ORIGINAL ARTICLE

A phase contrast cytomorphometric study of squames of normal oral mucosa and oral leukoplakia: Original study

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ABSTRACT

Oral leukoplakia represents the most common potentially malignant oral disorder, representing 85% of such lesions. The worldwide prevalence of leukoplakia is 1.5- 4.3%. Leukoplakia is often associated with carcinogenic exposures, such as from use of tobacco, alcohol or betel nut. The level of risk for malignant transformation of leukoplakia is associated with lesion histology. The overall malignant transformation rates for dysplastic lesions range from 11% to 36%, depending on the length of follow-up. Exfoliative cytology is a simple and minimally invasive method. Phase contrast microscope, an essential tool in the field of biology and medical research provides improved discrimination of cellular details. **Aims:**To study and compare the cytomorphological and cytomorphometric features of squames obtained from the mucosa of normal individuals, tobacco habituates with and without clinically evident leukoplakia. To assess the role of phase contrast microscopy as an alternative and easy method of cytological evaluation of wet and unstained smears. **Materials and Methods:** Fifty cases from each group were taken. Fixed, unstained smears were viewed under phase contrast microscope and were evaluated morphologically and morphometrically for nuclear and cellular diameters. **Results:** The study showed a significant increase in the mean nuclear diameter and decrease in the mean cellular diameter. **Conclusion:**Cytomorphometric changes could be the earliest indicators of cellular alterations. This indicates that there could be a cause-effect relationship between tobacco and quantitative alterations.
Key words: Cytomorphometry, exfoliative cytology, leukoplakia, phase contrast microscopy

INTRODUCTION

Leukoplakia is the most common potentially malignant disorder defined by Warnakulasuriya *et.al*, 2007 as- "recognizable white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer".[1-3] Many etiological factors have been implicated in leukoplakia, but tobacco plays a major role. With regard to tobacco chewing, leukoplakia was diagnosed among 6.1% of people who chewed betel-tobacco as well as smoked and among 1.8% of betel-tobacco chewers. The risk for leukoplakia was estimated to be 60 times higher in daily chewers as compared to non-chewers.[4] Leukoplakia is a clinical diagnosis and may show dysplastic features histopathologically. Biopsy still being the gold standard for confirmatory diagnosis, can be supplemented by exfoliative cytology as an adjunct, with an advantage of being painless, non-invasive and very well accepted by patients causing little discomfort.[5,6] Oral smears from areas of leukoplakia may reveal the degree of epithelial atypism and early malignant changes. Screening populations for the early detection of precursor lesions is an attractive strategy to reduce the burden of OSCC (Oral squamous cell carcinoma).[7]

The rationale of oral exfoliative cytology is based on examining the cells that are physiologically desquamated or abraded from the surface of the oral mucosa. Alterations in these cells can serve as reliable indicators of dysplastic or neoplastic changes.[5] Exfoliative cytology is a method that gives better insight into the nuclear and cellular details of individual cells.[8]

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Qualitative analysis and quantitative cytomorphometric assessments of exfoliated buccal cells have shown measurable changes in cells obtained from malignant and premalignant lesions. Quantitative parameters such as nuclear size, cell size, nuclear to cytoplasmic ratio, nuclear shape, nuclear discontinuity, optical density and nuclear texture can be evaluated collectively in order to improve its diagnostic sensitivity. Of these parameters, the nuclear size, cytoplasmic size and their ratio have been shown to be significant in the evaluation of oral lesions.[5,8,9]

Though these features can be appreciated using light microscopy, the visualization of viable cellular specimens is difficult, and hence, different forms of phase microscopy have been devised, wherein contrast is enhanced by manipulation of the optical path.[10]

Phase contrast microscopy provides improved observation of the cellular details by rendering the differences in refractive indices between regions of the specimen visible in the form of differences in intensity resulting in high-contrast images. Thus, phase contrast microscopy can prove to be a useful method to study morphologic changes in epithelial cells. The cells can be observed without the changes induced by staining procedures, and the slide preparation is quick and requires very little effort, and it can be immediately visualized and evaluated, which is an advantage over light microscopy.[11]

Thereby, we have conducted a study to identify cytomorphometric features of exfoliated keratinocytes that could signify an impending dysplastic change. Due to the increasing interest in the cytological diagnosis, the present study was undertaken to assess the cytopathological changes and morphometric changes of cell diameter (CD) and nuclear diameter (ND) of squames from oral leukoplakia under phase contrast microscope, as it appears to offer a distinct advantage over light microscopy for quick, comprehensive and quantitative assessment of the study material.

MATERIALS AND METHODS

Oral smears were obtained from the oral mucosa of healthy individuals, tobacco habituates with and without clinically evident leukoplakia from the outpatient department of Krishnadevaraya College of Dental Sciences and Hospital, Bangalore, Karnataka.

Patient selection

The study groups were divided as follows:

- Group I/Control Group: 50 cases with no history of tobacco related habits and no associated lesions
- Group II: 50 cases with history of tobacco related habits in any form with no evident clinical lesion
- Group III: 50 cases with history of tobacco habits in any form and clinically evident leukoplakia.

Inclusion criteria

Habituates of tobacco in any form for more than five years in the age group of 3rd to 6th decade of life. For group III, histopathologically diagnosed cases of leukoplakia were included.

Exclusion criteria

Individuals with no other known local/systemic disorders, medically-compromised and immunocompromised patients, patients with anemia and blood dyscrasias, patients aged <20 years and >60 years were excluded from the study to avoid cellular changes associated with these conditions.

The smears were obtained after obtaining a written consent from the patient and were fixed in 95% alcohol for 24 h. One slide was stained using the Papanicolaou staining (PAP Stain) method, other slide was unstained, unmounted and used for morphometry. Biopsies were performed for group III to confirm the diagnosis.

Cytomorphological features

For each case, the representative unstained and unmounted slide was taken and the cytomorphological features were evaluated [Table 1].

Cytomorphometric analysis

Exfoliated cells were visualized using a phase contrast microscope with camera attachment. For each case, areas with uniformly spread exfoliated cells were selected, and each cell was viewed under 20× and 40× magnifications for assessing the morphological features. For morphometry, photographs were taken under 10× magnification and 100 cells were chosen, where in the nuclear diameter and cell diameter was calculated using automated image analysis software [Figures 1-3]. Only clearly defined cells were considered for measurement whereas clumped, folded cells and unusually distorted nuclei were avoided. The mean nuclear and cellular diameters in both the planes were measured (microns) and a mean for the 100 cells was calculated.

One way ANOVA (Analysis of Variance) was used for comparing the parameters for multiple groups. Comparison of the mean nuclear and cellular diameter values between groups was made using Tukey's multiple post-hoc procedure.

RESULTS

The study groups with gender distribution showed female predominance for group I and male predominance for group II and III. Distribution of study subjects by types of

Table 1: Cytomorphological features evaluated

Cytoplasmic characteristics
Cytoplasmic borders
a) Poorly defined
b) Well defined
c) Regular and even
d) Irregular and uneven
Cytoplasmic area
a) Scanty
b) Moderate
c) Abundant
Nuclear characteristics
Shape of the Nucleus
a) Spherical
b) Oval
c) Irregular
Size of the Nucleus
a) Small
b) Medium
c) Large
Nuclear outline
a) Poorly defined
b) Well defined
Nuclear-Cytoplasmic ratio
a) Normal
b) Moderately increased
c) Highly increased
Perinuclear Halo
a) Absent
b) Present

clinical cases showed homogenous leukoplakia (60%) and non-homogenous leukoplakia (40%).

Nuclear diameter

The mean value of the nuclear diameter in group III smears were the highest when compared with those of the other two groups [Figure 4].

A pair wise comparison of three categories (I, II, III) with respect to nuclear diameter in μm showed a *P* value of 0.0000 (<0.05), which was highly significant [Table 2].

Cellular diameter in various study groups

The mean value of cellular diameter in group III smears was lowest when compared with those of the other two groups [Figure 5].

A pair wise comparison of three groups (I, II, III) with respect to cellular diameter in μm showed a *P* value of 0.0000 for group I vs. group II and group I vs. group III and a *P* value of 0.0450 for group II vs. group III. All values were < 0.05, which was highly significant [Table 3].

DISCUSSION

Oral leukoplakia represents the most common potentially malignant disorder of the oral cavity.[3] There is a strong male predilection (70%) except in regional populations in which women use tobacco products more than men do, this was consistent with our study.

All major forms of tobacco use such as cigarettes, cigars, pipes and smokeless tobacco are known to cause oral cancer. Oral smears from areas of leukoplakia may reveal a degree of epithelial atypism and early malignant change.

Previous studies have stated the use of unstained and unfixed smears for observation under phase contrast microscope.[10] We modified the technique by fixing the smears and using them unstained. This modification was done to preserve the material for a longer time as immediate observation and diagnosis would be difficult in certain situations as in screening camps.

Cytomorphometric changes under the phase contrast microscope

Nuclear diameter

This refers to the maximum width of the nucleus. Due to the increase in DNA synthesis in the cells exposed to carcinogenic agents, significant changes occur in the nuclear diameter in dysplasia and malignancy. We observed that the nuclear diameter appreciably increased from group I to II and from II to III.

Cell diameter

This refers to the maximum dimension of the cell. Variation in cell diameter is a common finding in the malignant lesions, and it was observed that cell diameter appreciably decreased from group I to II and similarly from group II to III.

Our above results were in accordance with the study conducted by Hande, Chaudhary (2010) to assess the effect of tobacco chewing on buccal mucosa by using cytomorphometry.[12-14]

Cytomorphological features observed under phase contrast microscope

The pattern of occurrence of the epithelial cells

These cells appeared as sheets or singly shed in smears. Direct scraping produces sheets of cells rather than isolated cells as seen in spontaneous exfoliation. The nature of the lesion also plays an important role, i.e. in dysplasia and carcinoma *in situ*, the dysplastic cells exfoliate singly and are scattered throughout the smear.[15] In our study, groups I, II and III showed 80%, 75% and 70% of cells spread in sheets and 20%, 25% and 30% of cells were arranged singly, respectively. A decrease in mutual cellular adhesion between atypical epithelial cells is an important criterion of malignancy,

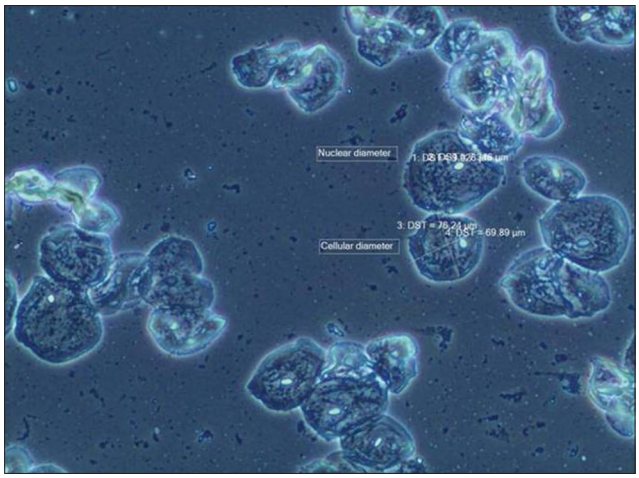

Figure 1: Phase contrast photomicrograph (×100) of exfoliated cells showing morphometric measurement of cellular and nuclear diameter for group I

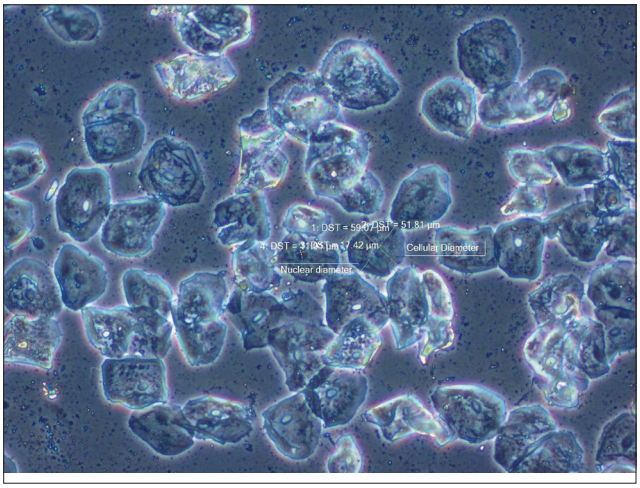

Figure 2: Phase contrast photomicrograph (×100) of exfoliated cells showing morphometric measurement of cellular and nuclear diameter for group II

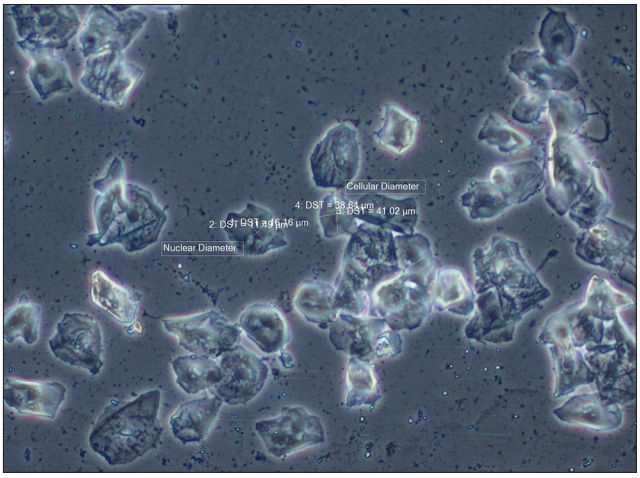

Figure 3: Phase contrast photomicrograph (x100) of exfoliated cells showing morphometric measurement of cellular and nuclear diameter for group III

Table 2: Pair wise comparison of three groups (I, II, III) with respect to nuclear diameter in µm by Tukey's multiple post-hoc procedures

Group	Mean difference	Std. error	*P* value	95% confidence interval	
				Lower	Upper
Group I *vs.* Group II	−0.84200*	0.1334	0.0000	−1.1578	−0.5262
Group I *vs.* Group III	−2.18200*	0.1334	0.0000	−2.4978	−1.8662
Group II *vs.* Group III	−1.34000*	0.1334	0.0000	−1.6558	−1.0242

*: The mean difference is significant at the 5% level (P<0.05)

Table 3: Pair wise comparison of three groups (I, II, III) with respect to cellular diameter in µm by Tukey's multiple post-hoc procedures

Group	Mean difference	Std. error	*P* value	95% confidence interval	
				Lower	Upper
Group I *vs.* Group II	5.38860*	0.9339	0.0000	3.1775	7.5997
Group I *vs.* Group III	7.63980*	0.9339	0.0000	5.4287	9.8509
Group II *vs.* Group III	2.25120*	0.9339	0.0450	0.0401	4.4623

*: The mean difference is significant at the 5% level (P<0.05),
Std: Standard deviation

which is the result of abnormalities in the expression of the intercellular adhesion molecules. Thus, tumor cells tend to shed singly and in abundance.[16]

Layer of epithelial cells

The layers of the epithelial cells from where they have been exfoliated were evaluated using PAP stain. The intensity and color of the cell helps in diagnosing the nature of the lesion. An abnormal amount of cytoplasmic keratinisation produces a glassy, deep orange stain as seen in hyperkeratinised cells exfoliated from a well-differentiated SCC. Generally, the cytoplasm is basophilic in the cells of immature or poorly differentiated carcinoma.[16]

In our study, 99% of cells for group I, II and III were obtained from the superficial layers. No cells were obtained from the parabasal and basal area. Thus, only the superficial cells were used to evaluate the morphometric changes. The changes occurring in the superficial cells help us to evaluate the changes that would be occurring in the deeper part of the epithelium.

Shape of the cells

The extreme variation in shape of the cytoplasm of cells is diagnostic. Many factors influence the shape, it may be related to the cellular composition i.e. the thickness and amount of cytoplasm, rigidity and thickness of the cytoplasmic membrane. Other factors are extrinsic i.e. sampling technique and the pressure exerted by the surrounding cells. The shape variation can also be the result of mechanical distortion or of cellular regeneration.[16]

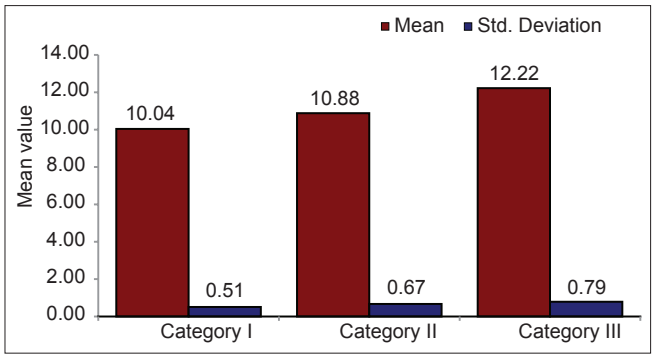

Figure 4: Comparison of three categories (I, II, III) with respect to nuclear diameter in μm

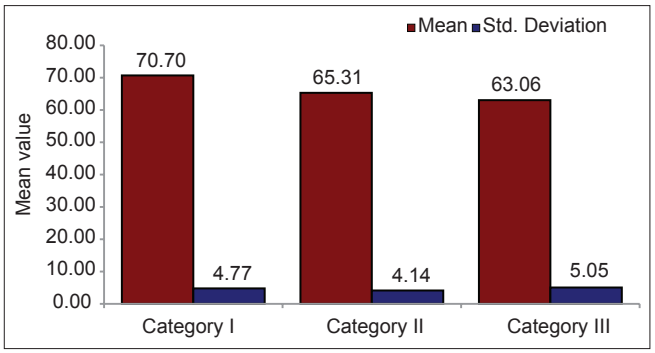

Figure 5: Comparison of three categories (I, II, III) with respect to cellular diameter in μm

When the cell shape was compared among the different study groups, most of the cells were polygonal to oval in shape in group I and II. However, in group III the cells showed an irregular shape.

Cytoplasmic borders

The cytoplasmic boundary is sharp, distinct and regular in some malignant cells (flattened, well differentiated, keratinizing, infiltrating SCC) or indistinct and heavy, as in the case of a thick, spherical, malignant cell with scanty cytoplasm (undifferentiated carcinoma). The irregularity of the cytoplasmic borders (if thin and frayed) can indicate that a portion of the cytoplasm has been lost traumatically.[16]

We observed that phase contrast microscopy provided a better visualization of the cytoplasmic borders. In Group I and II, 90% of the cases showed a distinct, well-defined, regular and an even border. However, in group III, 40% of the cases showed borders that were well-defined, irregular and uneven.

Cell area

It is the space occupied by the cell. The cell area is often found to be decreased in the dysplastic conditions due to the increase in the nuclear content. The amount of cytoplasm helps not only in the diagnosis of malignancy but also in determining the nature and degree of differentiation of the neoplasm. The cytoplasm of the dysplastic and malignant cells does not usually enlarge in the same proportion as the nucleus.[16]

In our study, group I cases showed abundant amount of cytoplasmic area whereas group II and III cases showed moderate amount of cytoplasm in exfoliated cells. An apparent cytoplasmic scantiness of exfoliated cells could result from a cytoplasmic torsion, traumatic partial loss or the position of the cells in relation to the viewing axis. The apparent cytoplasmic scantiness of the cells alone is not a dependable criterion of malignancy.

Nuclear changes

Nuclear changes are the most important criteria used for the cytologic diagnosis of carcinoma. Their features reflect the cells biological potential. No single structural change is diagnostic by itself. A combination of several changes is necessary for diagnosis.[16]

Nuclear shape

The variation in the nuclear shape (a distinctive feature of malignancy) is often due to the rapid rate of growth of the neoplastic cells, which become crowded against each other and have to occupy a continuously narrowing space. Some of the irregularities in the nuclear shape are due to abnormal mitosis producing an irregularity in the number and shape of the chromosomes.

An artificial variation can occur in benign cells as a distortion resulting from poor cellular fixation, trauma, degeneration, etc., by itself, irregularity in nuclear shape, if not extreme, is not sufficient for diagnosis.[16]

In our study, majority of the nuclei were spherical in shape in group I, whereas mixed populations of spherical and oval nuclei were seen in groups II and III. In group III, along with the oval and spherical, irregular shapes were also seen.

Nuclear size

This variation is observed in the cells of most malignant neoplasms. The size of the nucleus is important for a relative comparison when the cells are in the form of sheet or acinus, rather than appearing singly. In addition, the size of the nucleus differs in cells originating from the various strata's of the epithelium. Pseudo variation may result from the position of the cell on the slide in relation to the axis of observation and physiologic variation can be seen according to their function.[16] Increased nuclear size in cells has been reported in aging, in systemic disorders such as diabetes and anemia and in localized disorders of the mouth. The relatively larger nuclei generally associated with smoking seem to be consistent with a topical mechanism.[17]

In our study, 99% of the cases from group I showed small

nucleus and medium-sized nuclei were seen in groups II and III.

Nuclear outline

The nuclear outline could be well defined or poorly defined with regular or irregular borders. In our study, the nuclear outlines were distinct when visualized under a phase contrast microscope. In groups I, II and III, most of the cases showed a well-defined nuclear outline. The nuclear borders were regular in groups I and II and irregular in group III.

Nuclear-cytoplasmic ratio

It is the ratio of the size of nucleus to the size of cytoplasm of the cell. The size of the nucleus is compared to the size of its cytoplasm to determine the N/C ratio. It indicates the maturity of a cell. As a cell matures, the size of the nucleus generally decreases and N/C ratio decreases. N/C ratio is found to be increased in malignant neoplasms and is a more consistent finding than the nuclear and cytoplasmic changes alone. Most nuclear hypertrophy may be explained by the frequent hyperploidy of malignant cells. Variations could also be due to edematous swelling, poor fixation and air drying or if the cells are in contact with a hypotonic solution. Nuclear enlargement can also be due to irradiation, regeneration, chemotherapy, administration of alkalinizing agents, cautery and viral infections, thereby increasing the N/C ratio.[16]

In our study, group I cases showed normal N/C ratio, group II and III cases showed moderately increased N/C ratio, which was in accordance with many studies.[8,18]

Perinuclear halo

This morphological change could be assessed only by using phase contrast microscopy, and we observed that the exfoliated cells in group I showed absence of perinuclear halo, whereas exfoliated cells of group II and III showed perinuclear halo. It was increased among the cells of group III.

CONCLUSION

Exfoliative cytology along with cytomorphometric analysis can aid in motivating individuals to withdraw the use of tobacco, as the acceptance in reliability of measurable values increases. We emphasize that cytomorphology is an invaluable parameter to assess the influence of tobacco on oral mucosa. Phase contrast microscopy revealed better cellular details and therefore, can be used to assess the cytological changes. Thus, phase contrast microscopy can be used as a better diagnostic tool since it is quicker and cost effective in cytomorphometrical studies.

Our study, thus, elucidates the importance of early recognition of cellular alterations for identification of individuals who require early intervention even in the absence of visible changes of mucosal surface. Further studies with larger sample sizes should be encouraged to confirm these findings, and a cytomorphometric grading system using phase contrast technique should be formulated to further explore the advantages of this technique.

REFERENCES

1. Warnakulasuriya S, Johnson NW, van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med 2007;36:575-80.
2. Van der Waal I. Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. Oral Oncol 2009;45:317-23.
3. Silverman S Jr. Observations on the clinical characteristics and natural history of oral leukoplakia. J Am Dent Assoc 1968;76:772-7.
4. Prabhu SR, Wilson DF, Daftary DK, Johnson NW. Oral diseases in the Tropics. 1st ed. Oxford: Oxford University Press; 1993. p. 104-16, 402-15, 430-5.
5. Khandelwal S, Solomon MC. Cytomorphological analysis of keratinocytes in oral smears from tobacco users and oral squamous cell carcinoma lesions--A histochemical approach. Int J Oral Sci 2010;2:45-52.
6. Reddy CR, Kameswari VR, Prahlad D, Ramulu C, Reddy PG. Correlative study of exfoliative cytology and histopathology of oral carcinomas. J Oral Surg 1975;33:435-8.
7. Bremmer JF, Braakhuis BJ, Ruijter-Schippers HJ, Brink A, Duarte HM, Kuik DJ, *et al.* A noninvasive genetic screening test to detect oral preneoplastic lesions. Lab Invest 2005;85:1481-8.
8. Bhavasar RS, Goje SK, Hazarey VK, Ganvir SM. Cytomorphometric analysis for evaluation of cell diameter, nuclear diameter and micronuclei for detection of oral premalignant and malignant lesions. J Oral Biol Sci 2011;53:158-69.
9. Patel PV, Kumar S, Kumar V, Vidya GD. Quantitative cytomorphometric analysis of exfoliated normal gingival cells. J Cytol 2011;28:66-72.
10. Curl CL, Bellair CJ, Harris PJ, Allman BE, Roberts A, Nugent KA, *et al*. Quantitative phase microscopy: A new tool for investigating the structure and function of unstained live cells. Clin Exp Pharmacol Physiol 2004;31:896-901.
11. Shahi SK, Ranga S, Khurana SK, Talib VH. Phase contrast microscopy: Utility in obstetrics and gynaecology. Indian J Pathol Microbiol 1999;43:123-4.
12. Hande AH, Chaudhary MS. Cytomorphometric analysis of buccal mucosa of tobacco chewers. Rom J Morphol Embryol 2010;51:527-32.
13. Ramaesh T, Mendis BR, Ratnatunga N, Thattil RO. Cytomorphometric analysis of squames obtained from normal oral mucosa and lesions of oral leukoplakia and squamous cell carcinoma. J Oral Pathol Med 1998;27:83-6.
14. Einstein TB, Sivapathasundharam B. Cytomorphometric analysis of the buccal mucosa of tobacco users. Indian J Dent Res 2005;16:42-6.
15. Shabana AH, el-Labban NG, Lee KW. Morphometric analysis of basal cell layer in oral premalignant white lesions and squamous cell carcinoma. J Clin Pathol 1987;40:454-8.
16. Naib ZM. Cytopathology. 4th ed. New York City: Little Brown and Company; 1996. p. 149-60.
17. Hillman RW. Kissin B. Oral cytologic patterns in relation to smoking habits. Some epithelial, microfloral, and leukocytic characteristics. Oral Surg Oral Med Oral Pathol 1976;42:366-74.

18. Usta U, Berberoglu U, Helvaci E, Altaner S, Sut N, Ozdemir C. Evaluation of cytological alterations in normal-appearing oral mucosal epithelia of smokers and non-smokers via AgNOR counts and nuclear morphometry. Trakya Univ Tip Fak Derg 2008;25:110-6.

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