Cytomorphometric analysis and morphological assessment of oral exfoliated cells in type 2 diabetes mellitus and healthy individuals: A comparative study

ABSTRACT
Context: Oral exfoliative cytology is a simple, nonaggressive technique that is well accepted by patients. Therefore, it is an attractive option, which aids in the diagnosis and observation of epithelial atypias associated with oral mucosal diseases.

Aims: The aim of this study was to evaluate and compare the quantitative and qualitative alterations in exfoliative smears from type 2 diabetics and healthy individuals.

Patients and Methods: The study includes 30 type 2 diabetics and 30 healthy persons of both sexes. PAP and hematoxylin and eosin (H and E) stained smears were prepared from buccal mucosa (BM), tongue (T), floor of the mouth (FOM), and palate (P). Under a light microscope, 50 clearly defined unfolded epithelial cells were quantitatively evaluated for cellular area (CA), nuclear area (NA), and cellular-to-nuclear area ratio (CA:NA) and assessed for morphological features.

Statistical Analysis: Collected data was manually entered into the Statistical Package for the Social Sciences version 13.5 for analysis. Student's t-test was used at 95% confidence interval.

Results: Quantitative assessment of the overall mean CA was less, mean NA was more, and mean CA:NA was less in diabetics than that in healthy persons at all the four sites. Diabetic oral cells showed qualitative cytoplasmic and nuclear alterations: cytoplasmic vacuoles, karyorrhexis, karyolysis, pyknosis, peri-nuclear halo, binucleation, nuclear vacuoles, inflammation, and microbial colonies.

Conclusion: Oral cytology from type 2 diabetics is associated with detectable cytomorphic changes with alteration in size of the cell and nucleus, which is site specific, indicating epithelial cell degeneration in cytoplasm and nucleus.

Key words: Cytomorphometry; diabetes; oral exfoliative cytology; oral mucosa

Introduction
Type 2 diabetes mellitus (DM) is world’s fifth most common chronic condition and sixth leading cause of mortality among elderly.[1] The World Health Organization (WHO) has estimated India to be home to the largest number of diabetics than any other country and this trend is supposed to continue in future.[2] In a developing country like India, majority of people with diabetes are in the age range of 45–64 years, whereas in developed countries they are found to be of more than 64 years of age. Globally as well as in the Indian scenario, the prevalence of DM is higher in men than that in women.[3]
Although the exact etiology of DM is obscure, there are multiple causes involving genetic and environmental factors. These causes could be associated with family history, urbanization, aging, increased obesity prevalence, stress, and physical inactivity. DM is a group of metabolic disease in which the defect lies in the deficient insulin secretion, insulin action, or both which leads to chronic hyperglycemia. In diabetic patients, chronic hyperglycemia can result into various systemic as well as oral complications. Therefore, healthcare professionals including dentists should be well versed with its clinical manifestation. A number of clinical manifestations and oral lesions associated with DM have been reported in literature. These can vary from minimal nonspecific changes such as traumatic ulcer, candidiasis, and xerostomia to alarming more defined changes such as periapical abscess, gingivitis, and periodontitis. Although none of them are its pathognomic indicator, biopsy becomes mandatory for diagnosis of such pathologies in DM. However, in DM, changes in these pathologies may occur due to variations in blood glucose level, which render the biopsy of these pathologies of no use. In these cases, oral exfoliative cytology accounts as a more appropriate tool because it is easy, simple, and noninvasive as compared to conventional intervention techniques. Moreover, exfoliative cytology can be performed from multiple sites in the same patient in single/multiple visits.

Thus, the present study was conducted to evaluate cytomorphometric and morphological changes in oral epithelial cells by exfoliative cytology in diabetic patients from four sites, namely, buccal mucosa, tongue, floor of mouth, palate (BM, T, FOM, P, respectively). Various cytomorphometric parameters such as cellular area (CA), nuclear area (NA), and cellular-to-nuclear area ratio (CA:NA), as well as morphological (cytoplasmic and nuclear) alterations have been assessed in PAP and hematoxylin and eosin (H and E) stained cytosmears. Eventually, to assess these cellular changes occurring in the oral mucosa of type 2 DM patients and to establish the role of exfoliative cytology as an adjunct in DM diagnosis.

**Patients and Methods**

Institutional ethical committee clearance was obtained prior to the beginning of the prospective study. Patient’s personal, medical, and dental history were recorded. On the day of the cytological examination, random blood glucose level for each case was estimated. PAP stain was used for quantitative analysis whereas both H and E and PAP were used for qualitative analysis. Inclusion criterias for DM were patients falling under any of these diagnostic criterias (1) Fasting blood glucose ranging from 130 to 200 mg/dl, (2) Random blood glucose of ≥ 200 mg/dl, (3) Postprandial blood glucose of ≥ 200 mg/dl or diagnosed type 2 diabetes for not more than 5–10 years with good health and oral hygiene, without any oral lesions or diabetics taking medication either in form of oral hypoglycemic or insulin. Inclusion criterias for healthy individuals were adult healthy individuals with no history of diabetes or any other illness. Exclusion criterias were medical history/records with pathological states systemic diseases/disorders other than type 2 diabetes, malignancy, nutritional deficiency, and reduced immune competency; physiological states such as pregnancy and lactation; habits such as smoking, alcohol, and tobacco chewing.

A total of 60 cases were considered for the study, which included study group of type 2 diabetic patients (Group 1) and control group of healthy individuals (Group 2). Study and control groups were further subdivided according to gender as Group 1A, Group 1B, and Group 2A; Group 2B consisted of 15 males and 15 females each of age ranging 40–70 years from whom smears were prepared from keratinized (T, P) and non-keratinized (BM, FOM) mucosa.

Patients were asked to rinse the oral mucosa with saline; the mucosa was cleaned and dried with a gauze swab to remove surface debris and excess saliva. Eight smears (two from each site) were prepared by a wet wooden spatula by gentle scraping and then transferred to clean dry glass slides. The slides were then fixed in 95% ethyl alcohol for 15 minutes. Out of the eight smears, four each were stained in PAP and H and E stains.

In each PAP-stained smear, 50 clearly defined unfolded cells with adequate staining were selected by systematic sampling, moving the microscope stage from left to right followed by down and across in order to avoid repetition of cells from different fields at 40×. CA and NA were calculated with the help of Image J analysis. In each PAP and H and E stained smears- binucleation, inflammation, cytoplasmic vacuolation, karyorrhexis, karyolysis, pyknosis, microbial colonies, and Candida were assessed.

The collected data was manually entered into the Statistical Package for the Social Sciences version 13.5 (IBM, Delaware, USA) for analysis. Then the data was subjected to descriptive and inferential statistics to generate mean and standard deviation. For intergroup and intragroup gender comparison of means, Student’s t-test was used at 95% confidence interval.

**Results**

The mean CA, NA, and CA:NA ratio of study and control group of
four sites are presented in Table 1. Statistical analysis of the data obtained showed that the mean CA was less, NA was more and CA:NA was less in Group 1 for all the four sites. All the results were statistically significant except for CA for P [Table 1].

On intergroup gender comparison of diabetic males and females (Group 1A, Group 1B) with control males and females (Group 2A, Group 2B) a lower mean CA was noted. However, the result was significant only for T and FOM between intergroup males and for BM, T, and FOM for intergroup females. Moreover, the mean NA was more and the mean CA:NA was less and the results were significant when intergroup gender comparison was done. Overall, the result was statistically significant except of CA for the sites BM and P in males and for P in females [Tables 2 and 3].

Table 1: Inter-group comparison of cytomorphic parameters from all the four sites

| Cytomorphic Parameters | Site  | Group 1 (Mean±SD µm²) | Group 2 (Mean±SD µm²) | P     |
|------------------------|-------|-----------------------|-----------------------|-------|
| CA                     | BM    | 3586.52±375.95        | 3778.51±252.38        | 0.024 |
|                        | T     | 2589.82±360.99        | 3029.39±185.85        | <0.001|
|                        | FOM   | 2845.96±95.35         | 3116.69±201.94        | <0.001|
|                        | P     | 2649.79±119.46        | 2684.60±206.42        | 0.428**|
| NA                     | BM    | 86.87±5.74            | 62.42±3.01            | <0.001|
|                        | T     | 73.75±3.08            | 54.64±2.40            | <0.001|
|                        | FOM   | 88.54±1.73            | 58.72±3.04            | <0.001|
|                        | P     | 72.47±2.24            | 53.97±2.19            | <0.001|
| CA:NA                  | BM    | 40.97±5.29            | 60.70±5.30            | <0.001|
|                        | T     | 35.18±5.34            | 55.67±4.58            | <0.001|
|                        | FOM   | 32.15±1.19            | 53.18±4.21            | <0.001|
|                        | P     | 36.60±2.10            | 49.76±3.67            | <0.001|

**P value less than 0.05

Table 2: Intergroup gender comparison between type 2 diabetic males and control males

| Cytomorphic Parameters | Site  | Group 1A (Mean±SD µm²) | Group 2A (Mean±SD µm²) | P     |
|------------------------|-------|-----------------------|-----------------------|-------|
| CA                     | BM    | 3652.32±494.73        | 3823.20±246.37        | 0.24**|
|                        | T     | 2640.06±322.97        | 2981.33±170.87        | 0.002 |
|                        | FOM   | 2861.23±82.37         | 3093.83±167.60        | <0.001|
|                        | P     | 2640.96±107.4         | 2598.5±153.34         | 0.38**|
| NA                     | BM    | 87.08±6.76            | 62.43±2.26            | <0.001|
|                        | T     | 73.43±2.32            | 56.17±2.2             | <0.001|
|                        | FOM   | 88.60±1.73            | 58.22±1.88            | <0.001|
|                        | P     | 72.48±2.39            | 53.56±1.51            | <0.001|
| CA:NA                  | BM    | 41.23±7.14            | 61.35±5.06            | <0.001|
|                        | T     | 36.02±4.91            | 53.16±3.82            | <0.001|
|                        | FOM   | 32.30±0.91            | 53.14±2.59            | <0.001|
|                        | P     | 36.46±1.87            | 48.55±3.40            | <0.001|

**P value less than 0.05

Table 3: Intergroup gender comparison between type 2 diabetic females and control females

| Cytomorphic Parameters | Site  | Group 1B (Mean±SD µm²) | Group 2B (Mean±SD µm²) | P     |
|------------------------|-------|-----------------------|-----------------------|-------|
| CA                     | BM    | 3520.71±196.81        | 3733.82±258.77        | 0.017 |
|                        | T     | 2539.57±400.26        | 3077.45±193.38        | <0.001|
|                        | FOM   | 2830.68±107.45        | 3139.54±235.07        | <0.001|
|                        | P     | 2658.62±133.62        | 2770.71±221.04        | 0.104**|
| NA                     | BM    | 86.65±4.73            | 62.39±3.69            | <0.001|
|                        | T     | 74.06±3.75            | 53.12±1.48            | <0.001|
|                        | FOM   | 88.48±1.78            | 59.22±3.87            | <0.001|
|                        | P     | 72.45±2.14            | 54.36±2.69            | <0.001|
| CA:NA                  | BM    | 40.7±2.61             | 60.04±5.63            | <0.001|
|                        | T     | 34.32±5.76            | 58.17±3.90            | <0.001|
|                        | FOM   | 32±1.43               | 53.21±5.47            | <0.001|
|                        | P     | 36.73±2.37            | 50.96±3.63            | <0.001|

**P value less than 0.05
In intragroup gender comparison, for diabetics, the result was statistically insignificant. The result was nonsignificant in control as well, except CA of P and for T in case of NA and CA:NA [Tables 4 and 5]. This reflected that in diabetics gender has no effect on morphometric changes in the cell but, the control group showed some variation in all the parameters.

On intergroup comparison of qualitative changes, it was observed that cytoplasmic vacuolization [Figure 1a] was seen in all the sites except P in diabetics and was absent in all the sites in the control group. Nuclear degenerative changes such as karyorrhexis [Figure 1b], karyolysis [Figure 1c], pyknosis [Figure 1d], perinuclear halo [Figure 1e], and nuclear vacuolization [Figure 1f] were present in Group 1, however, none were present in Group 2. Binucleation [Figure 1g] was seen in both the groups with higher rate of occurrence in Group 1. Percentage distribution of inflammation [Figure 1h] was more in Group 1 for all the four sites, as compared to Group 2. Microbial colonies [Figure 1i] were present on all the sites in both the groups, however, its frequency of occurrence was more in Group 1. Candida was not found in any of the groups.

### Discussion

Aim of the present study was to identify the oral epithelial changes using exfoliative cytology through cytomorphometry and morphological methods from BM, T, FOM, and P in type 2 diabetic patients and comparing them with the normal

| Cytomorphometric Parameters | Site | Group 1A (Mean±SD µm²) | Group 1B (Mean±SD µm²) | P   |
|-----------------------------|------|------------------------|------------------------|-----|
| CA                          | BM   | 3652.32±494.73         | 3520.71±196.81         | 0.34*** |
|                             | T    | 2640.06±322.97         | 2539.57±400.26         | 0.45** |
|                             | FOM  | 2861.23±82.37          | 2830.68±107.45         | 0.39** |
|                             | P    | 2640.96±107.4          | 2658.62±133.62         | 0.69** |
| NA                          | BM   | 87.08±6.76             | 86.65±4.73             | 0.84** |
|                             | T    | 73.43±2.32             | 74.06±3.75             | 0.58** |
|                             | FOM  | 88.60±1.73             | 88.48±1.78             | 0.85** |
|                             | P    | 72.48±2.39             | 74.25±2.14             | 0.97** |
| CA:NA                       | BM   | 41.23±7.14             | 40.70±2.61             | 0.79** |
|                             | T    | 36.02±4.91             | 34.32±5.76             | 0.39** |
|                             | FOM  | 32.30±1.43             | 32±1.43                | 0.50** |
|                             | P    | 36.46±1.87             | 36.73±2.37             | 0.73** |

**P value less than 0.05

| Cytomorphometric Parameters | Site | Group 2A (Mean±SD µm²) | Group 2B (Mean±SD µm²) | P   |
|-----------------------------|------|------------------------|------------------------|-----|
| CA                          | BM   | 3823.29±246.37         | 3733.82±258.77         | 0.34*** |
|                             | T    | 2981.33±170.87         | 3077.45±193.38         | 0.16** |
|                             | FOM  | 3093.83±167.60         | 3139.54±235.07         | 0.54** |
|                             | P    | 2598.5±153.34          | 2770.71±221.04         | 0.01 |
| NA                          | BM   | 62.43±2.26             | 62.39±92.69             | 0.97** |
|                             | T    | 56.17±2.2              | 53.12±1.48             | <0.001 |
|                             | FOM  | 58.22±1.88             | 59.22±3.87             | 0.37** |
|                             | P    | 53.56±1.51             | 54.36±2.69             | 0.32** |
| CA:NA                       | BM   | 61.35±5.06             | 60.04±5.63             | 0.50** |
|                             | T    | 53.16±3.82             | 58.17±93.90             | 0.001 |
|                             | FOM  | 53.14±2.59             | 53.21±5.47             | 0.96** |
|                             | P    | 48.55±3.40             | 50.96±3.63             | 0.07** |

**P value less than 0.05
mucosa of healthy individuals. Mean of the parameters CA, NA, CA:NA along with morphological features were calculated and assessed. Furthermore, intergroup and intragroup gender comparisons were performed.

Quantitative assessment showed less mean CA, more mean NA, and less mean CA:NA in diabetics than that in controls for all the four sites with significant results except for CA for P.

Similar results have been reported by few authors, however, the difference in mean CA in their studies was statistically not significant. Although in the present study there was statistically significant decrease in mean CA in diabetics, according to some authors, there was no statistical difference in CA when comparison was made between diabetics and controls. There are various explanations for the maintained CA in both the groups.

Type 2 DM is a psychosomatic and an age-related disease, which consequentialy causes decreased perfusion of tissues and cell turnover. Decreased tissue perfusion occurs by narrowing of blood vessels, which ultimately causes ischemia and atherosclerosis resulting in less nutrition to the cell. Moreover, decreased cell turnover gives the cell a stressed environment. Normally, a cell maintains proper proportion and quantities of different cellular constituents by genetic and enzymatic regulation. Hence, in a cell the enzymes which are inactive can be activated as per requirement.

In a stressful condition such as DM, most of the ATPs in cell are depleted and a considerable amount of c-AMP is found as a breakdown product of ATP. The c-AMP acts as an enzyme activator for phosphorylase enzyme and controls intracellular ATP concentration, as well as maintains function of the cell in a stressed condition. This c-AMP instantly activates the glycogen splitting enzyme and phosphorylase liberating glucose molecule, which rapidly metabolizes and provides energy for replenishment of ATP storage. Hence, ATP storage helps in the maintenance of CA, which is a compensatory mechanism of cell function.

Another parameter assessed was NA, which was increased in diabetics. First reason for its increase is sustained hyperglycemia which increases glycation of proteins, lipids, and nucleic acids, causing accumulation of advanced glycation end products in the walls of large vessels as well as in the basement membrane of microvasculature. This leads to ischemia and atherosclerosis which in turn causes progressive narrowing of the vessel lumen, decreased tissue perfusion, and decrease in cell turnover. This causes delay in the keratinization process of the epithelium. This delay in the cell differentiation process leads to an increase in the number of cells which usually have a large nucleus.

Second factor could be xerostomia due to reduced salivary flow. Decreased salivary flow is related to hyperglycemia, dehydration, medicaments (diuretics), membranopathy of ducts, and secondary to adverse hormonal, microvascular, and neuronal changes. Xerostomia ultimately causes increased oral mucosa trauma which leads to cell loss. Therefore, the activity of the basal cells is enhanced to replenish the lost cells by increasing the proportion of actively dividing cell compartment, which constitutes cell with prominent and large nucleus.

Third factor can be the increased glucose concentration in cell which favors cell growth because glucose has a pivotal role in metabolic processes. Hence, an actively growing cell presents with a prominent and large nucleus.

A study by Suvarna et al. mentioned that, in an actively growing cell, if there is an increase in NA, there should be a concomitant increase in the CA. However, this was contradicted by Frost, according to him, in an actively growing cell the cytoplasmic volume decreases whereas nuclear contents increases because it is undergoing replication. This gives an appearance of larger nucleus in proportion to cytoplasm. Some authors suggested that inflammation can cause an increase NA, but typically in young cells.

The present study showed variation in cell and nuclear size among the four sites under investigation; however, a close correlation was found in some of the results of CA and NA between BM and FOM and T and P in both the groups. This correlation may account for the similarity in the type of mucosa, i.e., nonkeratinized (BM, FOM) and keratinized (T, P).

According to Cowpe et al., it is not possible to predict cell size from nuclear size and vice versa. In their study, CA:NA value displayed a significant variation with age and sites; thus, they considered it to be an unsatisfactory criteria. However, Patel et al. and Franklin et al. have emphasized the need for CA:NA. They reported that it is a suitable parameter to discriminate among study population and also has an advantage of relating nuclear volume to cellular volume, which possibly represents the significant changes that occur in a cell.

In the present study, the intergroup gender comparison for all the parameters was carried out. In diabetic males and
females, the mean CA was comparatively less than that in control males and females. However, the result was significant only for T and FOM between intergroup males and for BM, T, and FOM between intergroup females, respectively. Moreover, the mean NA was more and the mean CA:NA was less, and the results were significant when intergroup gender comparison was made. Overall, the result was statistically significant except of CA for BM and P in males and for P in females. Palate being the most difficult site for scraping could be the reason behind inconsistent results.

The statistically nonsignificant result was found in intragroup gender comparison in diabetics. Further, the majority of the parameters did not show a significant result on intragroup gender comparison in control except for P in case of CA, and for T in case of NA and CA:NA. This reflected that, in diabetics, gender has no effect on morphometric changes in the cell, but the control group showed some variation in all the parameters.

A sex-related survey by Cowpe et al. found, on normal oral squames showed that there was no significant variation in CA, NA, and CA:NA between control males and females. Contradictory to the present study, Patel et al. observed some morphometric changes on gender comparison, which could be due to sexual dimorphism and hormonal differences.

Qualitative degenerative changes were observed and assessed in cytoplasm and nucleus, and were found to be prominent in diabetics compared to the control group. Cytoplasmic vacuoles, karyorrhexis, karyolysis, pyknosis, perinuclear halo, and nuclear vacuoles were present only in diabetics. We could not find Candida in any of the smears of study population.

Occurrence of morphological changes in the oral mucosa of diabetic patients are multifactorial. Although type 2 DM is an age-related disease, it is also governed by numerous factors such as hereditary, diet, lifestyle, pschycologial, social conditions. Long-term hyperglycemia produces the advanced glycation end product which is important in the pathogenesis of DM and also participates in cellular aging.

Cellular aging leads to progressive decline in the proliferative capacity and lifespan of cells. The effects of continuous exposure to exogenous factors causes progression of morphologic alterations, cellular and molecular damage, which includes irregular lobed nuclei, pleomorphism, and cytoplasmic vacuolization. An aging cell also shows nuclear degeneration such as karyolysis which presumably occurs secondary to DNase activity; pyknosis which is characterized by nuclear shrinkage, condensed chromatin, and increased basophilia; and karyorrhexis where there is fragmentation of pyknotic nucleus, along with nuclear vacuolization and perinuclear halo.

DM also causes reduced epithelial cell proliferation and turnover, as well as reduction in the stimulatory effect of insulin and IGF-1. Reduction in cellular nourishment associated with microvascular changes are other possible explanations for these morphological alterations. Moreover, side effect of oral hypoglycemics produces changes such as lactoacidosis, cellular swelling, coarse nuclear chromatin, and altered nuclear size.

Some authors believe that inadequate metabolic control of diabetes is related to certain oral manifestations. Others believe that it might be due to altered immunological response in diabetes such as lower chemotaxis and phagocytosis, and due to the involvement of microcirculation because of reduction in blood supply. This makes diabetic patients more prone to oral infections and alterations.

Morphological alteration due to xerostomia results in a dry, atrophic mucosa accompanying mucositis as well as opportunistic infections with an increase in inflammatory response to microbial colonization.

It is a well-established fact that there is a neutrophil chemotactic defect in DM. In an attempt to overcome this deficiency, a positive feedback mechanism results in an increased inflammation. Adverse hormonal, microvascular, and neuronal changes may also serve as the cause behind increased inflammation. According to Amir et al., hyperglycemia being the major sequel of type 2 DM exacerbates the inflammatory processes and promotes the accumulation of RAGE and TLR4 ligands within the oral mucosa through induction of advanced glycated end product formation and colonization of microbes.

Although in the present study we did not find any Candida, studies by Guggenheimer et al. and Jajaram et al. suggested that diabetics are at a high risk of Candidal infection due to an immunocompromised state.

Type 2 DM adversely affects the morphology of oral mucosa, which compromises tissue function to favor the occurrence of oral infections and epithelial cell degeneration; hence, exfoliative cytology being a simple and noninvasive and with the advancement in the field of computer-aided morphometry can be used as a diagnostic tool. Thus, the present study determines that the mean CA, NA, and CA:NA and various other morphological changes can be used as an effective tool in the diagnosis of DM.
Conclusion

In type 2 diabetics, exfoliative cytology can prove to be a more patient friendly technique as compared to invasive techniques of glucose monitoring from blood. Thus, it can be employed as a tool of mass screening for DM in public health programs. Further, the knowledge of quantitative and qualitative changes in oral mucosa by exfoliative cytology in type 2 DM patients could contribute to the understanding of the disease process at a cellular level. A better understanding of this disease process may help in correlating the degree of cellular changes with the progressive stages of type 2 DM. This could further help in establishing the role of exfoliative cytology as a viable indicator for the diagnosis of type 2 DM through assessment of oral mucosal changes.

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Conflicts of interest

There are no conflicts of interest.

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