ORIGINAL ARTICLE

# A Comparative Cytomorphometric Analysis of Buccal Mucosal Smears of Cigarette Smokers and Naswar (Nicotiana Tabaccum) Users

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**ABSTRACT**

**Objective**: To compare the cytomorphometric changes in buccal mucosal cells of cigarette smokers, naswar users and non-users/smokers.

**Study Design:** Cross Sectional Comparative.

**Place and Duration of Study**: PGMI, Lahore & Islamic International Dental College, Islamabad: between July 2013-January 2014.

**Materials and Methods***:* Cellular diameter CD, nuclear diameter ND and nuclear to cytoplasmic ratio N/C ratio were assessed in buccal smears taken with wooden spatula from clinically normal mucosa of smokers, naswar users and control group. The sample size was 99 male subjects of ages 15yrs-60yrs, divided into three groups (33 each group) i.e., M as control group, S as smoker group and N as naswar users' group. Slides were stained with three stains Hematoxylin and Eosin Stain, Giemsa Stain and Papanicolaou Stain. The data was collected through random sampling. The cytomorphometric variables were measured by using stage and ocular micrometers. The cytopathological variables as inflammatory cells, mitotic figures, pleomorphism and hyperchromatism were also studied. The results were then statistically analyzed using SPSS version 18.

**Results**: The mean cellular diameter of group M, S and N was 43.8µm, 54.3µm and 42.7µm respectively. The mean nuclear diameter of M, S and N was 9.97µm, 12.6µm and 11.8µm respectively. And the mean N/C ratio of group M, S and N was 1:4.4, 1:4.3and 1:3.5 respectively.

**Conclusion:** The comparative changes assessed by this study depicts cause effect relationship with smoking and naswar use. Association of these changes with dysplasia or pre-malignancy needs further verification with the help of specific immune markers.

**Key Words:** *Cytomorphometry, Tobacco, Naswar, Oral Neoplasms, Smokers.*

## Introduction

Oral squamous cell carcinoma (OSCC) is one the most common malignant alteration of squamous epithelium encountered in oral cavity. It is one of the sixth most common malignant tumors in world and third most common in Pakistan.[1] There are multiple causative factors for OSCC among which smoking, use of areca nut, betel quid or pan and tobacco chewing, naswar, pan masala, gutka are considered to be major risk factors among Pakistani population.[2] Although visibly detectable due to the accessibility of the oral cavity, oral cancer has a high morbidity and mortality because it is diagnosed typically at an advanced stage when it is finally clinically visible. Early diagnosis is crucial for better prognosis.[3] Exfoliative cytology is used to investigate the effect of tobacco on the oral epithelial cells.[4] It is one of simple non-invasive investigative tool for early diagnosis of dysplastic changes in oral lesions.[5] Quantifiable methods based on the measurement of variables as nuclear diameter ND, cellular diameter CD and nuclear to cytoplasmic ratio N/C ratio can accelerate the sensitivity of exfoliative cytology for

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the early diagnosis of oral premalignant and malignant lesions, because these methods are precise, objective and reproducible.[4] Simple non-invasive techniques like exfoliative cytology can be employed as a chairside technique and in mass screening programs for identification of cellular changes in oral mucosa of individuals with tobacco habits. Various studies have been conducted among different population across the globe to see the effect of tobacco and its products on oral mucosal cells using this noninvasive tool.[6-9]

The purpose of present study was to compare the cytomorphometric changes in buccal mucosal cells of cigarette smokers, naswar users and non-users/smokers among Pakistani population and to analyze the cause–effect relationship between tobacco uses and quantitative alterations made.

## Materials and Methods

This was Cross-Sectional comparative study which was carried out at Post Graduate Medical Institute, Lahore and Islamic International Dental College, Riphah International University, Islamabad. The Duration of the study was six months. The sample size was 99 in total, calculated by WHO calculator; divided into three groups of 33 each as M (control group), N (naswar users) and S (smoker group). Non-Probability Convenient sampling technique was used. A written consent proforma was filled and submitted by all the subjects included in the study, as per the requirements of the Ethical Review Committee. The study was approved by the institution's ethical review board. The letter number was: IIDC/IRC/2017/02/001.

Male subjects above 15 years of age were included in all the 3 groups. Among the naswar users, subjects using naswar for atleast three years or more and 3-4 times /day were included; as for the smoker groups individuals smoking 1 pack/day (20 cigarettes) for at least 3 years or more were included. While control group included the subjects with no history of tobacco use in any form. Subjects with poor oral hygiene, any pre-existing premalignant or malignant lesion, denture wearers, tobacco users of any form were excluded. Also, individuals suffering from chronic debilitating diseases or undergoing any drug therapies that may cause oral mucosal changes were also excluded.

Data was collected from the subjects by requesting them initially to rinse their mouth with water. Then smears were taken using a moistened wooden spatula from the buccal mucosa of all the subjects. Three glass slides per each subject were marked with their reference number and alphabet of their particular group. The smears were taken and immediately fixed in using Cyto-fixi spray (95 % alcohol).[10]

Slides of each subject were stained Hematoxylin and Eosin stain, Papanicolaou stain and Giemsa stain respectively. Cellular diameter (CD) and nuclear diameter (ND) of the 100 cells in each smear were measured by using pre calibrated ocular micrometer fixed in eye piece of microscope on 40 x. The ocular micrometer was pre-calibrated with the help of stage micrometer.[11] The following equation shows the calibration.

100 div on ocular micrometer = 30 divisions on stage micrometer (one div =10µm)

= 30×10

100 div on ocular micrometer = 300 um

1 div on ocular micrometer = x

x =3 µm

In each case 100 clearly defined cells, from left to right in N direction were analysed for variables CD, ND and N/C ratio using ocular micrometer.

Cytopathological variables i.e., Inflammatory cells, presence of mitotic figures, pleomorphism, hyperchromatism were also studied under microscope.

Data analysis was carried out using Statistical Package for Social Sciences (SPSS) version 18. One way anova, post hoc tukeys test was used to compare means of CD, ND and N/C among the three groups. p value of 0.05 or less was taken as statistically significant.

## Results

After completing the performas the readings were compared in all three groups and all three stains. For each variable i.e. CD, ND and N/C ration mean was calculated in every group and then was compared and statistically analysed. Summarizing the cytomorphometric results in table I, the cellular diameter is highest in smokers on pap stain. Nuclear diameter ND shows a significant increase in smokers and naswar users on all three stains as compared to the control group.

The highest cellular diameter (55.22µm±3.36)

among all three stains was found to be in the smokers who smoked for more than 10 years. Likewise, in Naswar users the highest cellular diameter was recorded as 45.46 µm±1.65 in those subjects who had been using naswar for more than 10 years. Moreover, among all three stains CD was highest in smokers who consumed 2packs/day showed a value of 54.58 µm±2.95 as compared to others who smoked less cigarettes. Similarly, for naswar users, CD was found to be highest in those consuming naswar for 5-6 times per day i.e., 44.09 µm±2.02. One way anova was applied to compare cytomorphometric findings among groups with respect to frequency of smoking or naswar use /day and duration of use, however in both cases results were statistically insignificant with p-value more than 0.05.

When variance analysis was conducted to analyse any difference in the nuclear diameter between the three groups, a statistically significant difference was found (p = 0.000). The intergroup Post-hoc Tukey analysis revealed that the difference in the nuclear diameter between the smoker's group (12.68 µm± 0.91) and the control group (9.97 µm± 0.80) was significant. However, the difference in the nuclear diameter between the Naswar users (11.80 µm± 2.23) and the smoker group (12.68 µm± 0.91) was not significant (p > 0.005). While the difference in the ND between the naswar users' group (11.80 µm± 2.23) and the control group (9.97 µm± 0.80) was significant (p=0.001). The mean difference between CD, ND, and N/C ratio in all three stains, among the three groups, S, N and M was found to be statistically highly significant i.e., p=0.001 on ONE WAY ANOVA. While on post hoc Tuckey test between S and N, CD and N/C ratio were highly significant p=0.000 while ND was not significant. While between S and M, CD and ND both were significant and showed p=0.000 while N/C ratio was not significant. In comparison between N and M, ND and N/C ratio were found to be significant while CD was not significant.

The cytopathological variables such as presence or absence of inflammatory cells, pleomorphism, hyperchromatism and mitotic figures were also studied on all three stains. Although all the variables were studied on all three stains but the results were same for each one of them with a very negligible difference, thus for convenience of understanding the results observed on H&E stain only, are presented here. Out of 33 cases of each group, inflammatory cells were present in 23 (70%), 17 (51%) and 7 (21%) cases of naswar users, smokers and control group respectively. Chi square test was used, and statistically significant results were found between the groups with a p value of <0.05 With regard to pleomorphism, hyperchromatism and mitotic figures, none was found in any of the studied groups.

## Discussion

Oral squamous cell carcinoma is caused by use of different forms of tobacco.[3] Commonly oral cancers are diagnosed at advanced stages, which results in its poor prognosis decreasing the survival rates among the patients. Oral exfoliative cytology has been proven to detect early changes in the cells even before the onset of the clinical lesion, and this technique is inexpensive and easy with high sensitivity rates and diagnostic values.[4,5] In the present study, cytomorphometric and cytopathological variables were studied on buccal cells using exfoliative cytology and compared among three groups i.e. naswar users, cigarette smokers and control group.

With regard to cellular diameter, variable results are shown by various studies conducted worldwide, like Hande and Chaudhary in 2010 conducted a study using cytomorphometry and showed that systemic and external factors affect the cytomorphometric variables such as ND, CD and N/C ratio. As the CD is increased in smokers in the present study, it may be due to any factor which is caused by smoking cigarettes. The results of the present study, i.e, increase in the CD of smokers as compared to the control group, contrasts with the other studies carried out like in case of Sumit babuta (2014) and Goregen, (2011).[3,6] Whereby in a study conducted by Ramesh et al. (1999) CD was decreased in cigarette smokers.[7] Similarly, a study conducted by Ogden et al. (1997) also showed a decrease in CD of tobacco users.[1]

However, the results of buccal mucosal smears of naswar users showed strikingly similar results with study conducted by Hande and Chaudhary in 2010 showing decrease in CD.[8] Ramaesh et al in 1998 and Ogden et al in 1997 also showed a decrease in CD of tobacco chewers in their respective studies.[1,8] The decrease in the CD in the present study can be an

early detection of dysplastic or malignant change.[9]

The next variable studied in this study was nuclear diameter, both smokers and naswar users group showed an increase in ND in comparison with the control group. This may be due to various reasons including use of tobacco or increase in DNA content as stated by Hande and Chaudhary in 2010.[8] Other studies which have been conducted in the past on the same subject showed the similar results which are consistent with the findings of the present study i.e; increase in nuclear diameter of tobacco users, be they smokers or naswar users.[3,4,10,11]

Ogden et al observed in 1997 that 5 % average increased in nuclear diameter of smokers when compared with those of the non-smokers.[1] While a study conducted by Goregen in 2011 showed an increase of 16.5 % increase in ND of smokers as compared to non-smokers which was attributed to smoking.[8] None of the groups showed significant relationship between ND and duration or frequency of smoking or naswar use. This finding contrasts with the results of a studies which were carried out by Saranya, 2014 and Goregen, 2011.[3,12,13]

The third cytomorphometric variable studied in the study was N/C ratio, highest N/C ratio was observed in the naswar user group. This finding is due to the fact the ND in naswar user group was higher than the control group while their CD was smallest among the three groups (smokers, naswar users and control group) due to which the N/C ratio has automatically increased. carried out a study which showed that N/C ratio helps us to show the precise relationship in the altered cellular and nuclear diameter.[14] N/C ratio in the smokers was also higher when compared with the control group which could be because of increased CD and ND in the respective group. Increase in the N/C ratio can be indicative of an early dysplastic change because in squamous cell carcinoma the N/C ratio is increased to 1:1 from 1:4.[14] This result of the present study is also consistent with the results of previous studies conducted by multiple authors in the past decade.[6,12,13]

## Conclusion

Changes in cellular diameter, nuclear diameter and N/C ratio was observed among smokers and naswar chewers when compared to control group which might indicate a cause affect relationship. The results seen in this study are limited to a reactionary change with smoking and naswar use. The relationship of these changes with pre-malignancy or malignancy needs further research with the help of more accurate methods as immuno-markers

**Table I: Comparison of Cytomorphometric Readings in Three Stains H & E, PAP, Giemsa, in Smokers, Naswar Users and Control Group.**

| Stains | CONTROL | | | SMOKERS | | | NASWAR USERS | | |
|---|---|---|---|---|---|---|---|---|---|
| | CD µm | ND µm | N/C ratio | CD µm | ND µm | N/C ratio | CD µm | ND µm | N/C ratio |
| H &E | 43.81±2.01 | 9.97±0.80 | 1:4.4±0.41 | 54.38±3.31 | 12.66±0.92 | 1:4.3±0.39 | 42.72±8.00 | 11.80±2.23 | 1:3.5±0.68 |
| PAP | 43.81±2.01 | 9.97±0.80 | 1:4.4±0.37 | 54.41±3.29 | 12.68±0.91 | 1:4.3±0.39 | 42.72±8.00 | 11.80±2.23 | 1:3.5±0.69 |
| GIEMSA | 43.81±2.01 | 9.97±0.80 | 1:4.4±0.37 | 54.36±3.32 | 12.63±0.91 | 1:4.3±0.38 | 42.72±8.00 | 11.80±2.23 | 1:3.6±0.28 |

S= smoker, N= naswar user, M =control group.

CD= Cellular Diameter, ND= Nuclear Diameter, N/C= Nuclear /Cytoplasmic ratio

**Table II: Result of Post Hoc Tukey Test Among Groups on H & E, Pap, and Giemsa Stain**

| Dependent Variable | Group | Group | Significance Value H & E | Significance Value Pap | Significance Value Giemsa |
|---|---|---|---|---|---|
| Cellular diameter | S | N | .000 | .000 | .000 |
| | S | M | .000 | .000 | .000 |
| | M | N | .664 | .664 | .664 |
| Nuclear Diameter | S | N | .049 | .047 | .061 |
| | S | M | .000 | .000 | .000 |
| | N | M | .000 | .000 | .000 |
| N/C ratio | S | N | .000 | .000 | .000 |
| | M | S | .650 | .676 | .541 |
| | M | N | .000 | .000 | .000 |

S= Smoker, N= Naswar User, M =Control Group.

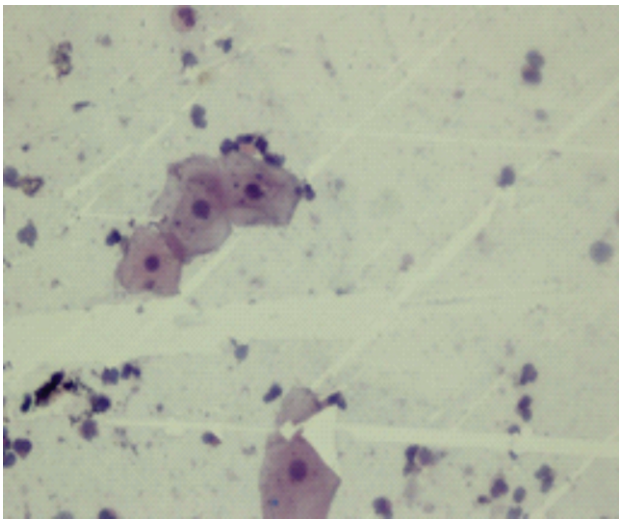

**Fig. 1: Inflammatory cells in Naswar users at 40x**

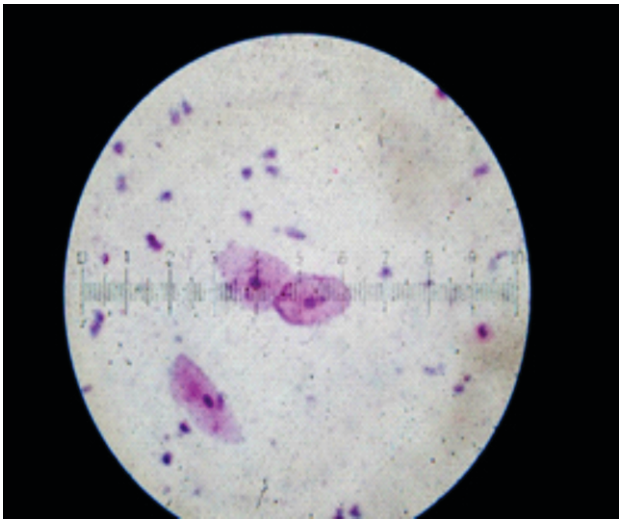

**Fig. 2: Cells Stained with Pap Stain in Naswar Users With Ocular Micrometer At 40x**

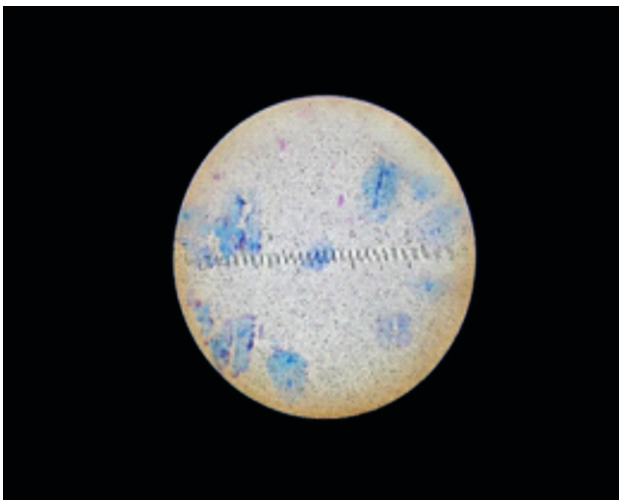

**Fig. 3: Cells Stained with Geimsa Stain In Naswar Users With Ocular Micrometer 40x**

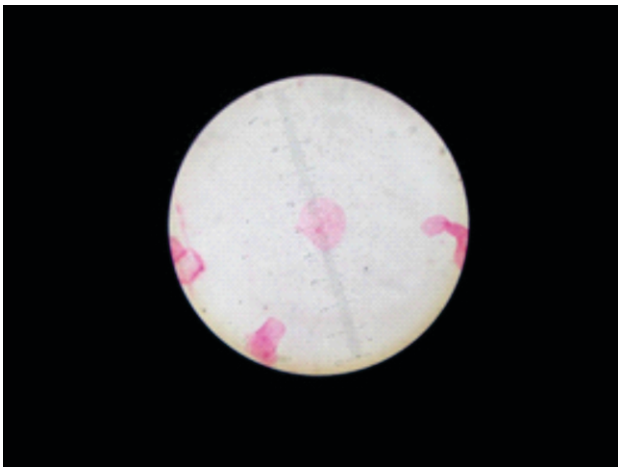

**Fig. 4: Cells Stained with H&E Stain in Naswar Users with Ocular Micrometer At 40x**

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**CONFLICT OF INTEREST**

Authors declared no conflicts of Interest.

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**DATA SHARING STATMENT**

The data that support the findings of this study are available from the corresponding author upon request.