Original Article

Cytomorphometric Study of Changes in Buccal Mucosal Cells in Alcoholics

Abstract

Background: Chronic alcohol consumption carries a high risk for oral and pharyngeal cancers among persons who have never smoked. Excessive alcohol consumption displays cytogenetic changes in oral mucosa cells. Cytomorphometric analysis of oral mucosal cells helps in the early detection of cytomorphological transformations in alcoholics before and after the onset of carcinoma. **Materials and Methods:** A prospective, hospital-based, comparative study was done after written informed consent. Smears were obtained from the clinically normal buccal mucosa of 102 randomly selected alcoholic patients attending the medicine outpatient department aged above 25 years who consumed a minimum of 45 ml alcohol per day for at least 10 years and of 102 nonalcoholics as control. The slides were immediately fixed in absolute methanol and stained by the Papanicolaou (Pap) staining technique. PAP-stained smears were examined under the light microscope. Using the image J 1.47 image analysis software, a morphometric analysis of around 50 cells/case was done. **Results:** A statistically significant increase in mean cytoplasmic area ($P < 0.001$), mean nuclear area ($P < 0.01$), and cell-to-nuclear parameter ratio ($P < 0.001$) was seen in the alcohol group in comparison with the control group. **Conclusion:** Prolonged consumption of alcohol produces cytomorphometric changes in buccal mucosal cells before the onset of premalignant lesions.

Keywords: *Alcoholism, carcinogenesis, computer-assisted, image processing, oral neoplasm*

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Introduction

Mouth cancer is one of the top six-most common cancers and is also one of the ten principal causes of mortality globally.[1,2] Prolonged alcohol consumption carries a high risk for carcinoma in the upper gastrointestinal tract and is a primary cause for oral and pharyngeal cancers among persons who are not smokers.[3-5] Experimental studies on rodents subjected to prolonged alcohol consumption have shown hyperplasia in the tongue. However, the exact role of ethanol in the development of cancer is poorly understood.[6-8] The carcinogens such as acetaldehyde and free radicals are produced during the metabolism of ethanol. There is enough proof in the literature to collaborate that acetaldehyde is mainly responsible for the development of carcinoma. Acetaldehyde formed from the alcohol binds to nuclear DNA and proteins in the cells thereby, damaging the DNA and causing destruction of folate resulting in increased proliferation of cells.[3] Almost all oral cancers are detected at the late stages which can result in dismal prognosis and early death of the patient.[9,10] Significant improvements were not seen in the survival rates even with diagnostic and therapeutic breakthroughs.[11] Therefore, early detection of oral cancers plays an important role in the successful treatment of the patients.[12]

Oral exfoliation cytology is an easy, noninvasive procedure that helps in microscopic analysis of oral mucosal cells collected from its exfoliation.[13] The quantitative methods based on the nuclear area (NA), cell area (CA), and CA: NA ratio increases the diagnostic accuracy of exfoliative cytology in the preliminary diagnosis of mouth cancers.[14] This test has been used as a cancer evaluation and diagnostic technique with acceptable sensitivity and specificity. This method is gaining popularity with advancements in imaging and computerized techniques. The utilization of quantitative methods helps in the authentication of cytomorphometric analysis.[10,15] The cytogenetic changes can be seen in the oral mucosal cells of persons

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who are constantly exposed to a high amount of alcohol.[16] There are very few studies substantiating the cause-effect relationship between alcohol and oral cancer. Hence, this study intended to analyze the cytomorphological parameters as well as to evaluate the potential cytomorphological transformations in alcoholics.

Materials and Methods

The protocol of our study was approved by the Ethics Committee on Human Research at our institute.

Group of participants with normal oral mucosa

Male patients were randomly selected in the medicine outpatient department. A detailed clinical history with a predetermined protocol was obtained, and all patients were thoroughly examined including the oral cavity. Participants with systemic diseases such as diabetes mellitus, obvious oral mucosal lesions, smokers, and previous neoplasms, on anti-cancer drugs and radiation therapy, were excluded from this study.

Based on clinical history, patients were divided into two groups: group I (control) – patients who had never consumed alcohol, smoked or chewed tobacco and Group II (alcoholics) –patients who consumed a minimum of 45 ml alcohol in a day for at least 10 years. The daily alcohol consumption was calculated by multiplying the number of drinks consumed weekly by the average alcohol content of a glass of beer, wine, whiskey, and Sarayam (locally distilled alcohol) divided by 7 days. A conversion factor of 0.8 was used to convert alcohol by volume to alcohol by weight.[16] Of the 220 subjects examined, 204 were included in the study: 102 in the control group (Group I) and 102 in the alcohol group (Group II).

Written consent was obtained from all the subjects before taking the cytological smears. The smears were obtained from the clinically normal buccal mucosa of both control and cases. The participant was requested to clean the mouth with drinking water. Under aseptic precautions, scrapings were taken from the inner side of the cheek after applying firm pressure with a wooden spatula. The scrapings were taken on to the glass slides to prepare smears. The smears were quickly fixed in absolute methanol and after fixation, it was stained by Papanicolaou (Pap) stain and examined under the light microscope.

On microscopic examination, cells with distinctly defined cellular and nuclear outlines in the field of vision were selected. The degenerated, folded, or partially visible cells were not included for the analysis. A high-resolution camera on the microscope was used to take a 640 × 400-pixel digital image in both low and high magnification. The image J 1.47 image analysis software was used for the morphometric analysis of around 50 cells/case [Figures 1 and 2].[17] The mean nuclear and cytoplasmic area, perimeter, diameter, and cell to nuclear (C/N) ratio was recorded, and their median

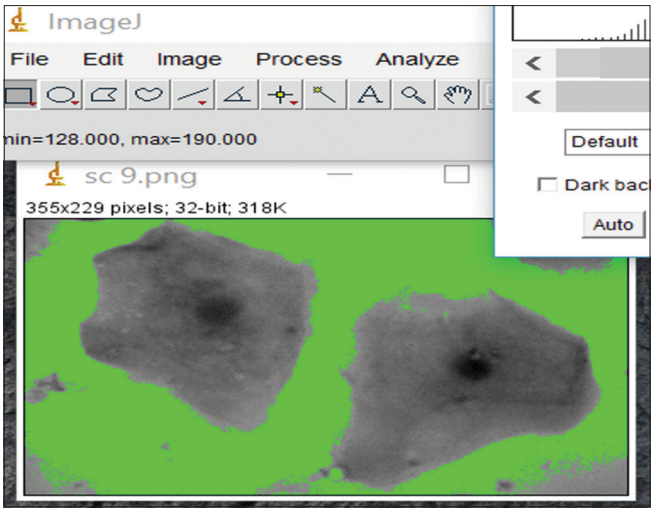

Figure 1: Morphometry using image J 1.47 image analysis software of well-spread cells of the control group, exfoliated buccal mucosal cells in a pap smear

values were expressed in square micrometers for the area and micrometers for perimeter and diameter.

The following statistical parameters were calculated in each group: cell area (CA), cell perimeter, maximum cell diameter, minimum cell diameter, NA, nuclear perimeter, maximum nuclear diameter, minimum nuclear diameter, and cell to nuclear parameter ratio. The data were represented by median and inter-quartile range (IQR). The data were analyzed using the SPSS trial version 20. The Shapiro–Wilk test was used to analyze the normality of the data. The data were represented by median and IQR. Since the data were not normally distributed, the Mann–Whitney test was used for comparing the test and control parameters. Cytomorphometric values of the parameters were expressed in microns. AR form factor (area divided by ð/4 × longest axis × shortest axis) was calculated.

Results

In the present study, all were males and most of the alcoholics were in the age group of 36–45 years (38%), followed by 46–55 years (33%), 56–65 years (23%), 26–35 years (4%), and 66–75 years (2%). The exposure to alcohol for a median of 21 years was observed.

Table 1 shows that a statistically significant increase in NA ($P < 0.001$) with a median of 1521 μm^2 and IQR of 629 μm^2 in test group in comparison to control group with a median of 912 μm^2 and IQR of 233 μm^2. A statistically significant ($P < 0.001$) decrease in nuclear diameter with a median of 85.7 μm in the test group in comparison to the median of 150.7 μm in the control group was noted. A significant increase in perimeter, circularity, AR, and solidity of the cell was seen in comparison with the control group. Nonetheless, no significance in the values of the roundness of the cell between the two groups was noted.

Table 2 shows the cellular morphometric parameters of the test and control group. A statistically significant ($P < 0.001$) increase in the cell area, diameter, perimeter, and solidity of the test group in comparison to the control group was noted. The median cell area of the test group was 33529.33 μm^2 more than the control group.

Table 3 showing C/N parameter ratio, the C/N area is significantly ($P < 0.001$) increased in the test group with a median of 38.8 μm^{2}and IQR 16.7 μm^2 in comparison to control group with a median of 27.7 μm^2 and IQR 11 μm^2 [Figure 3]. The table highlights the significant ($P < 0.001$) decrease in circularity and solidity in the C/N ratio [Figure 4]. The nucleus to cytoplasmic (N/C) ratio was calculated by subtracting NA from the CA and further dividing the NA by the subtracted area. The N/C ratio of 0.036 and 0.025 was noted in the control and alcoholics group, respectively. Although there was no significant change in the N/C ratio between the two groups, there was a statistically significant increase in the cell, cytoplasmic, and nuclear parameters in alcoholics of clinically normal buccal mucosa without much change in the N/C ratio.

Discussion

An introduction of the quantitative cytomorphometric technique of smear analysis has increased the diagnostic flow cytometry, and cytomorphology accuracy of oral exfoliative cytology in the detection of oral cancer.[13] In normal oral mucosa, diversity in the size of the cells and nuclei can be seen due to the differences in the development and maturation of individual cells. So it is difficult to have a criterion for nuclear and cytoplasmic

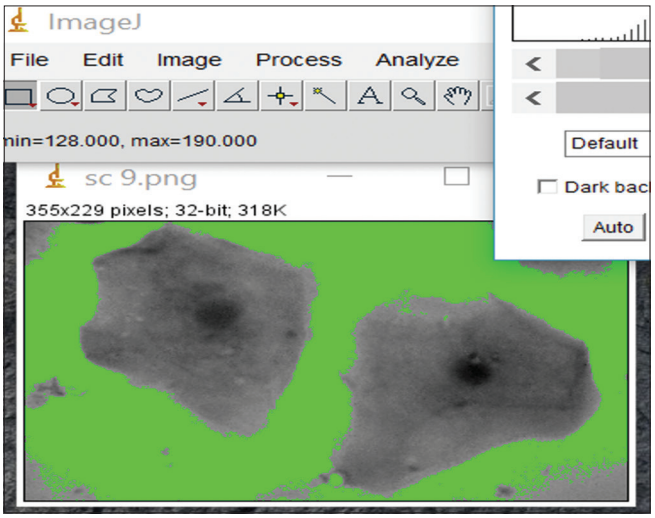

Figure 2: Photography of measurement of the cell using image analysis software under ×40 of test group, exfoliated buccal mucosal cells in Pap smear

Table 1: Nuclear morphometric parameters in alcoholics and nonalcoholics

Morphometric Parameters	Group				*P*
	Control		Test		
	Median	IQR	Median	IQR	
Mean nuclear area	912.000	233.000	1521.000	629.056	<0.001
Mean nuclear diameter	150.763	55.048	85.703	21.723	<0.001
Minimum nuclear diameter	118.000	52.333	65.619	30.76	<0.001
Maximum nuclear diameter	175.9997	72.667	103.222	20.111	<0.001
Mean nuclear perimeter	255.593	138.108	294.233	125.739	0.023
Circular nuclear	0.174	0.154	0.257	0.180	0.001
AR nuclear	1.322	0.269	1.386	0.214	0.049
Round nuclear	0.758	0.143	0.749	0.104	0.418
Solidity nuclear	0.719	0.101	0.774	0.094	<0.001

Mann–Whitney U test; $P<0.05$ is significant. IQR: Inter-quartile range

Table 2: Cellular morphometric parameters in alcoholics and nonalcoholics

Morphometric parameters	Group				*P*
	Control		Test		
	Median	IQR	Median	IQR	
Mean cell area	26185.000	10303.000	59714.333	25751.429	<0.001
Mean cell diameter	175.632	59.502	107.601	18.012	<0.001
Minimum cell diameter	104.000	61.333	64.714	26.089	<0.001
Maximum cell diameter	219.333	48.000	132.722	26.912	<0.001
Mean cell perimeter	1751.863	877.467	3755.554	1388.038	<0.001
Circular cell	0.096	0.083	0.075	0.050	0.005
AR cell	1.579	0.621	1.719	0.377	0.133
Round cell	0.636	0.221	0.625	0.101	0.855
Solidity cell	0.835	0.098	0.747	0.052	<0.001

Mann–Whitney U-test; $P<0.05$ is significant. IQR: Inter-quartile range

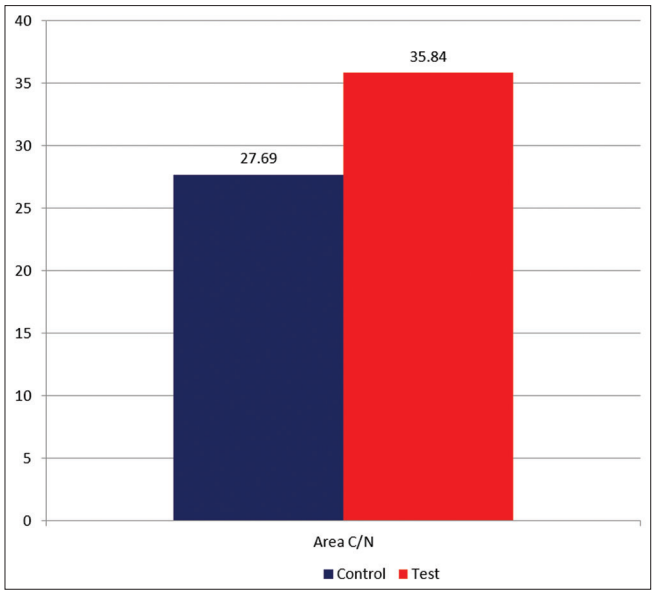

Figure 3: Correlation of control with alcoholics for cell/nuclear ratio

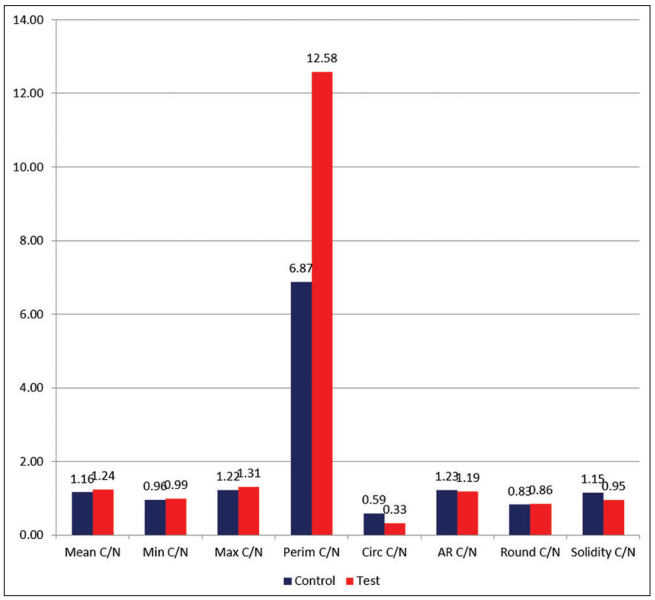

Figure 4: Correlation of control with alcoholics for various cell/nuclear ratio parameters

Table 3: The cell to nuclear parameter ratio in alcoholics and nonalcoholics

Morphometric Parameters	Group				*P*
	Control		Test		
	Median	IQR	Median	IQR	
Area C/N	27.690	11.023	35.841	16.712	<0.001
Mean C/N	1.160	0.491	1.239	0.227	0.185
Minimum C/N	0.955	0.529	0.986	0.203	0.421
Maximum C/N	1.218	0.386	1.308	0.251	0.035
Perimeter C/N	6.868	3.665	12.580	5.759	<0.001
Circular C/N	0.593	0.476	0.325	0.210	<0.001
AR C/N	1.226	0.435	1.190	0.336	0.709
Round C/N	0.826	0.293	0.856	0.156	0.898
Solidity C/N	1.150	0.187	0.953	0.141	<0.001

Mann–Whitney U-test; $P<0.05$ is significant. C/N: Cell to nuclear, IQR: Inter-quartile range

changes in exfoliated cells. However, significant variations can be observed by comparing the normal group with that of the alcohol group.[18, 19] Quantitative methods like DNA cytophotometry, flow cytometry, and cytomorphology. Which helps to distinguish the proliferative activity of the cell populations of oral mucosal cells, cannot be used on normal mucosal cells. The normal oral cells which are nonreplicating have a diploid DNA distribution. A variety of DNA profiles such as diploid, hyperdiploid, polyploid, and aneuploidy can be seen in a malignant cell. Thus, exfoliative cytology combined with imaging analysis is a preferred technique before the onset of dysplasia and malignancy.[20,21] The majority of the false-negative results could be due to the cytological analysis of keratinized surface in patients with leukoplakia. Hence, that explains the limitation of exfoliative cytology to reliably detect the morphological changes in leukoplakia which happens during the early onset of carcinogenesis.[22]

The consequence of exclusive ethanol intake on oral mucosa is poorly understood. The damage incurred by the DNA with oncogene expression of oral keratinocytes may be due to the acetaldehyde, a carcinogenic metabolite of alcohol. Alcohol results in space between epithelial cells, thereby helping the other carcinogens to penetrate the oral mucosa. Epidermal growth factor secreted by oral salivary glands helps in protecting the oral mucosa against the damage caused by acids. Ethanol reduces the epidermal growth factor secretion by deranging the salivary gland, leading to oral ulcerations. Heavy alcohol consumption can lead to nutritional deficiencies and further prevent the usage of antioxidants by the body, leading to the formation of cancers.[23,24] Consumption of alcohol in the form of beer, wine, or liquor is a principal risk factor for cancers of the oral cavity. The ethanol content of a variety of alcoholic beverages is the same that is 10–14 g per-drink. However, the liquid volume differs (beer, 3.3 dl; wine, 1.5 dl; and liquor, 0.4 dl). The reason why alcohol concentration varies by beverage type (beer, 3.4 g/dl; wine, 9.2 g/dl; and straight (undiluted) liquor (26.7 g/dl). Few studies have suggested that the risk for oral cancer is due to the consumption of the high amount of alcohol (in grams), rather than the concentration of the beverages. Everyday ethanol consumption of more than 45 ml can have a carcinogenic effect on the body. Hence, the total amount and the duration of alcohol consumed are the significant factors in the development of oral cancer rather than the composition or type of alcoholic beverage.[23,25] In the present study, the alcohol group consumed a minimum of 80 ml/day and an average amount of about 430 ml/day. After an extensive literature search, we could only find a few studies done exclusively on cytomorphometric changes of alcohol on buccal mucosal cells. As early as 1999, Ogden

et al. noted a statistically significant reduction in mean cytoplasmic area ($P < 0.001$) and mean NA ($P < 0.01$) in alcoholics when compared to controls.[25] In 2006, Reis *et al*. study on cytological alterations in oral mucosa after chronic exposure to alcohol concluded that the ethanol group showed a significant increase in the frequency of abnormal nucleus/cytoplasm ratio, pyknosis, karyorrhexis, and karyolysis in exfoliated cells from the buccal mucosa when compared with the control group. However, it was not statistically significant.[26] Similarly, a study conducted by Batista *et al*. in 2007 also showed a significant increase in both cytoplasmic and NAs in normal buccal mucosa of ethanol group. No statistical difference was observed in the NA/CA ratio ($P > 0.05$).[27] In the present study, the mean cytoplasmic and NA was significantly increased in the ethanol group but not the mean nuclear to cytoplasmic ratio. Hence, suggesting an early significant increase in cytoplasmic area and cell/nuclear ratio before the increase in nuclear/cytoplasmic ratio what we see in dysplasia. The nuclear morphology changes from round to spindle over a while and asymmetry of the nuclear membrane can happen as a result of abnormal clumping of chromatin to the inner surface of neoplastic nuclei. The highly altered nuclear morphology will show a high degree of pathogenicity.[28] The analysis of cell shape has evolved from basic microscopic visual evaluation to more progressive methodologies that make use of sophisticated computer hardware and software for data analysis. In previous studies, only the size and changes in the cells have been studied. In the present study, there was a significant change in roundness and solidity of the cell as well as the nucleus in alcoholics suggestive of abnormality in the shape of the cell. The solidity of the cells in the control group was maintained, whereas in alcoholics, it was very low ($P < 0.001$). AR factor of a cell suggestive of convexity of a cell was also significantly increased in alcoholics.[17] Image analysis of exfoliative cytology also plays a pivotal role in providing information in other conditions such as diabetes mellitus, oral malignancies, effects of smoking, and iron content in beta-thalassemia.[29,30]

Conclusion

Significant cytomorphometric changes in oral mucosa were seen due to the stimulation of alcohol. We suggest the utilization of image analysis software for cytomorphometric evaluation in exfoliative cytology as a screening tool for the evaluation and observation of cellular and nuclear parameters including solidity and AR factor in cytological smears of normal buccal mucosa in alcoholics. The method is noninvasive, well-tolerated by a patient with high precision, objectivity, and reproducibility, and hence it can also be used among at-risk population screening. However, there is a paucity of studies in literature, so further research is necessary to substantiate the relative effects of alcohol on buccal mucosal cells.

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Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Gupta PC, Sinor PN, Bhonsle RB, Pawar VS, Mehta HC. Oral submucous fibrosis in India: A new epidemic? Natl Med J India 1998;11:113-6.
2. Razavi SM, Sajadi S. Epidemiological study of oral and perioral cancers in Isfahan. Dent Res J 2007;4:18-25.
3. Seitz HK, Becker P. Alcohol metabolism and cancer risk. Alcohol Res Health 2007;30:38-41, 44-7.
4. Seitz HK, Stickel F, Homann N. Pathogenetic mechanisms of upper aerodigestive tract cancer in alcoholics. Int J Cancer 2004;108:483-7.
5. Fioretti F, Bosetti C, Tavani A, Franceschi S, La Vecchia C. Risk factors for oral and pharyngeal cancer in never smokers. Oral Oncol 1999;35:375-8.
6. Carrard VC, Filho MS, Rados PV, Chaves AC, Lauxen Ida S. Quantification of silver-staining nucleolar organizer region in epithelial cells of tongue of mice after exposure to, or intake of, alcohol. Alcohol 2004;34:233-8.
7. Carrard VC, Pires AS, Mendez M, Pasquali AB, Badauy CM, Lauxen IS, *et al*. Exploring the mechanisms of alcohol-related damage in oral mucosa-is oxidative stress associated with the increase in cell proliferation in rat tongue epithelium? Pharm Biol 2013;51:160-9.
8. Homann N, Jousimies-Somer H, Jokelainen K, Heine R, Salaspuro M. High acetaldehyde levels in saliva after ethanol consumption: Methodological aspects and pathogenetic implications. Carcinogenesis 1997;18:1739-43.
9. Gupta PC, Metha FS, Pindborg JJ. Oral cancer in rural India. Lancet 1987;1:1087.
10. Sampaio HC, Loyola AM, Gomez RS, Mesquita RA. AgNOR count in exfolioative cytology of normal buccal mucosal effect of smoking. Acta Cytol 1999;43:117-120.
11. Hunter KD, Parkinson EK, Harrison PR. Profiling early head and neck cancer. Nat Rev Cancer 2005;5:127-35.
12. Orellana-Bustos AI, Espionoza-Santander IL, Franco-Martínez ME, Lobos-James-Freyre N, Ortega-Pinto AV. Evaluation of keratinisation and AgNORs count in exfoliative cytology of normal oral mucosa from smokers and non-smokers. Med Oral 2004;9:197-203.
13. Diniz-Freitas M, García-García A, Crespo-Abelleira A, Martins-Carneiro JL, Gándara-Rey JM. Applications of exfoliative cytology in the diagnosis of oral cancer. Med Oral 2004;9:355-61.
14. Ogden GR, Cowpe JG, Wight AJ. Oral exfoliative cytology: Review of methods of assessment. J Oral Pathol Med 1997;26:201-5.
15. Pektaş ZO, Keskin GO, Karslioğlu Y. Evaliuation of nuclear morphometry and DNA ploidy status for detection of malignant and premalignant oral lesions: Quantitative cytologic assessment and review of methods for cytomorphometric measurements. J Oral Maxillofac Surg 2006;64:628-35.
16. Webber LP, Pellicioli AC, Magnusson AS, Danilevicz CK, Bueno CC, Sant'Ana Filho M, *et al*. Nuclear changes in oral mucosa of alcoholics and crack cocaine users. Hum Exp Toxicol 2016;35:184-93.

17. Parmar D, Indupalli AS, Sawke N, Sawke GK. Cytomorphometric evaluation of alterations in buccal mucosal cells of smokers and non-smokers. Int J Bioassays 2014;3:1775-9.
18. Cowpe JG. Quantitative exfoliative cytology of normal and abnormal oral mucosal squames: Preliminary communication. J R Soc Med 1984;77:928-31.
19. Sharma D, Sandhu SV, Bansal H, Gupta S. Distinct perturbations of oral squamous cell carcinoma patients: A quantitative cytomorphometric analysis. Int J Health Sci (Qassim) 2015;9:283-91.
20. Cowpe JG, Longmore RB, Green MW. Quantitative exfoliative cytology of abnormal oral mucosal smears. J R Soc Med 1988;81:509-13.
21. Patel PV, Kumar S, Kumar V, Vidya G. Quantitative cytomorphometric analysis of exfoliated normal gingival cells. J Cytol 2011;28:66-72.
22. Sudbo J, Kildal J, Risberg B, Koppang HS, Danielsen HE, Reith A. DNA content as a prognostic marker in patients with oral leukoplakia. N Engl J Med 2001;344:1270-8.
23. Feng L, Wang L. Effects of alcohol on the morphological and structural changes in oral mucosa. Pak J Med Sci 2013;29:1046-9.
24. Reidy JT, McHugh EE, Stassen LF. A review of the role of alcohol in the pathogenesis of oral cancer and the link between alcohol-containing mouthrinses and oral cancer. J Ir Dent Assoc 2011;57:200-2.
25. Ogden GR, Wight AJ, Rice P. Effect of alcohol on the oral mucosa assessed by quantitative cytomorphometry. J Oral Pathol Med 1999;28:216-20.
26. Reis SR, do Espírito Santo AR, Andrade MG, Sadigursky M. Cytologic alterations in the oral mucosa after chronic exposure to ethanol. Braz Oral Res 2006;20:97-102.
27. Batista AB, Hase ED, Grégio AMT, Ignácio SA, França BHS, Soares de Lima AA. Cytomorphometric study of normal buccal mucosa of alcoholic individuals. Pharmacologyonline 2007;1: 243-251.
28. Mohanta A, Mohanty PK. Nuclear pleomorphism-based cytopathological grading in human oral neoplasm. Russian Open Med J 2017;6:E0203.
29. Radhika T, Hussain S, Adithyan S, Jeddy N, Lakshmi S. Cytomorphometric evaluation of oral exfoliated cells − Its correlation with age of an individual. J Orofac Sci 2019;11:84-8.
30. Sahay K, Rehani S, Kardam P, Kumra M, Sharma R, Singh N. Cytomorphometric analysis and morphological assessment of oral exfoliated cells in type 2 diabetes mellitus and healthy individuals: A comparative study. J Cytol 2017;34:27-33.