

Effects of immunosuppressive drugs on oral mucosa in patients with Behçet's disease: cytomorphological and cytopathological assessment

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Background/aim: The aim of this study was to investigate cytomorphological and cytopathological changes in oral exfoliated smears collected from immunosuppressed patients with Behçet's disease (BD) using stereological methods.

Materials and methods: For cytometric analysis, mucosal cell smears were obtained from the buccal mucosa and the floor of the mouths of BD patients treated with immunosuppressive drugs and from healthy volunteers. All mucosal smears from the patients and the healthy volunteers were stained using the Papanicolaou method and examined cytopathologically under light microscopy and cytometrically via the stereological nucleator method.

Results: The cytometric analysis revealed 3 types of mucosal cells, with numbers of particularly pink cells lower in the aphthous areas of the patients with BD compared to the healthy controls ($P < 0.05$). The nuclear volumes (NVs) and cytoplasmic volume (CVs) were significantly higher in the BD patients ($P < 0.05$), but the NV/CV ratio was higher only in the drug-use patient groups ($P > 0.05$). There was lower apoptotic activity in the nondrug-use patients with BD and in the immunosuppressive-taking BD patients.

Conclusion: The findings suggest that quantifiably morphological and morphometric changes in oral mucosa can be detected by stereological techniques. Changes in these parameters may indicate malignant transformation in the oral mucosa.

Key words: Behçet's disease, oral cytology, stereology, quantitative cytometry

1. Introduction

Behçet's disease (BD) is an inflammatory disorder characterized by recurrent oral ulcers, genital ulcers, and skin lesions. The etiology of BD is unknown, and a viral etiology has not been confirmed up to now. The disease was first described in 1937 by a Turkish dermatologist, Hulusi Behçet, in 3 Turkish patients with oral and genital ulcerations and hypopyon uveitis (1). BD seriously affects all organs of the body, depending on the system involved (2,3), and it has a high prevalence along the ancient Silk Road countries, including Turkey (4). Recently, many studies have focused on the pathogenesis and clinical findings of BD (1–5). The diagnosis of BD is based on oral and genital ulcers, uveitis, and skin lesions. In the treatment of BD, topical and systemic corticosteroids are commonly used as antiinflammatory agents. Controlled studies of the use of

such agents to treat BD are lacking, but their long-term use is limited as a result of significant side effects, which requires the simultaneous use of other immunosuppressive drugs, such as calcineurin inhibitors (cyclosporine and tacrolimus), colchicine, azathioprine, interferon alpha, thalidomide, and pentoxifylline (1,5,6). Some studies have reported the effects of immunosuppressive drugs on the oral mucosa cells (7,8). Such effects may be dosage-related, with gingival squamous cell carcinomas most frequently reported (9,10).

Diagnoses of oral cancers include incisional or excisional biopsy of lesions in oral mucosa, and histopathologic analysis of biopsied material. Oral exfoliative cytology has been used as a diagnostic method in the diagnosis of precancerous and cancerous cells (8). It is a simple method for the morphological and

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cytological assessment of exfoliated cells from the oral mucosa (11). It is also a cheap and easy procedure for the diagnosis of lesions in oral mucosal smears and can determine cytomorphometric alterations at an early stage (12–14). The higher frequency of premalignant exfoliated cells from patients taking immunosuppressive drugs has been observed in previous studies (8–10), indicating the changing of nuclear and cytoplasmic volumes as an indicator for the risk of oral squamous cancer.

In the assessment of exfoliative cytology, quantitative techniques are more objective and accurate and are based on an evaluation of quantitative values, such as variations in the size of the nucleus and the cytoplasm, and changes in the nucleus/cytoplasm ratio may enhance the diagnostic sensitivity of many diseases (8,15). Stereology is one of the most reliable methods of making a morphometric assessment. Stereological methods are based on fundamental principles of geometry and statistics and can be applied to morphometric analysis of many different materials or tissues (8).

Disturbances in cellular homeostasis often trigger programmed cell death (16). It was suggested that degenerative alterations in oral epithelium cells are indicative of apoptosis (karyorrhexis, pyknosis, and condensed chromatin) (17,18). As well as the micronucleus, nuclear alterations are indicative of apoptosis (19–22).

The aim of the present study was to investigate histopathological and quantitative cytological changes of oral mucosa cells collected from patients with BD who were taking immunosuppressive drugs. Cytomorphological and cytopathological alterations were compared in BD patients taking immunosuppressive drugs and in healthy control subjects to diagnose oral cancer at an early stage due to long-term immunosuppressive drug usage.

2. Materials and methods

The study consisted of 38 patients (17 women and 21 men) with BD and 21 healthy volunteers (9 women and 12 men). The ages of the volunteers and the patients ranged from 31 to 58 years. The characteristics of the study group are presented in Table 1. Mucosal smears were obtained from patients with BD from the Department of Dermatology and the Department of Ophthalmology of the Faculty of Medicine, Atatürk University, over a 2-year period. Healthy volunteers were selected from the Department of Oral Diagnosis and Radiology in the Faculty of Dentistry, Atatürk University. All procedures followed the tenets of the Declaration of Helsinki. Written informed consent was obtained, and a form was completed detailing the patients' name, age, sex, and relevant medical history. Patients with anemia, diabetes mellitus, radiotherapy, alcohol consumption, or a history of smoking were not included in the study.

Table 1. Cytomorphometric volumetric analysis of exfoliative cytology by groups: stained smears' nucleus volume, cytoplasm volume, and ratio of nucleus and cytoplasm volumes.

Groups	Number of patients	Nucleus volume (NV, μm^3)	Cytoplasm volume (CV, μm^3)	Nucleus/cytoplasm (NC/CV) ratio
Healthy controls' oral mucosa	21	376.34 ± 31.93 ^a	77,176.69 ± 8990.34 ^a	0.00510 ± 0.0003 ^a
Nondrug-use BD patients' aphthous areas	14	482.168 ± 47.83 ^b	98,189.21 ± 11,037.73 ^b	0.00517 ± 0.0011 ^a
Nondrug-use BD patients' oral mucosa	14	277.598 ± 51.72 ^a	72,033.13 ± 16,831.07 ^a	0.00383 ± 0.0006 ^a
Colchicine-use BD patients' aphthous areas	15	628.67 ± 34.43 ^b	78,566.04 ± 2673.32 ^a	0.00753 ± 0.0022 ^b
Colchicine-use BD patients' oral mucosa	15	416.07 ± 28.05 ^a	67,000.28 ± 5899.17 ^c	0.00638 ± 0.0047 ^b
Colchicine + cyclosporine A-use BD patients' aphthous areas	9	479.92 ± 55.72 ^b	90,355.22 ± 6522.74 ^b	0.00692 ± 0.0016 ^b
Colchicine + cyclosporine A-use BD patients' oral mucosa	9	348.87 ± 94.17 ^a	72,641.22 ± 4967.45 ^{ac}	0.00514 ± 0.0001 ^a

Values are expressed as means ± standard deviations.

^{abc}: Different superscripted letters in the same column indicate significant differences between groups with analysis of variance and Duncan's post hoc test ($P < 0.05$).

The patients were divided into the following groups: healthy control, nondrug-use BD patients, colchicine-use BD patients, and colchicine + cyclosporine A-use BD patients. All smears were taken from patients' oral mucosa and oral aphthous areas.

2.1. Specimen preparation

The subjects were instructed to gargle with normal saline. The oral mucosa was dried with a gauze swab to remove surface debris and excess saliva. Smears were obtained from both aphthous and nonaphthous areas of the mouth or tongue of the BD patients using a cytobrush and transferred to clean dry glass slides. The same procedure was used to obtain smears from the controls. The slides were then immediately sprayed with a commercial fixative containing 95% ethyl alcohol and stained using the Papanicolaou technique (23).

2.2. Cytopathological examination and apoptotic cell determination

The morphology, cytopathology, and apoptosis of the stained cells were examined under a light microscope. The apoptotic analysis protocol used was as suggested in previous studies (18–20). Apoptotic cells characterized by karyorrhexis, condensed chromatin, and pyknosis were scored as apoptotic cells (Figure 1). The apoptotic cell density was then examined in 20 randomly selected areas using an approximately 20× objective with an image analysis program (Kameram SLR, 1.4.1.0, Mikro Sistem Ltd., Turkey). The apoptotic cells in the stained smears were microscopically scored. The following scale was used: + = 5–25, ++ = 25–50, and +++ = ≥50. The scores of the smears for each patient were derived semiquantitatively using light microscopy. They were scored as follows: none = -, weak = +, moderate = ++, and strong = +++.

2.3. Stereological assessment

The smears were placed on a motor-driven stage attached to a microscope, and the cells were projected onto the monitor via a camera at a magnification of 200×. The

cytoplasmic volume (CV) and the nuclear volume (NV) of smears containing suspect cells from the buccal mucosa and the aphthae of the floor of the mouth of BD patients and from the mouth or the tongue of healthy volunteers were estimated using the nucleator method (Figure 1) (24).

The following formula was used:

$$V_{(N)} = \frac{4\pi}{3} l^n / n$$

where $V_{(N)}$ is the number-weighted volume, l is the length of intercepts, and n is the number of nucleator estimates.

As mentioned in the literature, there are specific requirements for obtaining full randomness in the section-sampling stage while estimating the volume of small objects by the nucleator method (8,24,25). However, these did not need to be applied in the current study because smears were used.

2.4. Statistical analysis

For statistical analysis, differences between the cytomorphometric values were tested by ANOVA and Duncan's post hoc test using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as mean averages ± standard deviations. $P < 0.05$ was considered to be significant.

3. Results

3.1. Cytopathological results

In the stained oral epithelial cell smears, we morphologically observed 3 types of cells: pink cells, large green cells, and small green cells. These cells were classified according to the size and the color of the cellular components. The pink cells were the most keratinized cells and were located in the superficial layer of the oral epithelium. The large green cells were intermediate keratinized cells and were located in the intermediate layer. The small green cells were nonkeratinized cells, and they were located in the basal layer of the epithelium (26).

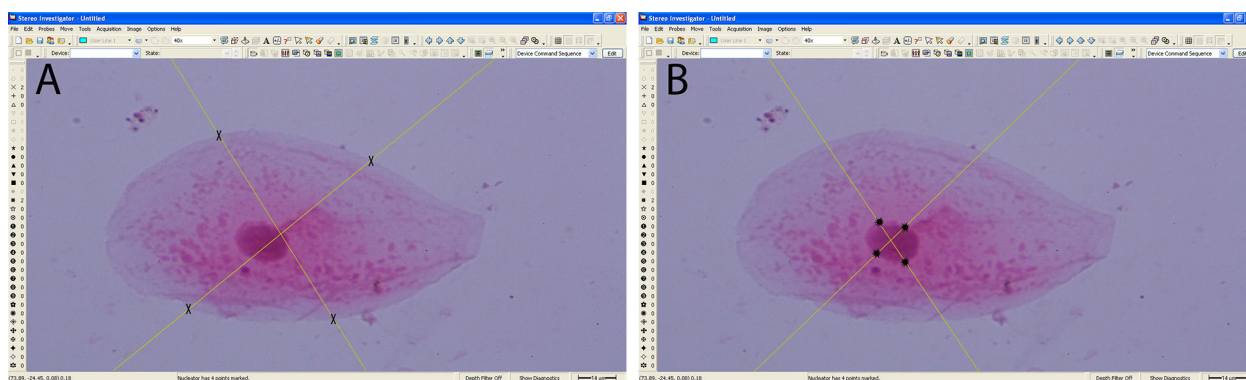


Figure 1. Illustration of the stereological estimation procedure: A) estimation of cytoplasm volume, B; estimation of nucleus volume via the nucleator method.

In the cytopathological examination of the smears from all the groups, the pink cells were observed more frequently in the healthy controls, whereas the large and small green cells were observed more often in the nondrug-use patients and occasionally in both the colchicine and cyclosporine groups. The distributions of the cell types are listed in Table 2 and Figure 2.

3.2. Apoptotic cell analysis

The semiquantitative apoptotic assessments according to degenerative nuclear changes in all the groups are presented in Table 2. A lower frequency of apoptotic cells was observed in both the aphthous areas and the oral mucosa (nonaphthous areas) of the patients with BD when compared to the healthy controls. The frequency of apoptotic cells was also higher in the oral mucosa of the healthy controls than in the aphthous areas of the immunosuppressive-taking BD patients. The highest apoptotic cell density was determined in the healthy control group.

3.3. Stereological results

The stereological examination revealed higher volumes of nuclei in the aphthous areas of the BD patients compared to the healthy controls, and the difference was statistically significant ($P < 0.05$). However, there was no significant difference between the NVs of the oral mucosa of the healthy controls and the BD patients ($P > 0.05$). When we compared the CVs among the groups, they were significantly different in the aphthous areas of BD patients and in the oral mucosa in the BD patients with or without immunosuppressive drug administration. The NV/CV ratios were increased in the aphthous areas and the increase was statistically significant in the drug-taking

group. Additionally, volumetric differences were found among the healthy controls and the BD patients either with or without immunosuppression in the nucleus, cytoplasm, and their proportions. We found a significant increase in the NVs of the aphthous areas and the oral mucosa of the patient group with and without drug use compared to the healthy controls ($P < 0.05$). However, there was no significant difference in the NVs in the aphthous areas of the nonimmunosuppressive-taking patients and the colchicine + cyclosporine A group ($P > 0.05$). In addition, the CVs of the aphthous areas of the nondrug-use and colchicine + cyclosporine A-use patients were higher than those of the other groups ($P < 0.05$). The NV/CV ratio was also higher in the colchicine and colchicine + cyclosporine groups ($P < 0.05$). The results of volumetric analysis for all groups are presented in Table 2.

4. Discussion

The present study investigated the exfoliative cytology of the oral mucosal cells of healthy controls and BD patients using immunosuppressive drugs. There was a statistically significant increase in the NVs and the CVs of the BD patients and healthy controls in the aphthous areas, nonaphthous areas, and/or oral mucosa. Furthermore, the NV/CV ratio was higher in the immunosuppressive-treated patients and this difference was statistically significant. Therefore, it appears that immunosuppressive treatment in patients with BD can affect the morphology of oral cells and activate apoptosis, potentially causing malignant transformation in patients with BD.

The use of immunosuppressive drugs can be associated with a number of adverse effects in many patients, and they can increase the risk of many diseases, such as

Table 2. Semiquantitative analysis of cytomorphological cell type and apoptotic cell density by groups.

Groups	Pink cells	Big green cells	Small green cells	Apoptotic cell density
Healthy controls' oral mucosa	+++	-	-	+++
Nondrug-use BD patients' aphthous areas	+	+++	++	+
Nondrug-use BD patients' oral mucosa	++	+	-	+
Colchicine-use BD patients' aphthous areas	+	++	++	+
Colchicine-use BD patients' oral mucosa	++	+	-	++
Colchicine + cyclosporine A-use BD patients' aphthous areas	+	+	+	+
Colchicine + cyclosporine A-use BD patients' oral mucosa	++	-	-	++

Cells densities were estimated as follows: none = -, rare = +, weak = ++, moderate = +++, strong = ++++.

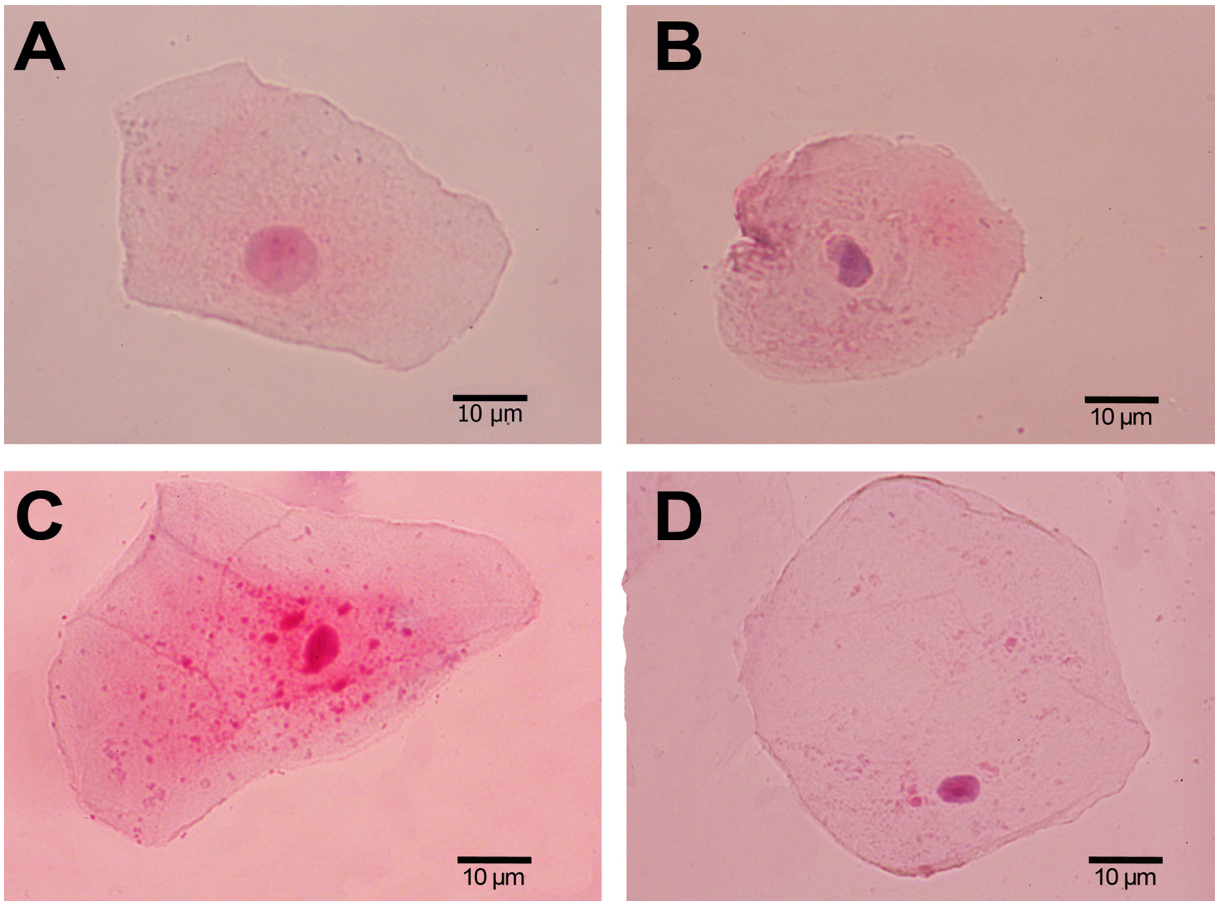


Figure 2. Exfoliated mucosal cells: normal mucosal cell (A), condensed chromatin (B), karyorrhexis (C), and pyknosis (D), with Papanicolaou staining.

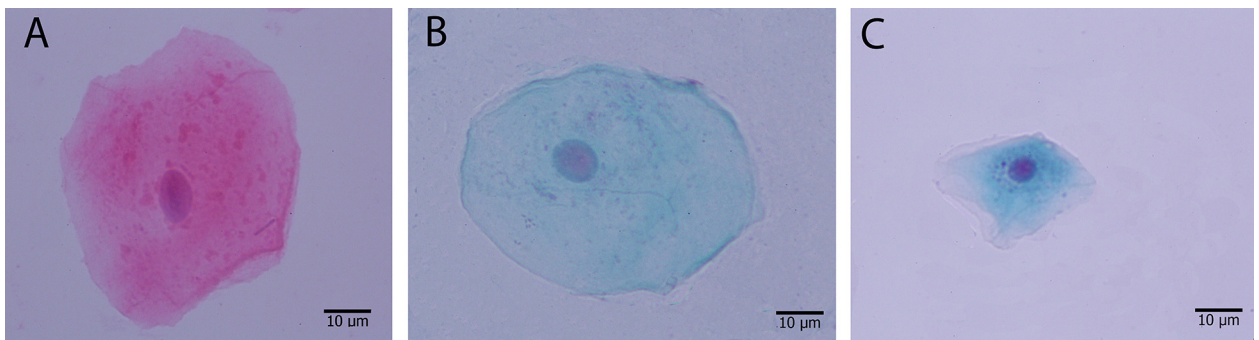


Figure 3. Illustration of exfoliative cells of oral mucosa stained smears: A) pink cell, B) large green cell, C) small green cell, with Papanicolaou staining.

hypertension, cancer (27,28), renal impairment (29–31), and skin atrophy (32,33). A number of studies have reported that immunosuppressive drugs (colchicine, cyclosporine, and infliximab) caused some pathologies, including aortic involvement, lower serum creatinine, and nephrotoxicity, in patients with BD (30,31,34). BD patients may be at a higher risk of such diseases because of

longer periods of drug administration. The present study suggests that long-term immunosuppressive drug use has cytopathological and cytomorphometric effects on oral mucosa cells.

As the induction of apoptosis results in DNA damage (35,36), we assessed the extent of oral epithelial cell apoptosis in exfoliated mucosal cells from

immunosuppressed patients with BD and healthy controls. The results revealed that the apoptotic cell density in the oral mucosa of the patients with BD was higher than in the long-term immunosuppressive drug-taking groups. In contrast, other studies suggested that apoptotic activation was lower in the peripheral lymphocytes (37), oral epithelium (21), and skin lesions of BD patients (38). The inhibitions of apoptosis in the immunosuppressive drug-taking groups may be related to the activation of proinflammatory cytokines, which are suppressed by immunosuppressive drugs (39).

In the current study, we performed a stereological analysis of the oral epithelium to determine malignant transformations associated with long-term immunosuppressive drug usage in patients with BD. In the cytomorphometric analysis, we observed a statistically significant increase in the NVs and CVs of the oral mucosa cells in the aphthous areas of the immunosuppressive-taking and nondrug-use groups ($P < 0.05$). Similarly, there was a significant increase in the mean NV/CV ratio in smears from the aphthous areas of the drug-use patients ($P < 0.05$). A previous study suggested that CVs and NVs were increased by cell functions and that the cellular CVs reached a maximum when cells were ready to divide (40). It was also noted that the increased proliferative capacity

of these cells has a negative impact on cell volumes (40). Cellular swelling is an important factor for proliferative changes in the cell cycle (32). In the present study, we demonstrated that immunosuppressive treatment in the patients with BD affected the NVs and CVs of oral cells and that these changes may point to a malignant process in the cell cycle.

In the current study, we also demonstrated that there are semiquantitative cytopathological changes in the cells of patients with BD and healthy controls (Table 1). The characteristics of the different cells in the stained smears are detailed in Table 1. In addition, there were larger numbers of nonkeratinized cells in the parabasal layer of smears from the aphthous areas of the BD patients. These cells were smaller than the other cells, but they had a larger nucleus (41). The cytomorphometric analysis revealed an increased NV/CV ratio in the aphthous areas of the BD patients. This will cause nuclei enlargement and change the NV/CV ratio of patients with BD aphthous areas.

In conclusion, BD patients taking long-term immunosuppressive drugs exhibit morphological, apoptotic, and cytomorphometric alterations in their oral mucosa. These qualitative and quantitative cytological alterations may reflect the premalignant condition of the oral cavity.

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