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Cytological and cytomorphometric characteristics of buccal mucosa cells from smokeless tobacco users

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Abstract

Background: Use of smokeless tobacco (ST) is increasing in many communities. We investigated whether ST alters the cytological and cytomorphometric features of buccal mucosa cells.

Methods: Twenty male participants who had used *Nicotiana rustica* Linn.-containing ST (Maras powder) for at least 10 years, and 20 healthy male controls who did not use ST, were included in this study. After rinsing the mouth with water, samples were taken using a toothbrush from the buccal mucosa of subjects in both groups. Samples were gently spread over a glass slide. After applying a cytofixative spray, the Papanicolaou method was used to stain the slides. The presence of dysplasia, dyskeratosis, parakeratosis, hyperkeratosis, hypergranulosis, karyorrhexis, and pyknosis was evaluated by light microscopy, as were the increment amount of candida, cocco-bacillus, and *Leptotrichia buccalis*. Cytomorphometric analysis was performed and at least 20 cells with well-defined borders were evaluated from each slide, and the cellular diameter (CD), nuclear diameter (ND), and nucleus/cytoplasm (N/C) ratio of the cells were analyzed using a 60× objective.

Results: Other than the presence of dysplasia and candida, all measured cytological parameters were significantly higher in the ST users than in the non-ST users. Furthermore, CD was lower while nuclear/cytoplasmic ratio was higher in the ST users than in those non-ST users.

Conclusion: Cytological changes associated with the use of ST, include dyskeratosis, parakeratosis, hyperkeratosis, hypergranulosis, karyorrhexis, pyknosis together with increase in the bacterial population of cocco-bacillus and *L. buccalis*. There were no significant differences in patients with dysplasia in spite of reduction of CD, increased nuclear size and N/C ratio.

KEYWORDS

buccal mucosa, cellular diameter, cytology, cytomorphometrics, Maras powder, nucleus/cytoplasm ratio, smokeless tobacco

1 | INTRODUCTION

Although smoking cigarettes is the most common form of tobacco use, it is also common for tobacco to be chewed or sequestered behind the lip, which are referred to as smokeless methods.[1] Smokeless tobacco (ST) products take different forms and names in different countries.[2–6] ST use is gradually increasing owing to expanding smoking bans in public areas and the fact that the products are inexpensive and readily available. Several countries have implemented penal sanctions for smoking and selling tobacco products.[7,8]

Maras powder (MP) is a Turkish ST. It is consumed in large amounts, especially in the southeastern Anatolia region, and particularly in Kahramanmaras and Gaziantep Provinces.[9] MP is a mixture of ash and powder obtained from the leaves of *Nicotiana rustica* Linn., which is also known as crazy tobacco.[1,10,11] The ash obtained by burning sticks of grapevine, oak, and walnut ash is added to tobacco to

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facilitate absorption by the oral mucosa, because it renders the environment alkaline. Ash/tobacco mixtures in ratios of 1:1 to 1:3 are sold in 10–20 g packets, and then slightly dampened with water. This mixture is generally placed between the cheek and gums, either directly or wrapped in cigarette paper.[12,13] It is usually removed after 5–10 min, but can remain in the mouth for up to 1–2 h.

The oral mucosa can be regarded as an indicator of the health and lifestyle habits of an individual. Although the etiology of oral squamous cell carcinoma (OSCC) is multifactorial, smoking tobacco is among the most common risk factors.[14] Tobacco typically contains large amounts of tobacco-specific nitrosamine (TSNA), which is a major carcinogen in several forms of ST.[15] *N. rustica* Linn. contains 5-fold to 8-fold more nicotine than *Nicotiana tabacum* and a higher level of TSNAs than cigars. In addition, the urinary level of cotinine, which is an indicator of tobacco consumption and nicotine intake, is threefold higher in MP smokers than in cigar smokers.[16]

To our knowledge, few cytological studies have investigated the effects of ST on the oral mucosa. Indeed, there have been no studies on the oral cytological effects of ST containing *N. rustica*, which has a greater carcinogenic effect due to its higher nitrosamine content. Therefore, this study aimed to determine the effect of MP use on the cytological and cytomorphometric parameters of the oral mucosa.

2 | MATERIAL AND METHODS

Twenty 45–76-year-old males who had used MP for at least 10 years, and twenty 42–70-year-old healthy male controls, who did not use MP, smoke cigarettes or take any medication, were included in this study. The study was approved by the Human Experiments Ethics Committee of Inonu University (Decision no. 2014/147). Written informed consent was obtained from all subjects.

A sample size of 68 subjects, 34 in each group, is sufficient to detect a clinically important difference of 20% between groups in detecting differences using a two-tailed z-test of proportions between two groups with 80% power and a 5% level of significance. The exclusion criteria were familial or personal history of oral cancers, history of cigarette use (at any time in the last 10 years), history of drug/alcohol abuse, and a history of type 1 or type 2 diabetes mellitus and local/systemic disorders.

Gum bleeding and tooth coloring were first evaluated. After rinsing the mouth with water, samples were taken from the mucosa using a toothbrush and spread onto microscope slides; samples from the mucosa of the lower lip were taken from the healthy group. A toothbrush was used because it causes minimal disturbance and enables a complete transepithelial smear to be obtained with minimal discomfort. Using moderate pressure, the brush was brushed in one direction over the area where ST is located.

The material from the brush was spread onto the two thirds of two clean, dry glass slides (Figure 1). After application of a cytofixative spray, the slides were subjected to Papanicolaou staining and examined under a light microscope.

All smears showed satisfactory staining quality with blue nuclei and orange cytoplasm. To prevent assessment bias, cytological smears were labeled in such a way that the examiner was blinded to the group assignment (case or control group) of each subject. Regarding adequacy criteria, slides with at least 30 well-preserved cells (i.e., not obscured by blood, exudate, or necrosis) from deep epithelial layers (intermediate or parabasal-basal) were considered adequate.[17]

Superficial, intermediate, and parabasal cells were mainly evaluated in all smear by using the following parameters: dysplasia grade, dyskeratosis, parakeratosis, hyperkeratosis, hypergranulosis, karyorrhexis, pyknosis, *Candida*, cocco-bacillus, and *Leptotrichia buccalis*. Inflammatory cells were not evaluated because they were scarce in both groups. Based on the below-mentioned criteria, presence, and absence of these findings were scored respectively as positive, and negative.

Parakeratosis, hyperkeratosis, and hypergranulosis are not specifically included in the Bethesda System terminology. However, they are used to describe morphologic findings.[18] Anucleated squames are the main feature of hyperkeratosis. Parakeratotic cells are found in orange or pink stains, and typically have centrally located, pyknotic nuclei. Parakeratosis, hyperkeratosis, and hypergranulosis also frequently coexist in this condition.[18] However, in our study, the presence of anucleated squames and parakeratotic cells stain orange or pink (typically centrally placed pyknotic nuclei), and cells containing cytoplasmic keratohyalin granules in at least 5 low power field on smear were considered as

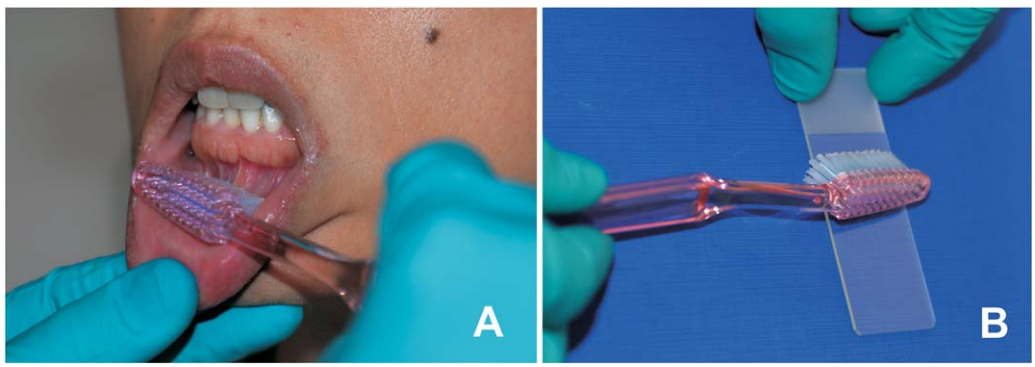

FIGURE 1 Oral brush biopsy procedure using tooth brush. A, Using moderate pressure, the tooth brush is brushed in one direction over the entire lesion multiple times. B, The material from the brush is spread on the two thirds of a clean, dried glass slide. [Color figure can be viewed at wileyonlinelibrary.com]

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hyperkeratosis, parakeratosis, and hypergranulosis, respectively.[19] Presence of more than three cells in the karyorrhexis and pyknosis were accepted as a positive. Presence of single or clusters dyskeratotic cells were also considered as positive. In a 10 high power field, the presence of more than five cells of *Candida*, cocco-bacillus, and *L. buccalis* were accepted as an increment. Cytological atypia was also evaluated using the criteria of the Bethesda system.[20] Based on Bethesda System, dysplastic cells are frequently seen as single cells, or in clusters, and sheeths. To decide whether a cell is dysplastic or not, nuclear characteristics of the cell have a determinative value. When compared with normal intermediate cell nucleus, nuclear growth, and hyperchromasia have a crucial importance for the identification of dysplasia. Dysplasia is cytologically graded based on the degree of similarity with corresponding normal cell type. Irregularities of nuclear membrane become more prominent with the grade of dysplasia. Nucleus/cytoplasm (N/C) ratio is higher in line with the severity of dysplasia.[20]

At least 20 cells with well-defined borders were evaluated from each slide by using the 60× objective lens of a trinocular model Olympus BX53 microscope, fitted with a camera (Olympus DP73) and the ProgRes version 8.0 image analysis software (cellSens, count & measure, 2015). Only clearly defined cells were included; clustered and folded cells were excluded from the analysis. The cell and nuclear outlines were monitored on the screen and the cellular diameter (CD) and nuclear diameter (ND) were automatically calculated. The nucleus-cell ratio was calculated by dividing the ND by the CD. Excel version 16 were used to analyze the data.

3 | STATISTICAL ANALYSIS

Calculations were performed using SPSS 21.0 for Windows. Data were presented Means ± SD. Mann-Whitney *U* test was used to compare continuous variable. A value of $P < 0.05$ was accepted as indicative of significance.

4 | RESULTS

The average age of the MP users and controls was 58.05 ± 9.09 and 55.95 ± 9.92 years, respectively. MP participated patients quitted smoking cigars for at least 10 years. However, most of MP users started using ST with the aim of giving up smoking and these patients had used MP ≥ 5 times/day for 10–50 years without smoking. The average period of exposure to ST was 18.9 years. The brown-black discoloration of the teeth and gingiva, and gingival recession in the area of MP application, were significantly greater in the users than in the controls (Figure 2).

Leukoplakia and mucosal erosion were seen in one case each. Brush cytology showed greater evidence of dyskeratosis, parakeratosis, hyperkeratosis, hypergranulosis, karyorrhexis, and pyknosis in the MP users than in the control subjects. Furthermore, the number of cocco-bacillus and *L. buccalis* was significantly ($P = 0.01$) higher in the MP users (Figure 3) than in the control subjects. We found no significant difference in low-grade dysplasia and *Candida* in MP users (except for only one user). The mean CD was slightly lower in the MP group than in the control group ($P < 0.01$). The mean ND was significantly higher in the MP group than in the control group ($P < 0.05$) while the mean N/C ratio was significantly higher in the MP group (Figure 4). The cytological and cytomorphometric findings of the MP user and control groups are summarized in Tables 1 and 2.

FIGURE 2 Brown-black discoloration of the teeth, gingival recession, and leukoplakia (arrow) in ST users. [Color figure can be viewed at wileyonlinelibrary.com]

5 | DISCUSSION

Approximately 30–40% of malignant neoplasms in the oral cavity are OSCC, which tend to exhibit early metastasis and a high rate of regional recurrence and are linked to exposure to tobacco.[21] MP, known locally as "crazy tobacco," is also called Hasankeyf, Turkish, Aztec, or East-Indian tobacco. Most ST products contain hazardous substances, such as nicotine, cadmium, and nitrosamines.[1] ST and some additives have been reported to have adverse effects on the oral mucosa, teeth and gingiva,[22] central nervous system,[23] cardiovascular system,[24] and digestive and urinary systems.[1] ST is also associated with a high prevalence of oral carcinoma, for example, in India. Early detection of premalignant or malignant oral lesions would improve the survival and morbidity of patients suffering from these conditions. However, in its early stages, oral cancer may appear innocuous. Therefore, there is interest in oral exfoliative cytology as a diagnostic and prognostic methodology for monitoring patients with oral premalignant and malignant lesions.[17]

To our knowledge, this is the first study in Turkey to evaluate early cytological and cytomorphometric detection of oral cancer in ST users. Previous studies focused on detecting oral premalignant and malignant lesions caused by cigarette smoking. However, little is known about the effect of ST on the oral mucosa. Al Shammari et al.[25] reported that inflammatory cell infiltrates, koilocytosis, candidiasis, and keratinization were more common (although not significantly so) among tobacco users than nontobacco users. In our study, the frequencies of detection of inflammatory cells and *Candida* were not significantly different between the control and ST groups.

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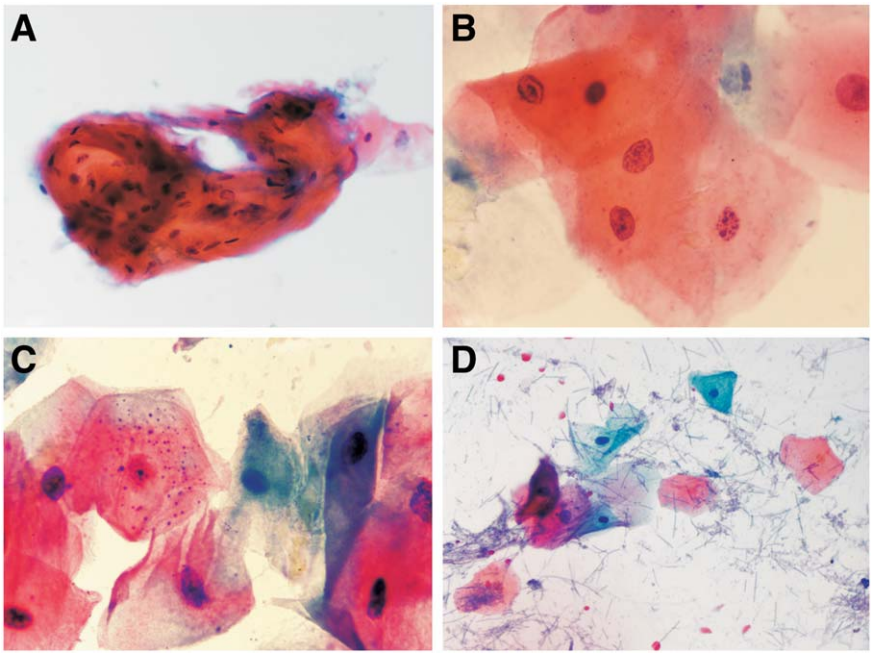

FIGURE 3 A, Dyskeratotic changes in the oral brush cytology of MP users (Pap, ×400); B, Karyorrhexis in the oral brush cytology of MP users (Pap, ×1000); C, Hypergranulosis in the oral brush cytology of MP users (Pap, ×1000); D, *L. buccalis* in the oral brush cytology of MP users (Pap, ×400). [Color figure can be viewed at wileyonlinelibrary.com]

ST influences the surface of the oral mucosa; the affected area or lesion may be white or yellowish-brown. In histological studies, the effects vary from hyperkeratosis to epithelial dysplasia.[26,27] In a study by Brima El.[2] reported that atypia, keratinization, inflammation, and infection were a higher rate in the ST group, a statistically significant difference even was not found. Desai et al.[3]; Goral et al.[4]; and Roberts[5] described keratosis and hyperkeratosis in most of the biopsy specimens. In our study, in accordance with these findings, ST users exhibited greater degrees of dyskeratosis, parakeratosis, hyperkeratosis, hypergranulosis, karyorrhexis, and pyknosis than control subjects.

Leptotrichia species (family Bacteroidaceae) are slow-growing, obligate anaerobic gram-negative members of the endogenous flora of the oral, intestinal, and genitourinary tracts. *Leptotrichia* species have been recently appreciated as causes of most human disease. Eribe et al.[28] reported that myriad pathological conditions are associated with *Leptotrichia*, including appendicitis, pneumonia, mucositis, and sepsis. There are also reports of chorioamnionitis, cavitary pneumonia, and septicemia in such cases. The numbers of *Bacillus* and *L. buccalis* were significantly higher in ST users in this study. *L. buccalis* is highly saccharolytic, and produces lactic acid, suggestive of participation in tooth decay.[28] Other effects of ST include gingival bleeding, gingival recession, tooth discoloration, gingivitis, and tooth eruption and decay.[29,30] It has also been reported to cause periodontal disease, delayed wound healing, and a decreased sense of taste and smell following long-term use.[19,20] Similar changes in our study may be associated with an increase in *L. buccalis*.

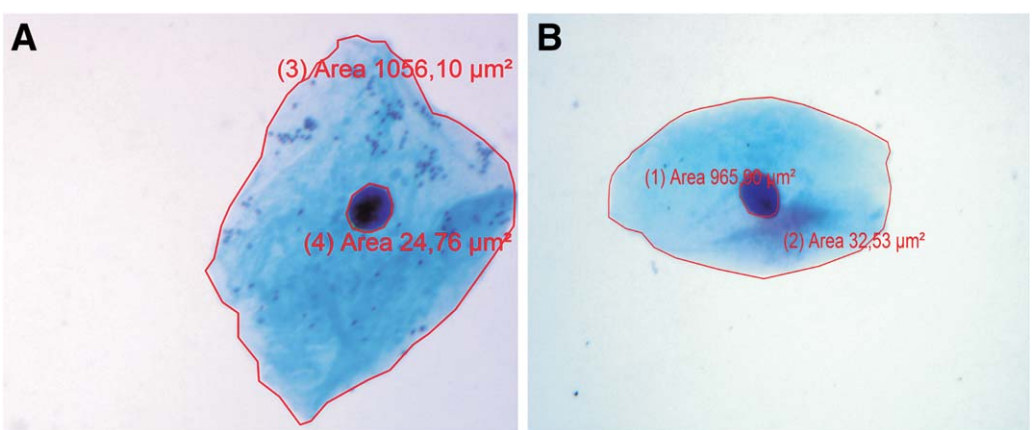

FIGURE 4 A, Cytomorphometric analysis in control group (Pap, ×600); B, Cytomorphometric analysis in MP group (Pap, ×600). [Color figure can be viewed at wileyonlinelibrary.com]

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TABLE 1 Cytological findings of Maras powder user and control group

Cytological Results	Maras powder users	Control
Dyskeratosis	6	–
Parakeratosis	13	–
Hyperkeratosis	16	4
Hypergranulosis	11	5
Karyorrhexis	8	–
Pyknosis	6	–
Increment of Coke Bacillus	13	6
Increment of *L. Buccalis*	9	–
Increment of Candida	1	–
Low-Grade Dysplasia	1	–

L. buccalis endotoxin is more potent than that of *Escherichia coli* and *Salmonella*. The organism has also been recovered from peritoneal fluid and from the blood of patients with neutropenia, HIV, leukemia, and endocarditis.[28] This suggests that *L. buccalis* may be involved in the etiology of many diseases seen in ST users, including genotoxic damage,[11] chronic respiratory diseases,[31] attention deficit hyperactivity disorder,[32] atrial conduction abnormalities,[33] and carotid intima media thickness.[34,35] Eribe et al.[28] suggested that *L. shahii* and *L. wadei* are involved in the pathogenesis of OSCC. Thus, the greater numbers of *L. buccalis* in ST users may play a role in the development of premalignant and malignant oral lesions. However, further work on this issue is needed.

As far as we know, firstly Weinmann performed the first relevant study on oral epithelium.[36] Although later on, despite conduction of many studies on this subject, and knowing that alterations in epithelial cells are indicators of dysplastic or neoplastic changes, exfoliative cytology has been always the subject matter of debates concerning the determination of premalignant, and malign lesions.[21,37] Therefore, cytomorphometric analysis may be more acceptable method because of the reliability of measurable values.

Previous studies using exfoliative cytology based on nuclear and cellular alterations could differentiate dysplastic and malignant from normal cells, and detect oral tissue alterations associated with tobacco use. Quantitative cytomorphometric parameters such as the ND, CD, and N/C ratio are useful for the diagnosis of oral lesions. Ramesh et al. reported a positive correlation between the ND and CD of the normal buccal mucosa and lesions with no epithelial dysplasia and a nonsignificant correlation in lesions with epithelial dysplasia and SCC lesions. The authors suggested that ND and CD could be useful in the diagnosis of premalignant and malignant oral lesions.[21,38] Hegde,[37] Sharma et al.,[39] Mohanta et al.,[40] and Khandelwal et al.,[41] suggested that cytomorphometric analysis of keratinocytes and a decrease in the CD of premalignant lesions facilitate early diagnosis of OSCC. Our work and that of Udayashankar et al.[21] and Ogden et al.[42] are consistent with these findings.

TABLE 2 Summary statistics of mean nuclear area, cellular area, nuclear/cytoplasmic ratio, and *P* values between the groups (area ± standard deviation in μm^2)

Group	Control group	MP group
Mean cellular area ± SD	1051,8231 ± 196,56	685,5364 ± 274,371
Mean nuclear area ± SD	24,7470 ± 5,551	28,6796 ± 3,809
Nuclear/cytoplasmic ratio	0,02353 ± 0,0035	0,04183 ± 0,0089

Cowpe et al.[43] reported that as an early indication of dysplastic changes, cell areas decrease, and indicated that cell areas decrease before decrease in nuclear areas underlying the tissues undergoing malign transformation. Also in our study, NDs were not so much different when compared with CDs between the control, and ST groups. Besides, Cowpe et al.[43] stated that malignant alterations can be detected by calculating the ratio between nuclear, and cellular sizes. However, they emphasized that it was difficult to determine normal baseline criteria based on age, and the affected region in exfoliative oral squams. Therefore, increase in the number of studies on oral exfoliative cytology dependent on the region affected, and age may contribute to determine baseline criteria in normal oral squams.

The CD was significantly lower in the ST group than in the control group, and so could be used as a marker for field alteration in oral cancer at the microscope level. The ND was slightly higher in the ST group than in the control group, in agreement with the work of Udayashankar et al.[21] and Ramesh et al.[38] This may have been due to exposure of the oral mucosa to a high concentration of carcinogens, such as reduced pyridine alkaloids, areca-derived nitrosamines, and N-nitrosamines.[21]

Contrary to precancerous lesions, the association of ST with OSCC has been comparatively well documented on an epidemiological base.[6,44–53] However, there are two different opinions on this matter. First, ST induces malignant carcinoma development over 20–50 years. Second, the relationship between leukoplakia and oral cancer development remains unclear.[26] The mean duration of ST use in our study was 18.9 years. For this reason, we could only detect dysplasia in one case.

In a study by Sharma et al., a total of five groups were investigated: control, ST, smokers, and combined (ST + smokers), and OSCC groups. One-to-one comparisons among these first four groups did not reveal any difference, while comparison of each one with OSCC group yielded significant differences as for CD, ND, and N/C.[39] Einstein et al.[54] reported that a significant difference between ST, and control groups with respect to ND, and CD was not detected, but a significant difference was found in the combined group. Significant increase in ND, and decrease in CD were found in the ST group relative to the control group (Hande et al.[55]). In a study by Ramesh et al.,[56] CD significantly decreased in the ST, and combination groups, but in the smokers group a significant decrease was not detected. However, a significant increase in the ND was found in the ST, smokers, and combination groups. In a study by Camilleri et al.[57] an increase in N/C ratio was detected during progression from benign to malignant stage. While Khandelwal et al.[42] proposed that cytomorphometric analysis of keratinocytes might be used as an aiding method in the early diagnosis of OSCC. Acharya et al.[58] detected increased ND values in the ST group

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relative to the control group, but lower than those found in the OSCC group. In many studies, even though different results were obtained among benign groups (ST, smokers, and combination groups), increase in ND/CD ratios were found in almost all OSCC groups.[42,43,55–57] Therefore, it has been reported that quantitative evaluation of oral exfoliative cytology can be used as a reliable aiding method in the determination of progressive behavior in the transition to malignant transformation. Differences seen between studies may be related to the duration, frequency of tobacco use, kind of the tobacco products used, and the severity of their impact. Indeed, *N. rustica Linn* content of Maras powder contains five- to eightfold more nicotine than *Nicotiana tabacum* and a higher level of TSNAs than cigars.[10] For this reason, in our study, increase in ND and ND/CD ratio, and decrease in CD in the ST group might be detected when compared with the control group.

In conclusion, even though this study was primarily limited by its small sample size, prominent cytological alterations induced by ST use on oral mucosa are dyskeratosis, parakeratosis, hyperkeratosis, hypergranulosis, karyorrhexis, pyknosis, and increase in the bacterial population of cocco-bacillus, and *L. buccalis*. Cytomorphometrically, in ST users decrease in CD, and increase in ND, and N/C ratio differ significantly, while any change was not encountered as for dysplasia. Therefore, large scale studies are needed where both ST users are followed up for >20 years, and also the development of malignancy is investigated.

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CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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