

# Conjunctival impression cytology in non-proliferative and proliferative diabetic retinopathy

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## Abstract

• **AIM:** To examine the integrity of the ocular surfaces of subjects with and without diabetes with no conjunctival and dry eye signs and symptoms and compare conjunctival impression cytology findings in diabetics with non-proliferative and proliferative diabetic retinopathy.

• **METHODS:** Conjunctival impression cytology was performed on 43 eyes of 43 subjects with non-proliferative diabetic retinopathy (NPDR), 42 eyes of 42 subjects with proliferative diabetic retinopathy (PDR), and 30 eyes of 30 control subjects. Impression cytology specimens of each group were graded and scored in the range 0–3 according to Nelson's method.

• **RESULTS:** There were 45 (52.9%) women and 40 (47.1%) men. The mean age of the patients was 59.6±9.3y (range, 43–76y) in NPDR group and 58.0±8.8y (range, 41–85y) in PDR group. Cases with NPDR and PDR showed statistically significant higher impression cytology scores than control group ( $P<0.05$ ). There was no difference between the NPDR and PDR patients for impression cytology grading scores.

• **CONCLUSION:** It is determined that impression cytology grades are altered in patients with NPDR and PDR. Consequently, we suggest that there might be an association between the impression cytology grading scores and the severity of diabetic retinopathy

**KEYWORDS:** conjunctiva; diabetes; impression cytology; retinopathy

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## INTRODUCTION

Diabetes mellitus (DM) is the most common endocrine disorder and is a significant cause of morbidity and mortality. It is a chronic disease with long-term macrovascular and microvascular complications, including diabetic nephropathy, neuropathy, and retinopathy. The retinal manifestations of diabetes mellitus are broadly classified as either non-proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR) [1]. Besides retinopathy, DM can lead to various ocular complications such as cataract, glaucoma, keratopathy, refractive changes, palsy of the ocular motor nerves, and chronic inflammation of the lids. Among these, keratopathy associated with diabetes mellitus comprises superficial punctate keratopathy, recurrent corneal erosion, persistent epithelial defect, corneal hypoesthesia, and corneal endothelial damage [2-5]. Diabetes is also associated with loss of capillaries and macrovessel dilation in the conjunctiva, similar to well-known vessel changes in the retina [6]. Changes of tear function parameters in diabetes have been studied [2]. Saito *et al* [7] also reported that both corneal sensation and total or reflex tear secretion are reduced in individuals with diabetes. Nepp *et al* [8] reported that abnormal tear function tests were associated with poorer metabolic glucose control, panretinal argon laser photocoagulation, and PDR.

Conjunctival impression cytology is a safe, relatively simple, minimally invasive biopsy method of obtaining specimens from the conjunctival surface. This technique allows the investigator to assess epithelial cell morphology, examine cytoplasmic and nuclear characteristics and quantify the goblet cell population in the conjunctiva [9]. The present study investigated the conjunctival surface changes and correlated them with NPDR and PDR with no conjunctival and dry eye signs and symptoms by comparing the results with those in a control group.

## SUBJECTS AND METHODS

We prospectively evaluated 85 patients with diabetic retinopathy between March 2009 and October 2009. All of the study procedures were conducted in accordance with the Declaration of Helsinki, and informed consents were taken from all of the participants. This study was approved by The Ethical Committee of Ankara Training and Research Hospital. All patients were Turkish Caucasians. The presence of diabetes in all patients had been confirmed

by the corresponding internal medicine department. The status of retinopathy was assessed by fundus photography and confirmed with fluorescein angiography and optical coherence tomography. Early Treatment of Diabetic Retinopathy Study criteria were used to define various stages of diabetic retinopathy [10]. Diabetics with systemic or ocular co-morbidities that have simulating ophthalmic manifestations were excluded from participation. Any patient with proliferative retinopathies associated with systemic diseases or localized retinal vascular and/or ocular inflammatory diseases were excluded from this study. During the ocular examinations, lid margins, tarsal and bulbar conjunctiva, and cornea were particularly examined.

We excluded the cases who reported topical or systemic cyclosporine or ophthalmic steroids use within the previous 6 months. Other reasons for exclusion included previous punctal plug, contact lens wear, use of other topical treatments, active ocular infection, blepharitis, herpes keratitis within the previous 6mo, ocular surgery or trauma within the previous 6mo, intraocular or periocular injections of the bulbus oculi, punctate epithelial erosions of the cornea, other ocular surface diseases, or non-keratoconjunctivitis sicca inflammation, including atopic keratoconjunctivitis. None of the patients with diabetes had a history of Stevens-Johnson syndrome, chemical, thermal or radiation injury, or any ocular surgery that would create an ocular surface problem. The patients with diabetes have no signs and symptoms of dry eye.

The Schirmer's test was performed using a standardized kit containing a strip of filter paper  $5 \times 30\text{-mm}^2$  and placed on to the lower lid margin in a temporal position. The patient looked up and blinked normally for 5min before the strip was removed and the length of wetted paper measured. A value of  $<5$  mm was taken as abnormal. The stability of the tear film over the conjunctiva and cornea was assessed using a Burton's lamp with a cobalt blue filter and sodium fluorescein. A drop of 2% sodium fluorescein was applied to the eye, and the patient was asked to blink five times so that a film is formed over the cornea and bulbar conjunctiva. The patient was then asked to refrain from blinking during which time black spots or lines begin to appear indicating dry spots. The interval between the last blink and the first randomly distributed dry spot was taken as the tear break-up time. An average of three measurements was recorded. A value of  $<10$ s was taken as abnormal [11].

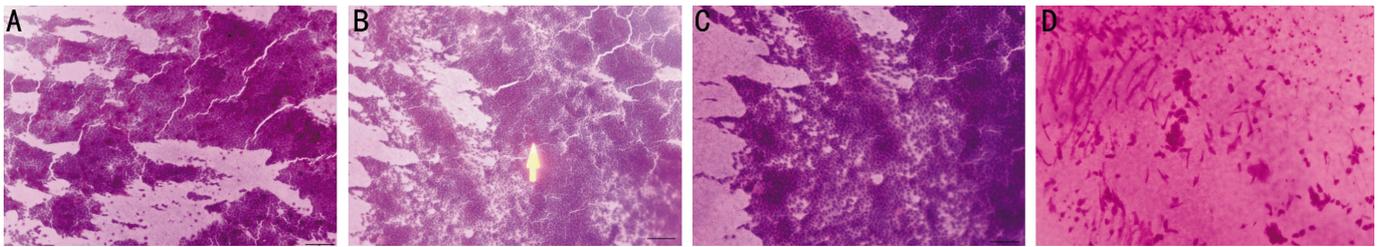
Impression cytology of the conjunctiva of the topically anaesthetized eye was performed according to the technique described by Nelson *et al* [12]. Small disks of cellulose acetate filter paper (MFS, Advantec MFS, Pleasanton, USA, pore size  $0.2 \mu\text{m}$ ) were cut into pieces approximately  $4 \times 5\text{-mm}^2$  in size, placed on the superior nasal interpalpebral conjunctiva 5 mm from the limbus, gently pressed for 5s, and then

removed. The specimens were placed in a fixative solution and stained with Papanicolau's modification of Gill's technique [13]. The specimens were examined with light microscopy by a pathologist who was masked to the history of each specimen. The examination employed the Nelson's method, and the appearance of the conjunctival epithelial cells and goblet cells (if present) was recorded [12]. Two observers, similarly masked, examined all the slides. All specimens were graded according to the following four-levels. Grade 0: the epithelial cells are small and round with eosinophilic-staining cytoplasm. The nuclei are large with a nucleocytoplasmic ratio of 1:2. The goblet cells are abundant, plump and oval with strongly Periodic Acid Schiff (PAS)-positive cytoplasm. Grade I: the epithelial cells are slightly larger than those in grade 0 and more polygonal, with eosinophilic-staining cytoplasm. The nuclei are smaller with a nucleocytoplasmic ratio of 1:3. The goblet cells are fewer in number; however, they still maintain their plump, oval shape with strongly PAS-positive cytoplasm. Grade II: The epithelial cells are larger than those in grade I and polygonal, occasionally multinucleated, with eosinophilic-staining cytoplasm. They have a nucleocytoplasmic ratio of 1:4 to 1:5. The goblet cells are markedly fewer in number and are smaller, less strongly PAS-positive and poorly defined. Grade III: the epithelial cells are larger than those in grade II and polygonal with basophilic-staining cytoplasm. The nuclei are small, pycnotic, or in many cells, completely absent. The goblet cells are completely absent.

The Kruskal-Wallis test was used to compare nonparametric variables among the three study groups. Wilcoxon-signed rank test as post-hoc test were done and  $P < 0.05$  was obtained using Wilcoxon-signed rank test in two (out of three) groups. Also, to ascertain the significant difference between-group, unpaired 2-tailed Student's *t*-test and Mann-Whitney *U* test was applied. The level of significance was set at  $P < 0.05$ . All statistical analyses of the study were performed by using Statistical Package for the Social Sciences (SPSS) for Windows Version 17.0 (SPSS, Inc., Chicago, IL, USA).

## RESULTS

The study subjects were 85 adults patients diagnosed with DM and 30 controls. There were 45 (52.9%) women and 40 (47.1%) men. Of these patients, 23 (53.5%) women and 20 (46.5%) men were within the NPDR group; and 22 (52.4%) women and 20 (47.6%) men were within the PDR group. The mean age of the patients was  $59.6 \pm 9.3\text{y}$  (SD, range, 43-76y) in NPDR group and  $58.0 \pm 8.8\text{y}$  (range, 41-85y) in PDR group. The time elapsed since the diagnosis of DM was  $11.4 \pm 2.8\text{y}$  for NPDR and  $13.0 \pm 3.7\text{y}$  for PDR. Healthy adults as controls 17 (56.7%) women and 13 (43.3%) men, mean aged  $54.1 \pm 6.9\text{y}$  (range, 37-69y) were also included into the study. There was no difference regarding the age ( $P = 0.20$ ) and gender ( $P = 0.31$ ) of the cases in the three studied groups.



**Figure 1 Impression cytology of conjunctival surface of cases** A: Impression cytology of normal conjunctival surface showing corneal epithelial cells (Grade 0). These cells are flat with a prominent nucleus and the nuclear cytoplasmic ratio is low; B: Impression cytology of the conjunctival surface of a patient with NPDR showing the epithelial cells are slightly larger and goblet cells are fewer in number (Grade 1); C: Impression cytology of the conjunctival surface of a patient with NPDR showing the epithelial cells are larger and goblet cells are markedly fewer in number (Grade 2); D: Impression cytology of the conjunctival surface of a patient with PDR showing dysplastic squamous cells with increased nucleus: cytoplasmic ratio and hyperchromatic nuclei (Grade 3) ( $\times 100$ , periodic acid Schiff staining).

The cases were separated into three groups based on the findings of clinical ocular examination. The first group included 43 patients with NPDR; the second group included 42 patients with PDR; the third group included 30 healthy adult subjects serving as controls with no history of systemic or ocular diseases.

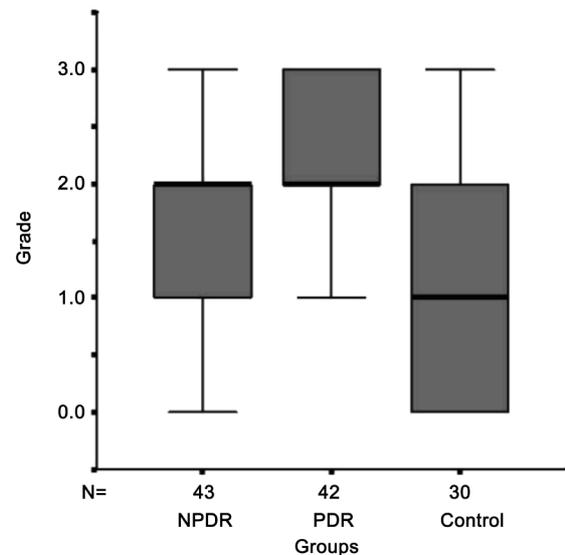
In the control subjects, grade 0 samples were observed mostly. In this sampling, normal goblet cell were clearly distinguishable due to their intense pink color, normal cells were flat with a prominent nucleus. The nuclear cytoplasmic ratio was low (Figure 1A). In the NPDR cases, grade 1 and 2 samples were determined as highly. The goblet cells were lower in number (grade I) but well formed (Figure 1B), and goblet cells were significantly lower in number (grade II) (Figure 1C). Grade II and III samples were observed more than others in the PDR samples. The goblet cells were not present and epithelial cells were larger and their nuclei were small in grade III (Figure 1D).

The results of Schirmer and tear break-up time tests were within normal limits in all 85 eyes of the diabetic patients and all 30 eyes of the healthy control subjects. Schirmer test I (test without topical anesthesia) was performed in both eyes simultaneously. The mean Schirmer test values were ( $15.68 \pm 5.11$ ), ( $16.24 \pm 6.75$ ), ( $15.58 \pm 4.92$ ) mm in patients with NPDR, PDR and in controls respectively. The mean break-up time values were ( $13.48 \pm 3.84$ ), ( $14.12 \pm 6.27$ ), ( $14.8 \pm 6.79$ ) seconds in NPDR, PDR and control groups respectively. There were no statistical significant difference in Schirmer test scores and break-up time values in the three studied groups ( $P > 0.05$ ). Impression cytology grading scores for all groups are summarized in Table 1. The conjunctiva samples showed significantly higher mean elevation impression cytology grading scores in NPDR ( $P = 0.001$ ) and PDR ( $P = 0.001$ ) patients, compared with controls. There was no significant ( $P = 0.2$ ) difference in impression cytology grading scores between NPDR group and PDR group. Mean values for the impression cytology grading scores for three groups were shown in Figure 2.

**Table 1 Impression cytology grading scores for all groups**

Groups	Grade 0	Grade I	Grade II	Grade III	Total
NPDR	2 (4.7)	14 (32.6)	19 (44.2)	8 (18.6)	43 (100.0)
PDR	2 (4.8)	8 (19.0)	21 (50.0)	11 (26.2)	42 (100.0)
Control	11 (36.7)	10 (33.3)	8 (26.7)	1 (3.3)	30 (100.0)
Total	15 (13.0)	32 (27.8)	48 (41.7)	20 (17.4)	115 (100.0)

NPDR: Nonproliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy.



**Figure 2 Mean values for the impression cytology grading scores for three groups. The alteration from the baseline in impression cytology grade was statistically significant in group PDR and NPDR ( $P < 0.05$  by Mann-Whitney  $U$  Test).**

## DISCUSSION

In the present study, we assessed tear break-up time, Schirmer tests, and conjunctival impression cytology in NPDR and PDR patients and compared the results with those in healthy subjects. Of these patients, 45 (52.9%) women and 40 (47.1%) men were available for the evaluation. The average age of the patients was (mean  $\pm$  SD)  $59.6 \pm 9.3$  y in NPDR group and  $58.0 \pm 8.8$  y in PDR group.

Diabetic retinopathy, the most common retinal vascular disease, is the leading cause of new-blindness in adults during the third through sixth decades of life<sup>[14]</sup>. Macro vessel

dilation and loss of capillaries, which are the well-known signs in the retina in DM, has also been observed in the conjunctiva. The effect of DM on conjunctival vessel tortuosity was less well documented [6]. Changes in retinal vascular morphologic features associated with DM have been attributed to decreases in blood velocity and flow, stimulated by changes in blood rheology, resulting in tissue hypoxia and excess perfusion [6]. Similar biological mechanisms may well exert an influence on vessels of the conjunctiva and may account for the apparent changes in vessel morphologic features detected. These vascular changes may lead to conjunctival hypoxia, which has been demonstrated previously among subjects with diabetic retinopathy, and may explain why patients with diabetes are at higher risk of conjunctivitis compared with those without diabetes [15,16]. Isenberg *et al* [15] measured conjunctival oxygen of 122 diabetic subjects to see whether worsening retinopathy was associated with changes in conjunctival oxygen tension. They determined a relationship between worsening of diabetic retinopathy and progressive conjunctival hypoxia. They emphasized the two occurrences could both be related to the severity of diabetes. So, we intended to assess the status of the conjunctiva by the impression cytology in NPDR and PDR cases.

Changes of tear function parameters in diabetes have been studied, but the results remain controversial [2]. In some study, total and reflex tear secretions were significantly reduced, but basal tear secretion and tear film BUT did not change [7]. However, another study reported a decrease in basal tear secretion and BUT [17]. Dogru *et al* [17] reported that BUT and basal tear secretion were decreased, especially in diabetes with poor metabolic control and peripheral neuropathy, but they were not related to the duration of diabetes or the stage of retinopathy, suggesting a neuropathy involving the innervation of the lacrimal gland. Saito *et al* [7] also reported that neither total or reflex tear secretion was correlated with the diabetic retinopathy stage. The mean Schirmer test values were (15.68 ± 5.11), (16.24 ± 6.75), (15.58 ± 4.92) mm in patients with NPDR, PDR and in controls respectively. The mean break-up time values were (13.48 ± 3.84), (14.12 ± 6.27), (14.8 ± 6.79) seconds in NPDR, PDR and control groups respectively. There were no significant differences among NPDR, PDR and control subjects regarding the Schirmer test values and the break up time of the tear film.

Impression cytology of the conjunctiva is an important diagnostic tool in investigating ocular surface disorders [18]. Since 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 [19]. Because of this, all samples were taken from the superior nasal bulbar conjunctiva in this study.

Kruse *et al* [16] determined the presence of DM was a modest risk factor for acute infectious conjunctivitis and they found a 1.24-fold increased risk of redeeming a prescription for a topical ocular antibiotic in individuals with DM, compared with population-based control subjects. Also, Bilen *et al* [20] concluded that the conjunctival flora in diabetic subjects differs from that in nondiabetic subjects. The emergence of conjunctivitis in patients with DM may be caused by conjunctival pathological changes showed in this study.

Nepp *et al* [8] reported the relationship between diabetic retinopathy and keratoconjunctivitis sicca with no laser treatment in diabetic eye in comparison to laser treatment. Most of the previous studies have suggested that impression cytology is altered in the diabetic cases [21]. Yoon *et al* [21] mainly focused on the correlation between the grade of diabetic retinopathy and ocular surface changes [2]. In their study, cases were divided into two groups according to the presence of NPDR and PDR. They reported 64.9% of the total eyes had an abnormal break-up time value and 35.1% of the total eyes had abnormal basal secretion test values. They showed higher grade of squamous metaplasia and lower goblet cell density in the diabetic patients than control subjects. No significant differences were found between the NPDR group and control subjects. However, there were statistically significant differences of these ocular surface parameters between the control and the PDR groups and also between the NPDR and the PDR groups [2]. In their study, the diabetic cases had functionally abnormal ocular surface. But in our study, we investigated the impression cytology grading scores of eyes with NPDR and PDR with normal Schirmer and tear break-up time value and compared them with each other and healthy subjects. To put it briefly, we investigated the cytological changes between the groups with PDR and NPDR who had all functionally normal ocular surfaces and compared them with control subjects. The mean impression cytology grading scores (mean ± SD) were 1.76 ± 0.12, 1.97 ± 0.12, and 0.96 ± 0.16 in NPDR, PDR and control groups respectively. Our impression cytologic analysis showed a higher grading scores in NPDR and PDR patients than in control group ( $P < 0.05$ ).

Based on the results of our present study, ocular surface changes including squamous metaplasia in the bulbar conjunctiva can be observed in cases with diabetic retinopathy. PDR patients showed higher grading scores than NPDR patients, but the difference was not statistically significant. The squamous metaplasia detected in our diabetic patients could at least partially be the result of a primary surface disease or of metabolic alterations of the conjunctival epithelial cells independent of tear film abnormalities [22]. Moreover, conjunctival hypoxia might also have played a role here. Ocular surface investigations with impression

cytology in Down syndrome patients have revealed a significantly higher rate of squamous metaplasia than in control individuals. This difference is thought to arise from a metabolic change in elements such as vitamin A [23]. In DM, there are more numerous and serious changes in metabolism of elements<sup>[14]</sup>.

Maumenee suggests that conjunctival keratinization may even be encountered when the tear film is normal<sup>[18]</sup>. Erda *et al* [24] have reported that Schirmer and BUT tests may reveal normal results in spite of the formation of metaplasia in conjunctiva. Goebbels [22] studied 86 eyes of insulin dependent DM for break up time, Schirmer's and impression cytology. He found no significant difference in results of break up time and unstimulated basal tear flow between study and control population. However statistically significant difference was found between two populations in terms of impression cytology results. Similarly, no statistically significant differences were found in Schirmer test scores and tear break-up time values between study and control population in present study.

There are several limitations in our present study. Firstly, the number of patients is small. Secondly, this study is insufficient in investigating other potential risk factors such as cornea touch sensitivity (aesthesiometry) in the development of keratopathy.

In conclusion, our study indicates that impression cytology grades are altered in patients with NPDR and PDR. However, Schirmer and tear break-up time values are not different between study and control subjects. These findings suggested that there may be an association between the impression cytology grading scores and the severity of diabetic retinopathy. Moreover, for early detection of corneal and conjunctival disorders in patients with DM, it seems reasonable to evaluate and follow up these cases for functional and cytological ocular surface investigations. Since some associations between diabetic retinopathy and ocular surface disorders were found, further investigations conducted to explain the mechanism of squamous metaplasia formation in these patients should be encouraged.

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