on Human Rights and Biomedicine, relevant provisions of the WHO, and Order of the Ministry of Health of Ukraine No.281 of November 1, 2000.*

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Abbreviations: *BAC, benzalkonium chloride; DED, dry eye disease; OSDI, Ocular Surface Disease Index;*

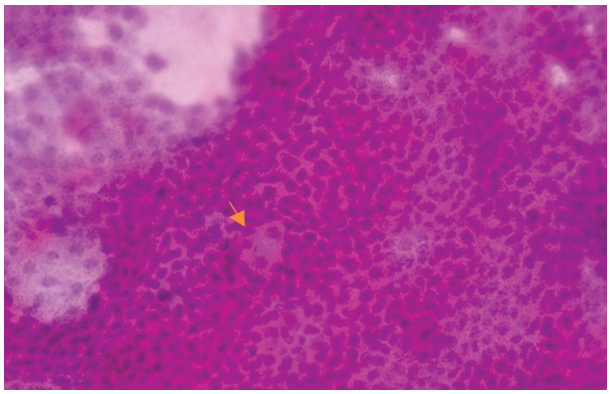

Fig. 1. Nelson's grade 1 squamous metaplasia. A sheet of round epithelial cells with interspersed polygonal cells (right-hand side) and goblet cells (arrow). Hematoxilin and eosin staining. Objective magnification, ×20.

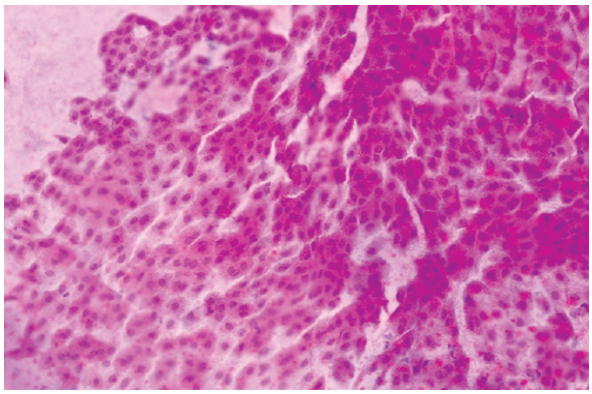

Fig. 2. Nelson's grade 2 squamous metaplasia. Polygonal and round cells with a loss of intercellular connections. Hematoxilin and eosin staining. Objective magnification, ×20.

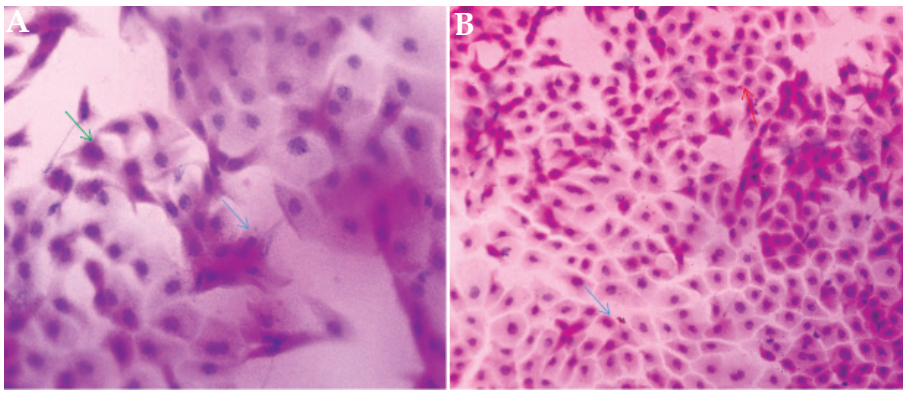

Fig. 3. Nelson's grade 3 squamous metaplasia. Wide polygonal seens can be seen, arranged mostly separately from each other. Note the cell with two nuclei (left blue arrow), and cells showing keratinization (right blue arrow) the cell with basophilic cytoplasm (red arrow). Hematoxilin and eosin staining. Objective magnification, ×40 and ×20.

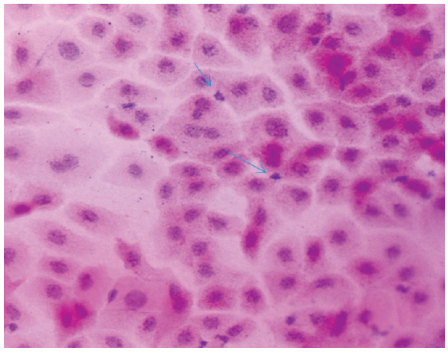

Fig. 4. Degenerative changes in the epithelial conjunctiva and lymphocytes (arrow). Hematoxilin and eosin staining. Objective magnification, ×40.

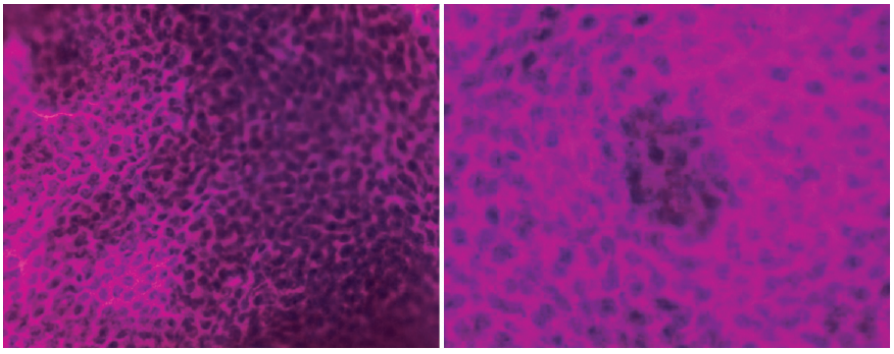

Fig. 5. Evidence of hyperplasia of deep layers of the conjunctival epithelium. Hematoxilin and eosin staining. Objective magnification, ×20 and ×40.

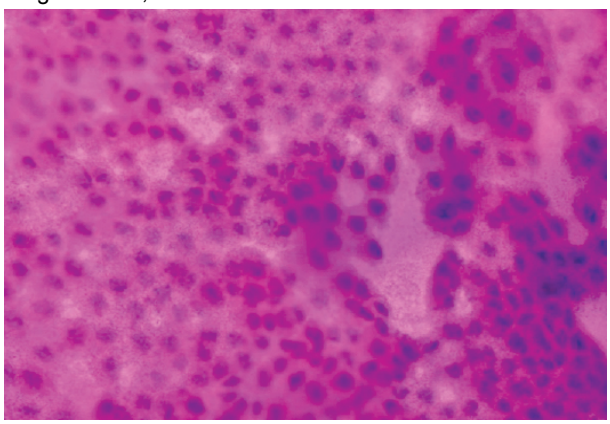

Fig. 6. A thinned layer of the conjunctival mucosa showing degenerative changes. Hematoxilin and eosin staining. Objective magnification, ×40.

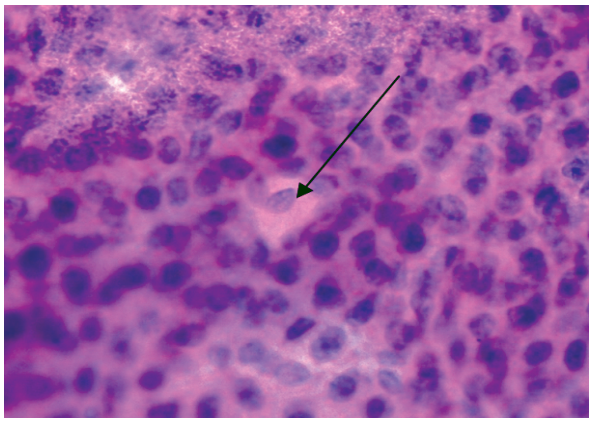

Fig. 7. Normal bulbar epithelial conjunctiva (Nelson's grade 0). An arrow points to a goblet cell. Hematoxilin and eosin staining. Objective magnification, ×40.