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Cytomorphometric evaluation of buccal mucosa in tobacco chewers

[1]Arpita Gupta, [2]Seema Gupta, [3]Rachna Agrawal, [4]Sunny Yadav, [5]Manish Kumar Singhal, [6]Rajesh Kumar Suri

[1]Senior Resident, Department of Anatomy, VardhmanMahavir Medical College and Safdarjung Hospital, New Delhi, India.

[2]Professor, Department of Anatomy, S.M.S. Medical College, Jaipur, Rajasthan, India.

[3]Senior Demonstrator, Department of Anatomy, Government Medical College, Bharatpur, Rajasthan, India.

[4]Senior Demonstrator, Department of Anatomy, S.M.S. Medical College, Jaipur, Rajasthan, India.

[5]Assistant Professor, Department of Pathology, S.M.S. Medical College, Jaipur, Rajasthan, India.

[6]Director Professor, Department of Anatomy, VardhmanMahvir Medical College and Safdarjung Hospital, New Delhi

***Corresponding Author**: **Arpita Gupta**

Email id: drarpita1808@gmail.com

ABSTRACT

Objective

This study was done to compare cytomorphometrical parameters of the buccal mucosal cells of tobacco chewers and non-chewers by non-invasive painless method.

Study design

We measured and compared nuclear diameter (ND), cellular diameter (CD) and nucleo-cytoplasmic ratio (ND:CD) in the buccal mucosal cells of controls (Group A n=40) and cases (Group B n=40).

Results

The mean nuclear diameter (in µm) of controls and cases was 7.6057±0.85434 and 10.2438±1.2453 respectively ($p<0.001$). The mean cellular diameter (in µm) of controls and cases was 68.8247±4.41425 and 53.3243±3.70648 respectively ($p<0.001$). The ratio of mean nuclear diameter and mean cellular diameter of controls and cases was 0.111±0.01236 and 0.194±0.03095 respectively ($p<0.001$). On application of Unpaired't' test, significant increase in nuclear diameter, significant decrease in cellular diameter and significant increase in nucleo-cytoplasmic ratio was observed in tobacco chewers as compared to tobacco non-chewers.

Conclusion

Changes in nuclear diameter and cellular diameter i.e. increase in nuclear diameter, decrease in cellular diameter and increase in nucleo-cytoplasmic ratio may be the earliest changes observed in buccal mucosal cells of cases chewing tobacco as compare to controls not chewing tobacco. This information may help in diagnosing the dysplasia and oral squamous cell carcinoma at early stage and monitoring its recurrence and follow-up.

Keywords: Nuclear diameter, Cellular diameter, Nucleo-cytoplasmic ratio, Cytomorphometric analysis, Tobacco chewers.

INTRODUCTION

Among various types of carcinomas in India, most common type is oral cancer[1]. Tobacco use either in chewing or smoking form and alcohol ingestion along with the rate of exposure can be ascribed as major causation factors for oral squamous cell carcinoma and most of the head and neck carcinomas [2].Chewable tobacco has carcinogens 100 times more concentrated than smoking tobacco[1, 3]. In India, apart from smoking, tobacco is commonly consumed as Gutkha which comprises of chewing tobacco and betel nut and Quid which is composed of tobacco and lime which are kept in oral cavity generally in the contact of buccal mucosa for a long time which leaks out carcinogens and causes precancerous lesions leading to carcinoma by acting on it [1]. In addition, lime shows additive effect contributing to cytogenetic damage of buccal mucosa leading to oral carcinoma by releasing reactive oxygen free radicals from betel nut [4].

Currently the diagnostic test of choice for oral premalignant and malignant lesions is biopsy which is very painful [5]. Exfoliative cytology is a simple, painless, non-invasive diagnostic technique which has proved to be of significance as an addition to biopsy in monitoring of suspicious lesions in the patient nervous of biopsy and in whom surgery is contraindicated, detecting dysplastic changes, diagnosing very early and minute tumors by observing the changes in normal cellular architecture, follow up of lesions to eliminate recurrence and can be done repetitively with little distress to the patients. These properties amplify the chances of detection of premalignant and malignant lesions in advance. The advantage of exfoliative cytology is that the smear prepared by it can be examined quantitatively and qualitatively [6, 7]

Various parameters such as cell size, nuclear size, nucleo-cytoplasmic ratio, nuclear shape, nuclear discontinuity, nuclear texture and optical density can be assessed jointly in order to favour the diagnosis [8]. Among these parameters, the cell size, nuclear size and their ratio have been shown to play major role in the assessment of oral lesions [9, 10]. The reduction in the cellular diameter and rise in the nuclear diameter are the two important morphologic changes that occur in the actively proliferating cells [11]. The present study has been done to observe and compare the cytomorphometrical data of buccal mucosal cells scraped from tobacco chewers with those of tobacco non-users.

MATERIALS AND METHODS

After taking ethical clearance, the study was conducted which comprised of 80 subjects divided into two groups: 40 cases (20 male + 20 female) with tobacco chewing habit were selected from MahilaChikitsalaya, Jaipur among attendants of patients admitted in labour ward/ postnatal ward. 40 controls (20 male + 20 female) without tobacco chewing habit and without any lesion were selected after matching for age and gender. Detailed history regarding quantity, duration and type of tobacco chewing was taken along with socio-demographic characteristics. Consumption of tobacco in any form for a minimum of 4-5 times per day for minimum period of 5 years was taken as criteria for tobacco chewing.

Subjects were asked to clean their mouth with water and cells were scraped from the clinically normal appearing buccal mucosa of both sides of the cases and controls using a gentle scraping motion with a premoistened wooden spatula. If there was any lesion in buccal mucosa in cases, then sample was taken from lesioned area. The sample was immediately smeared on the microscopic slides and fixed with 100% ethyl alcohol for 20-30 minutes to assure adequate fixation to maintain the cytomorphological characteristics. Slides were stained by using Rapid-Pap Kit (Bio Lab Diagnostics India Private Limited).

The stained slides were studied under 400X magnification of Research microscope (Nikon 90i). A total of 100 cells with well-preserved cytoplasm and intact nuclei (not smeared, clumped or overlapped) were included in the analysis and examined from each slide for each subject using zigzag method for screening of the slides. These cells were measured for nuclear diameter, cytoplasmic diameter and nucleo-cytoplasmic ratio by using Image J software (Scriptable Java app for scientific image processing). Nuclear and Cytoplasmic diameter of each of the 100 cells were measured along both X-axis (Figure 1) and Y-axis (Figure 2) along the maximum dimension observed. Then the mean of the values of X-axis and Y-axis

for each cell was calculated and considered the exact nuclear and cytoplasmic diameter for that cell. This information was then used for data collection and statistical analysis.

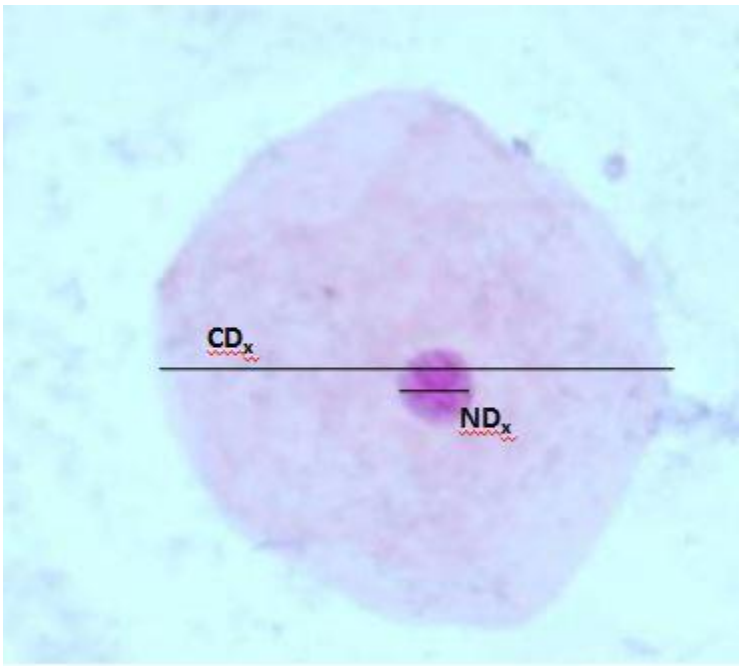

Figure 1: Cell showing measurement of cytoplasmic diameter and nuclear diameter in X-axis. CDx- Cytoplasmic Diameter in X-axis, NDx- Nuclear Diameter in X-axis.

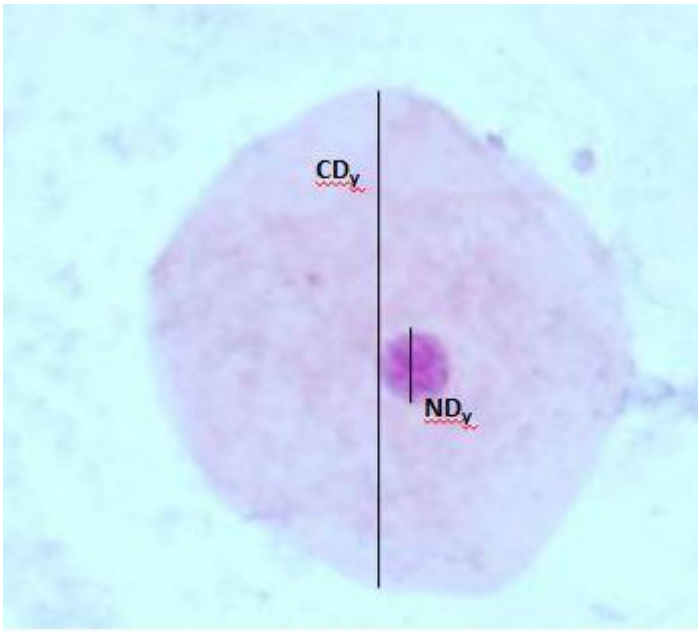

Figure 2: Cell showing measurement of cytoplasmic diameter and nuclear diameter in Y-axis; CDy- Cytoplasmic Diameter in Y-axis, NDy- Nuclear Diameter in Y-axis.

Continuous variables were expressed as mean and standard deviation while nominal variables as proportions. Comparison of continuous variables was done by using Unpaired t-test whereas Chi-square test was used for nominal variables. Correlation was found out by using Pearson correlation coefficient. All calculations were done by using Medcalc 14.0.0 version available online. $p<0.05$ was taken as significant.

RESULTS

All the cases in the study group had the habit of tobacco chewing for 5 years or more and minimum of 4-5 times/day. On comparison of mean nuclear diameter between tobacco chewers and non-chewers in table no.1, authors observed that mean nuclear diameter of tobacco chewers was 10.2438 µm while that of non-chewers was 7.6057 µm which was found statistically significant on application of unpaired't' test ($p < 0.001$).

Table No. 1:- Comparison of Mean Nuclear Diameter

Group	N	Mean	Std. Deviation	'p' Value*
Chewer	40	10.2438	1.2453	< 0.001
Non-Chewer	40	7.6057	0.85434	

On comparison of mean cellular diameter between the study groups in table no.2, the mean cellular diameter of tobacco chewers was 53.3243 µm while that of non-chewers was 68.8247 µm, which was observed statistically significant on application of unpaired't' test ($p < 0.001$).

Table No. 2:- Comparison of Mean Cellular Diameter

Group	N	Mean	Std. Deviation	'p' Value*
Chewer	40	53.3243	3.70648	< 0.001
Non-Chewer	40	68.8247	4.41425	

On comparison of ratio of mean nuclear diameter (ND) and mean cellular diameter(CD) between the study groups in table no. 3, authors observed that mean ND/CD ratio of tobacco chewers was 0.194 while that of non-chewers was 0.111 and the difference between them was observed to be statistically significant on application of unpaired't' test ($p<0.001$).

Table No. 3:- Comparison of Mean Nuclear Diameter and Mean Cellular Diameter Ratio (Mean ND/CD Ratio)

Group	N	Mean	Std. Deviation	'p' Value*
Chewer	40	0.194	0.03095	< 0.001
Non-Chewer	40	0.111	0.01236	

DISCUSSION

Oral cavity is a miniature screen of whole body and a lesion which is present in oral cavity may indicate the total health or habits of the individual. Tobacco related habits are highly prevalent among Indian people. It is well known that tobacco contains carcinogenic chemical constituents and cause various mucosal lesions ranging from chewers mucosa to potentially malignant lesions which may progress to oral cancer. Early detection and cessation in the habits can save people from life threatening tobacco related oral malignancies. The IARC identified alcoholic beverages, betel quid with tobacco and tobacco smoke as human carcinogens targeting on the oral cavity, pharynx larynx and oesophagus[12].

Before 1980s, cytology studies were constrained chiefly to the subjective or qualitative interpretation. But nowadays, with advance technologies, more trustworthy quantitative techniques like cytomorphometry, histometry etc., can be made using computer assisted image analyzer, which aims at reproducibility, enabling direct comparison from person to person. A combination of several abnormalities is necessary for proper diagnosis as single structural change is insufficient, for this, nuclear changes are one of the most important criteria for diagnosis of precancerous and cancerous lesions. Several studies in the past have used cell diameter and nuclear diameter parameters in morphometric analysis of cells [13,14,15,16,17]. The cytological and histological evaluation of a lesion collectively has been found to yield the maximum proportion of timely diagnosis of oral cancer. In our study tobacco chewers and non-chewers were evaluated and a correlation was obtained. With the advent of

image analysis techniques we feel that the application of such systems to our existing investigations would be extremely advantageous.

Atypia was consistently present with two or more of the following features: nuclear enlargement, amplified nuclear/cytoplasmic ratio, nuclear hyperchromatism, chromatin clumping with prominent nucleation, irregular nuclear membranes, bi or multi-nucleation, increased keratinization [18]. The reduction in cytoplasmic area has been found to be an early cytological indication of dysplastic change within a lesion [6, 10, 17]. When the mean cytoplasmic diameter of exfoliated cells from tobacco chewers (53.3243 µm) was compared with those from non-chewers (68.8247 µm), it was seen that tobacco chewers had a reduced cell diameter, when compared to that of non-chewers and this was highly significant (p value < 0.001).When the mean nuclear diameter of exfoliated cells from tobacco chewers (10.2438µm) was compared to the non-chewers (7.6057 µm), a highly significant increase in the nuclear diameter was seen (p value<0.001). This observation is consistent with previous studies [15, 19], where an increase in nuclear diameter was seen in oral leukoplakia lesions when compared to normal. The findings were similar as in smears observed by Ramaesh T et al[20] and in histometric studies done by Shabana AHM et al[15] suggesting significance of various nuclear and cellular parameters, nuclear and cytoplasmic area and nuclear to cytoplasmic ratio in the diagnosis of oral lesions. Due to amplified DNA content of the nucleus, the nuclear diameter increases which point out that tobacco use in any form brings about detrimental effects and alteration in the nuclear size, nuclear area of oral mucosal cells and shows dysplasia in histology [6]. However, some other authors in their studies have shown no significant changes in nuclear dimensions of cells from premalignant lesions when compared to that of normal oral mucosa [6,17,21].Similarly, when the cell diameter was compared between the two groups, reduction in the mean cytoplasmic diameter of the habit group which was statistically significant (p value< 0.001) was observed which could be an early indication of malignant change, which has also been reported in earlier studies [14, 6, 10].

Earlier studies showed that due to chronic irritation of oral mucosa caused by tobacco habits produces inflammatory changes which results in increase in the nuclear size[21]. This is also consistent with our study, which showed increased mean nuclear diameter (MND) of the tobacco chewers group (10.2438µm) as compared to the non-chewers (7.6057µm). In our study, tobacco chewers were considered for the subjects who chewed betel nut with tobacco or consumed tobacco in the raw or processed form. Significant increase in the nuclear size (ND), reduction in cell size (CD) and increase in ND:CD ratio have been reported to be early malignant changes seen in oral epithelial cells exposed to the habit of tobacco chewing suggest that this habit may be related to the development of oral premalignant and malignant lesions. These results tend to support the findings of epidemiological studies that tobacco chewing is a causative factor for oral cancer [14].

According to Goldsby et al [22], the mean nuclear size in epithelial cells varies from 7.6µm to 10.9µm and the cellular diameter varies from 29.7µm to 98.21µm. In our study, we found the MND, MCD and ratio between MND and MCD in controls to be 7.6057µm, 68.8247µm and 0.111 respectively. These values are well within the range given by Goldsby et al. In tobacco product users in our study, the exfoliated cells had nuclear diameter ranging from 8.09 to 12.09 µm with a mean of 10.2438 µm, the cell diameter which ranged between 44.29 and58.36 µm with a mean of 53.3243 µm and the nucleo-cytoplasmic ratio ranged between 0.143 and 0.239 with a mean of 0.194. Swetha Acharya assessed the tobacco chewing effects on the buccal mucosa by using cytomorphometry and observed progressive decrease in cell diameter, increase in nuclear diameter and increase in the nucleo-cytoplasmic ratio in smears from all tobacco (gutkha) chewers as compared with non-tobacco chewers [23]. Similar results were perceived in the study conducted by Saranya RS and Sudha S between controls and khaini users aged 25-50[24]. Present study showed similar noteworthy quantitative alterations in the tobacco chewers as compared to non-chewers.

CONCLUSION

Cell diameter and nuclear diameter have been found to be important subjective parameters, which can be used as a vital aid in creating a

cytopathologic distinction between cells from normal oral mucosa, tobacco chewers and premalignant lesions. This objective analysis of cells is better than the subjective assessment of size, which is less accurate and less reproducible. Histopathologic examination is necessary to completely characterize the lesion which may show a dense, chronic inflammatory infiltrate with epithelial changes ranging from atrophy supplemented by hyperkeratosis to dysplasia to frank malignancy.

In addition, it has been demonstrated that exfoliative cytology is appreciated for monitoring clinically suspicious lesions and malignant lesions after definitive treatment. This study, therefore, confirms only the cause–effect relationship between tobacco chewing and quantitative cellular and nuclear alterations. Thus, according to above discussion, mean nuclear diameter increases, mean cytoplasmic diameter decreases and mean nucleo-cytoplasmic ratio increases in the buccal mucosal cells of tobacco chewers as compared to tobacco non-chewers. To rule out the above possibilities, further studies are required including more sample size, standardized parameters and advanced techniques.

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