

## Original Article

# Ultrastructural analysis of oral exfoliated epithelial cells of tobacco smokers and betel nut chewers: A scanning electron microscopy study

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## ABSTRACT

**Background:** The study was undertaken to correlate epithelial surface pattern changes of oral exfoliated cells of tobacco smokers and betel nut chewers and also to compare them with patients of oral squamous cell carcinoma (OSCC) and healthy individuals.

**Materials and Methods:** In this cross-sectional study, a total of fifty persons were included in the study, out of which thirty formed the study group (15 each tobacco smokers and betel nut chewers) and twenty formed the control group (ten each of OSCC patients – positive control and ten normal buccal mucosa – negative control). Their oral exfoliated cells were scraped, fixed, and studied under scanning electron microscope (SEM). The statistical analysis was determined using ANOVA, Tukey honestly significant difference, Chi-square test, and statistical SPASS software,  $P < 0.05$ .

**Results:** OSCC, Individual cell modifications, intercellular relationships and surface characteristics observed by scanning electron microscopy between OSCC, tobacco smokers, betel nut chewers compared to normal oral mucosa have been tabulated.

**Conclusion:** In normal oral mucosa, cell surface morphology depends on the state of keratinization of the tissue. Thus, it could prove helpful in detecting any carcinomatous change at its incipient stage and also give an insight into the ultra-structural details of cellular differentiations in epithelial tissues..

**Key Words:** Analysis, betel nut, cells, chewing, exfoliated, oral, scanning electron microscopy, tobacco smoke, ultrastructure

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## INTRODUCTION

Environmental factors play an important role in the pathogenesis of carcinomas of the head and neck, and tobacco smoking is considered as one of the major risk factors.<sup>[1]</sup> Exposure to tobacco can be in the form of smoking or smokeless habits.<sup>[2]</sup> Betel quid chewing is an ancient practice common in many countries of Asia and among migrated communities in Africa, Europe, and North America.<sup>[3]</sup> The term areca nut is

used to denote the unhusked whole fruit of the areca nut tree, and term betel nut is used exclusively to refer to the inner kernel or seed which is obtained after removing husk.<sup>[4]</sup>

Histological changes of the oral mucosa have been reported in association with exposure to tobacco, and betel nut chewers along with exfoliative cytological

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techniques have also been applied to examine the effects of tobacco and betel nut chewing on the oral mucosa.<sup>[2]</sup> Exfoliative cytology, a simple, noninvasive diagnostic technique to microscopically examine shed or desquamated cells from the epithelial surface, usually the mucous membrane, could increase the chances of early detection of premalignant and malignant lesions.<sup>[1,5]</sup>

In the past, histopathological and cytological examinations of lesions were only used for the diagnosis of all carcinomas. However, recent advances in cancer research include the use of electron microscopy. Scanning electron microscopy (SEM) cannot contribute to routine diagnostic procedures, but it can add to the understanding of the biology of disturbances of epithelial differentiation in malignancy. Studies in the past under SEM have graded the malignant cells as abnormal on the basis of their size, shape, and general architectural and surface features.<sup>[6]</sup> The oral epithelium exhibits regional variations in the degree of keratinization. The surface ultrastructure has been described as specific to the type of keratinization; fully keratinized cells being described as having pitted surface appearance and nonkeratinized cells having surface micro-ridges.<sup>[6]</sup>

The present study was undertaken to correlate epithelial surface pattern changes of oral exfoliated cells of tobacco smokers and betel nut chewers and also to compare them with oral squamous cell carcinoma (OSCC) and healthy individuals, under SEM.

## MATERIALS AND METHODS

### Selection of subjects

This cross-sectional, analytical study was conducted in the Department of Oral Pathology and Microbiology and Birbal Sahani Institute of Paleobotany, Lucknow, India after ethical approval (Ethical No. CPGIDSH/IEC-2/0016/2013).

### Inclusion criteria

- Persons with habits of betel nut chewing 5-10gms per day for 5 years and above
- Persons with habits of tobacco smoking 5–10 packets per day for 5 years and above
- Persons between the ages of 25 and 65 years both males and females.

### Exclusion criteria

- Persons with combination habits of tobacco smoking and betel nut chewing
- Patients with any systemic disease and immunocompromised patients

- Patients with any oral potentially malignant disorder.

Exfoliated oral epithelial cells were taken only from the buccal mucosa of all the 15 patients into tobacco smokers group (Group III) ; the 15 patients in betel nut chewers group (Group IV); the ten patients with histologically confirmed OSCC in positive control group; (Group I) and the ten individuals with normal buccal mucosa in negative control group (Group II).

### Smear procedure

Cells were scraped from the buccal mucosa of all the four groups individually with the help of wooden spatula and were placed on coverslips and immediately fixed with 2.5% glutaraldehyde solution at pH of 7.4 for 2 h.

### Scanning electron microscopy

The specimens were mounted on aluminum stubs using double-sided adhesive tape; after drying under vacuum, they were coated with gold-palladium coating by Polaron Sputter for 2 min and then placed into the SEM (LEO 430 Oberkochen, Germany) after mounting over the metallic stub. Each specimen was scanned at an accelerating voltage of 15–30 kV at different magnifications. The areas selected for photography were those where maximum regularly shaped polygonal cells were observed with raised central contours indicating the position of cell nucleus; only the images of the representative areas were photographed. At higher magnification, the surface was seen to be composed of fine granular protrusions with a tendency to form numerous short discontinuous parallel micro-ridges along the cell surface. During visualization of specimens, the different surface morphologies of the cells of interest were marked in master chart of SEM findings of respective groups and appropriate images were stored and later photographed.

The normal samples (control group) were scanned first to form a base for the interpretation of samples for different SEM characteristics of exfoliated oral epithelial cells. Then, the samples of tobacco smokers and betel nut chewers were subjected to a preliminary scan to note the different SEM characteristics of exfoliated oral epithelial cells. Based on preliminary trial scan and review of literature, cell modifications, intercellular relationships, and surface characteristics such as micro-ridges, microvilli, and surface structures such as blebs, spherical, or cylindrical structures were looked in the cases of tobacco smokers and betel nut

chewers. Comparisons were made between the study and control groups.

The statistical analysis was determined using ANOVA, Tukey honestly significant difference, Chi-square test, and Statistical Package (window version 18.0: PSAAW).  $P < 0.05$  (significant) was determined and analysis was performed on SPASS software

## RESULTS

The present SEM study of surface exfoliated epithelial cells helped us analyze the general architectural and surface structural differences between tobacco smokers, betel nut chewers, patients with OSCC, and normal oral epithelial cells.

The cell modifications are observed in the groups and summarized in Table 1. The frequency (%) of cell modifications varied significantly ( $P < 0.05$  or  $P < 0.01$  or  $P < 0.001$ ) among the groups, except degenerative changes. The frequency of abnormal size variation ( $\chi^2 = 10.82$ ,  $P = 0.013$ ), aberrant forms ( $\chi^2 = 11.49$ ,  $P = 0.009$ ), and surface pattern irregularity ( $\chi^2 = 19.44$ ,  $P < 0.001$ ) was found significantly higher in Groups I, III, and IV as compared to Group II.

The intercellular relationships between the groups are summarized in Table 2. The frequency (%) of intercellular relationships varied significantly ( $P < 0.05$  or  $P < 0.01$  or  $P < 0.001$ ) among the groups,

except dense grouping of cells and loss of close intercellular adherence. The frequency of both surface pattern irregularity ( $\chi^2 = 9.50$ ,  $P = 0.023$ ) and absence of distinct cell borders ( $\chi^2 = 13.33$ ,  $P = 0.004$ ) was found significantly higher in Groups I, III, and IV than Group II. In contrast, the frequency of apparent cell cannibalism in both Groups III and IV was found to be significantly higher as compared to both Groups I and II ( $\chi^2 = 26.73$ ,  $P < 0.001$ ).

The SEM surface characteristics are summarized in Table 3. The frequency (%) of most of the SEM surface characteristics were found similar ( $P > 0.05$ ) among the groups, except cylindrical structures, surface evaginations, slender strands, and filopodia. The frequency of cylindrical structures was found significantly higher in Groups I, III, and IV than Group II ( $\chi^2 = 12.43$ ,  $P = 0.006$ ). In contrast, the frequency of surface evaginations was found significantly different and higher in Group I as compared to both Group III and IV ( $\chi^2 = 20.24$ ,  $P < 0.001$ ). Conversely, the frequency of both slender strands ( $\chi^2 = 17.39$ ,  $P = 0.001$ ) and filopodia ( $\chi^2 = 8.33$ ,  $P = 0.040$ ) was found significantly higher in Group I as compared to Groups II, III, and IV.

## DISCUSSION

This study demonstrated that general architectural and surface structural differences existed between tobacco

**Table 1: Individual cell modifications observed in groups by scanning electron microscope**

Individual cell modifications	Group I (control-P), <i>n</i> =10; %	Group II (control-N), <i>n</i> =10; %	Group III (tobacco chewers), <i>n</i> =15; %	Group IV (betel nut chewers), <i>n</i> =15; %
Abnormal size variation	6; 60.0	0	8; 53.3	9; 60.0
Aberrant forms	5; 50.0	0	10; 66.7	8; 53.3
Degenerative changes	4; 40.0	5; 50.0	9; 60.0	8; 53.3
Surface pattern irregularity	8; 80.0	0	12; 80.0	10; 66.7

**Table 2: Intercellular relationships observed in groups by scanning electron microscope**

Intercellular relationships	Group I (control-P), <i>n</i> =10; %	Group II (control-N), <i>n</i> =10; %	Group III (tobacco chewers), <i>n</i> =15; %	Group IV (betel nut chewers), <i>n</i> =15; %
Surface pattern irregularity	6; 60.0	0	8; 53.3	7; 46.7
Absence of distinct cell borders	7; 70.0	0	10; 66.7	8; 53.3
Dense grouping of cells	5; 50.0	5; 50.0	9; 60.0	8; 53.3
Apparent cell cannibalism	0	0	12; 80.0	10; 66.7
Loss of close intercellular adherence	0	0	0	0

**Table 3: Surface characteristics observed in groups by scanning electron microscope**

SEM surface characteristics	Group I (control-P), n=10; %	Group II (control-N), n=10; %	Group III (tobacco chewers), n=15; %	Group IV (betel nut chewers), n=15; %
Smooth surface	5; 50.0	7; 70.0	11; 73.3	10; 66.7
Granular surface	6; 60.0	5; 50.0	12; 80.0	8; 53.3
Discontinuous micro-ridges	7; 70.0	5; 50.0	11; 73.3	10; 66.7
Continuous parallel micro-ridges	6; 60.0	8; 80.0	8; 53.3	7; 46.7
Honeycomb of micro-ridges	6; 60.0	5; 50.0	13; 86.7	11; 73.3
Pitted surface	6; 60.0	5; 50.0	12; 80.0	13; 86.7
Finger-like microvilli	5; 50.0	0	6; 40.0	7; 46.7
Stubby club-shaped microvilli	6; 60.0	2; 20.0	10; 66.7	10; 66.7
Spherical blebs	4; 40.0	3; 30.0	11; 73.3	10; 66.7
Spherical structures	6; 60.0	2; 20.0	9; 60.0	7; 46.7
Cylindrical structures	4; 40.0	0	10; 66.7	9; 60.0
Surface evaginations	6; 60.0	2; 20.0	0	0
Slender strands	4; 40.0	0	0	0
Surface ruffles	3; 30.0	0	1; 6.7	1; 6.7
Filopodia	2; 20.0	0	0	0

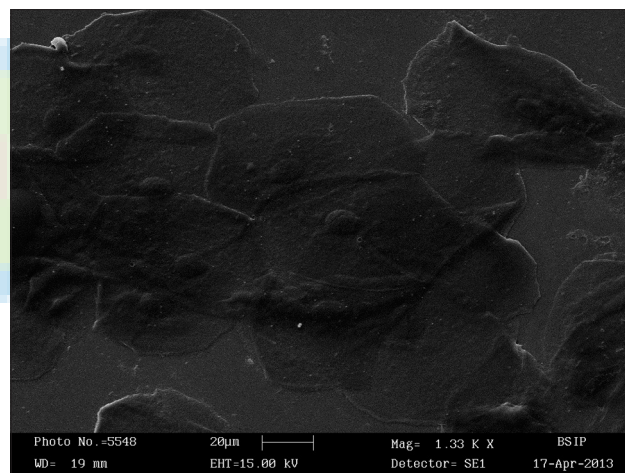
SEM: Scanning electron microscope

smokers, betel nut chewers, patients with OSCC, and normal oral epithelial cells and these could not be solely attributed to keratinizing changes.<sup>[7]</sup>

When changes in a cell as whole were observed, surface pattern irregularity was a characteristic feature for both tobacco smokers and betel nut chewers where cells often showed regional variation in surface appearance, whereas normal exfoliated oral epithelial cell showed regular polygonal outline [Figure 1].<sup>[6,7]</sup> The frequency of cell modifications varied significantly among the groups, except degenerative changes. Abnormal size variation, aberrant forms, and surface pattern irregularity were found higher in OSCC > tobacco smokers >betel nut chewers >normal mucosa.

Apparent cell cannibalism was a characteristic feature for both tobacco smokers and betel nut chewers and was found to be significantly higher as compared to both OSCC and normal exfoliated oral epithelial cells. Surface pattern irregularity and absence of distinct cell borders were found higher in OSCC > tobacco smokers > betel nut chewers > normal mucosa. There were greatly altered individual cell surface patterns for tobacco smokers' and betel nut chewers' cell surface when compared to normal and oral carcinomas.

Characteristic findings in tobacco smokers were cell surface covered by a honeycomb arrangement of micro-ridges (most), [Figure 2] granular surface (cell surface covered by many small granular

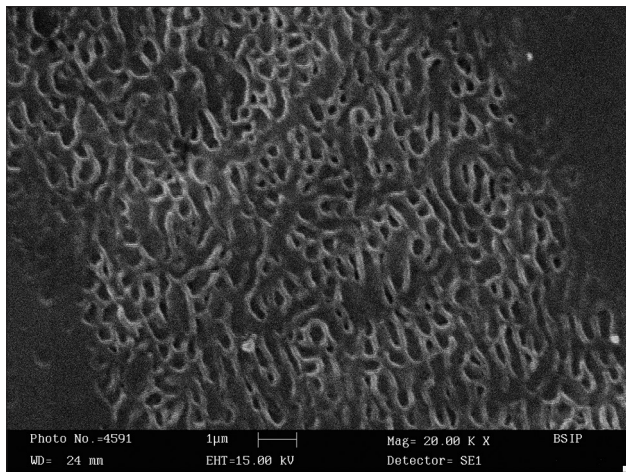


**Figure 1:** Scanning electron microscope of Group II showing cells with regular polygonal outline, distinct cell border, and granular surface (x1.33).

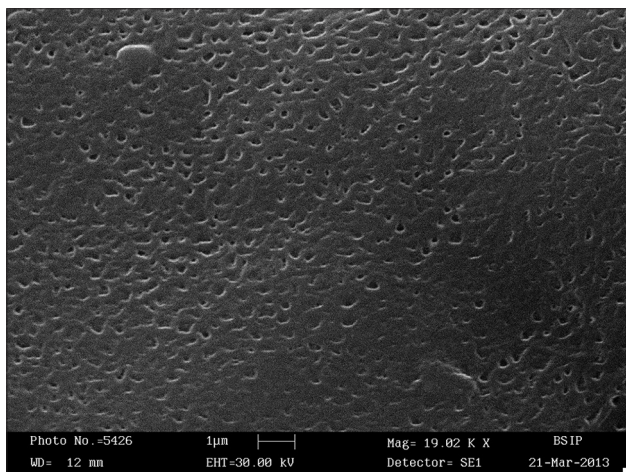
protrusions gave appearance of fine sandpaper), cell surface pitted with holes, smooth surface, cell surface with many small discontinuous parallel micro-ridges interspersed with granular protrusions, cell surface displaying numerous spherical blebs, cell surface covered by numerous stubby club-shaped microvilli, and cell surface with many associated cylindrical shaped structures of variable length in descending order.

In betel nut chewers, cell surface pitted with holes (most) [Figure 3], smooth surface, cell surface with many small discontinuous parallel micro-ridges interspersed with granular protrusions, cell surface





**Figure 2:** Scanning electron microscope of Group II showing honeycomb of micro-ridges ( $\times 20.00$ ).



**Figure 3:** Scanning electron microscope of Group III showing pitted surface pattern ( $\times 19.02$ ).

covered by numerous stubby club-shaped microvilli, and cell surface displaying numerous spherical blebs and cylindrical structures in descending order were observed. On comparison of these characteristic features of tobacco smokers and betel nut chewers with oral carcinoma, the most unique characteristic observed was cell surface with small discontinuous parallel micro-ridges being interspersed with granular protrusions whereas cell surface with continuous parallel micro-ridges was seen in normal cells.

To the best of our knowledge, there is no literature which compares these morphological features between tobacco smokers and betel nut chewers; thus, we could state that in this study the effects of tobacco smoking appeared to be more disruptive at its nascent stage.

Previous SEM studies of keratinized and nonkeratinized oral epithelium have shown that the two types of

epithelium show different surface structures. In the present study, the presence of micro-ridges arranged in parallel rows or an irregular honeycomb pattern appeared to be characteristic of an epithelium undergoing keratinization. Similar micro-ridges or micro-rugae have been observed in normal squamous cervical epithelium undergoing keratinization. Exfoliated cervical cells have also been observed to show similar micro-ridges.<sup>[7]</sup> On the other hand, nonkeratinized oral epithelium appeared to be characterized by a fine, granular surface pattern which exhibits a tendency to form short discontinuous parallel micro-ridges.

Thus, we could state that SEM gave an enhanced and lucid insight into the exfoliated oral epithelial cells of tobacco smokers and betel nut chewers, which revealed a modified architecture and altered surface characteristics not dependent on the site and histological differentiation of the lesion. To the best of our knowledge, after going through the literature available on PubMed, our study could be called the first of its kind in observing the morphological features of oral exfoliated epithelial cells of tobacco smokers and betel nut chewers and compared them with normal cells and cells obtained from OSCC patients. Thus, our study reinstates the fact that tobacco and betel nut do cause harm and ultrastructural changes in the oral mucosa even though they are not apparent early clinically in the oral cavity. Thus, it could be beneficial in detection of any early carcinomatous change and also give an insight into the ultrastructural details of cellular differentiations in epithelial tissues.

## CONCLUSION

The study could be concluded as individual cell modifications, intercellular relationships and surface characteristics observed by scanning electron microscopy between OSCC, tobacco smokers, betel nut chewers compared to normal oral mucosa have been tabulated and further it could prove helpful in detecting any carcinomatous change at its incipient stage and also give an insight into the ultra-structural details of cellular differentiations in epithelial tissues.

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Nil.

## Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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