Improvization of conventional cytology by centrifuged liquid-based cytology in oral exfoliative cytology specimen

ABSTRACT

Background: Exfoliative cytology is the microscopic examination of shed or desquamated cells from the epithelial surface. Centrifuged liquid-based cytology (CLBC) is a modified technique that was used in the current study.

Aims: To compare the efficacy of CLBC with conventional cytology in apparently normal mucosa after staining with Papanicolaou (PAP) stain.

Materials and Methods: Fifty cases of apparently normal mucosa from healthy subjects were selected for the study. The first sample was taken and spread on the slide by the conventional technique. The second sample was flushed out in a suspending solution, centrifuged, and the cell pellet obtained was used to make the smear. The stained smears were compared for seven parameters such as adequate cellularity, clear background, uniform distribution, cellular overlapping, cellular elongation, mucus, and inflammatory cells. Chi-square test was used for statistical analysis and $P \leq 0.05$ was considered to be statistically significant.

Results: There was a statistically significant difference between CLBC and conventional cytology with parameters such as adequate cellularity ($P = 0.001$), clear background ($P = 0.001$), uniform distribution ($0.005$), cellular overlapping, and cellular elongation ($P = 0$). The presence of mucus and inflammatory cells was minimal as the samples were collected from healthy subjects.

Conclusion: CLBC has better efficacy over the conventional method in all the parameters analyzed.

Key words: Centrifuged liquid-based cytology (CLBC); conventional technique; exfoliative cytology; Papanicolaou (PAP) stain

Introduction

Oral cancers are a major health problem in India, as oral cancers are the most common cancers in males and the third most common in females.[1] Early diagnosis greatly increases the probability of cure with minimum impairment and deformity.[2]

Exfoliative cytology is the microscopic examination of shed or desquamated cells from the epithelial surface, usually the mucus membrane and is a simple, safe, and reliable method. But limitations are seen such as clumping, overlapping of cells, along with the smears being obscured by blood, mucus, and other debris, which potentially leads to an increase in false negative results.[3]

Liquid-based cytology (LBC) was initially developed for cervical uterine cancer screening. As compared to conventional smears,
this technique reduces the number of unsatisfactory and false positive results with significant improvement in cytodiagnostic accuracy.\textsuperscript{[4]} Most LBC preparations showed a good quality of preparation including cytoplasmic and nuclear stainings with a clear background. However, LBC requires expensive automated devices and materials, which might not be affordable for many cytopathology laboratories with limited resources.\textsuperscript{[4]}

Centrifuged liquid-based cytology (CLBC) is a modification of LBC, which is a cost-effective yet efficient technique with better results in comparison to conventional cytology.\textsuperscript{[4]} This technique uses simple and readily available equipment, CLBC technique provides a clear background which is free of debris, blood and microbes, and a microbe-free background. It reveals clear, well-distributed smears with a thin, uniform distribution of cells. The residual sample can be used for advanced procedures such as immunohistochemistry, especially in laboratories with limited access to expensive automated systems.\textsuperscript{[5,6]}

This study was conducted on individuals in the age range of 19-26 years with no habit and with apparently normal mucosa, essentially to study the efficacy of CLBC over conventional cytology so that this technique can be applied in premalignant and malignant lesions as well.

Materials and Methods

The study sample for this comparative study was collected from 50 individuals with no habit and apparently normal oral mucosa. Two cytological smears were obtained from the lesion using a soft toothbrush. One smear was made using the conventional technique and fixed immediately in 95\% isopropyl alcohol. The second sample was collected with a soft toothbrush and flushed out in a suspending solution composed of 20 mL of 95\% ethanol, 6 mL acetic acid, and 74 mL normal saline. This was centrifuged for 15 min at 2,500 rpm. The obtained cell pellet was resuspended in 95\% isopropyl alcohol. A small amount of this sample was taken and spread over the glass slide evenly with the help of another glass slide and left for 1 h and then fixed in 95\% isopropyl alcohol. Gentle movement of one slide over the other did not cause any damage to the cells since a fluid medium existed. Both smears were further stained with Papanicolaou (PAP) stain. The subjects were informed with regard to the research objectives, methods, possible benefits, and potential risks, and a written consent was obtained from all the participants.

Evaluation of Smear Quality

Qualitative analysis of the smear obtained through conventional brush cytology and CLBC was made. Comparison between these two techniques was performed with respect to cellularity, cell distribution, cellular clumping, cellular morphology, the presence of blood, mucus, inflammatory cells, and microbial colonies.

All the slides were evaluated blindly by two independent observers and the information obtained was subjected to statistical evaluation by means of chi-square test. $P$ value $\leq 0.005$ was considered to be significant.

Results

There were considerable differences evident between the two techniques, particularly with respect to essential requirements of a cytological smear [Table 1].

A statistically significant difference was obtained between the two techniques with respect to adequate cellularity, clear background, uniform distribution, cellular overlapping, and cellular elongation and these established that CLBC method was better than conventional method with respect to the above parameters.

Adequate cellularity

Of the 50 cases, adequate cellularity was seen in 33 cases (66\%) using CLBL method [Figure 1a] in contrast to 16 (32\%) cases with the conventional method [Figure 1b]. The presence of more than 40 cells per high power field was considered as adequate cellularity (when 40 or more cells were seen in a given slide, it was considered to be “present” and when there were cells less than that number it was considered to be “absent”).

Clear background

A clear background was seen in 40 (80\%) cases using CLBL method [Figure 1a] in comparison to only 24 (48\%) cases showing a clear background in the conventional method [Figure 1b]. When the cell morphology and cell outline were not hampered with background staining in more than 70\% of the cells, it was considered as clear background (when 70\% of the cells in a given slide showed clear cell details, it was

Figure 1: (a) CLBC technique: Photomicrograph showing less cellular clumping, elongation, and overlapping with uniform distribution of cells (Pap stain, x100). (b) Conventional technique: Photomicrograph showing cellular clumping, elongation, and overlapping with less uniform distribution of cells (Pap stain, x200)
considered as “clear background present” and when less than that percentage of cells showed clear cell details it was considered as “absent”).

**Uniform distribution**
The CLBC method [Figure 1a] gave us better results in 18 (36%) cases in comparison to the conventional method, 6 (12%) cases [Figure 1b] in terms of uniform distribution of cells, which was statistically significant. When there was uniform distribution of more than 70% of the cells in a given slide, it was considered as uniform distribution of cells. In the conventional technique, scant cells were present in the center and a majority of cells were pushed to the periphery (when 70% of the cells in a given slide showed uniform distribution, it was considered as “uniform distribution present” and when less than that percentage of cells showed uniform distribution it was considered to be “absent”).

**Cellular overlapping and cellular elongation**
The conventional method revealed significant cellular clumping with cellular overlapping in 40 cases (80%) and cellular elongation in 35 cases (70%) [Figure 1a], which were high when compared to CLBC technique where cellular overlapping was seen in 19 cases (38%) and cellular elongation was seen in just 2 cases (4%) [Figure 1b]. When the clarity of the cell morphology and cell outline was hampered in more than 70% cells, it was considered as cellular overlapping and cellular elongation (when 70% of the cells in a given slide showed cellular overlapping and elongation, hampering the cell details, it was considered as “cellular overlapping and elongation present” and when less than that percentage of cells showed cellular overlapping and elongation it was considered to be “absent”).

The presence of mucus and inflammatory cells was minimal as the samples were collected from healthy subjects.

**Discussion**
Exfoliative cytology is an advantageous diagnostic procedure because it is noninvasive, relatively painless, and inexpensive, and requires a minimum of technical skills. Despite its advantages, it has certain disadvantages such as inadequate sampling and false negative results. It is well-established that only a small percentage of the harvested epithelial cells obtained during conventional smear are transferred to the glass slide and there is always a possibility of potential source for false negative smears. It is shown that a maximum of only 20% of the cells collected on a variety of collection devices can be mechanically transferred to the flat surface of a glass slide.

| Criteria                  | Conventional method (%) | CLBC method (%) | Chi-square test | P value |
|---------------------------|-------------------------|-----------------|-----------------|---------|
| Adequate cellularity      | 16 (32)                 | 33 (66)         | 11.56           | 0.001   |
| Clear background          | 24 (48)                 | 40 (80)         | 11.11           | 0.001   |
| Uniform distribution      | 6 (12)                  | 18 (36)         | 7.895           | 0.005   |
| Cellular overlapping      | 40 (80)                 | 19 (38)         | 18.231          | 0       |
| Cellular elongation       | 35 (70)                 | 2 (4)           | 46.718          | 0       |
| Mucus                     | 1 (2)                   | 0               | 1.01            | 0.315   |
| Inflammatory cells        | 2 (4)                   | 2 (4)           | 0%              | 1       |

In a study conducted by Shaila et al., slides prepared by the conventional wooden spatula method in normal oral mucosa were disregarded due to either excessive clumping or scarcity of cells. Ogden et al. found less cell yield and cell dispersion, whereas Ahmed et al. found a reduced amount of cells done on normal oral mucosa in the conventional method in comparison to the LBC method.

LBC offers significant advantages over the conventional exfoliative cytology. LBC technology removes most mucus, protein, and red blood cells with the use of glacial acetic acid, distributes cells evenly, improves cell morphology, optimizes sample fixation, provides improved and unbiased sampling, controls cellular density, enhances nuclear detail, reduces scanty preparations, and eliminates air-drying artefacts in oral samples. In a study in Brazil, the liquid-based preparations resulted in higher specimen resolution as well as presented a better cytological morphology for pemphigus vulgaris, squamous cell carcinoma, herpes simplex virus lesions, and fungal infections. But LBC requires expensive automated devices and materials, and trained users for interpretations, which might not be affordable for many cytopathology laboratories in countries with poor resources.

The revolutionary modification of the cervical smear method by using LBC with a significant improvement in cytdiagnostic accuracy with increased sensitivity is CLBC.

In this study, we have evaluated the efficiency of the inexpensive CLBC method relying on cytocentrifugation. This is the first study on normal oral cytology comparing the conventional and CLBC methods in healthy volunteers and it can serve as a reference for future studies with oral lesions.

The cells collected from the buccal mucosa with the help of a brush were initially flushed in a liquid medium and then centrifuged. Each of the components of the reagent has a definite role. Isopropyl alcohol acts as a good fixative in cytological smears. This is important to preserve the morphology of the cells, as much as possible, in the condition...
in which they were present before being sampled. Glacial acetic acid acts as a lysing agent and helps in the lysing of erythrocytes. Lysing of erythrocytes prior to slide preparation results in smears that are easier to interpret because of better visualization of epithelial cells and thus, it enhances the clarity of the background. Physiological saline is iso-osmolar, which maintains the cells in a proper osmolarity condition in order to avoid any osmotic shock and prevent the destruction of epithelial cells. Centrifugation at 2,500 rpm for 15 min with the sample dispersed in the reagent causes sedimentation of the cells at the bottom forming the cell button, whereas all the debris and mucus form the supernatant solution that can be discarded.

We found statistically a significant difference with various parameters such as adequate cellularity, clear background, uniform distribution, cellular overlapping, and cellular elongation in our study in CLBC in comparison to the conventional technique.

Kujan et al. in their study on apparently normal oral mucosa using LBC technique found adequate cellularity in 98% of the cases. However, as LBC is expensive the present method can be adopted as it provides better cellularity than the conventional smear technique using limited resources. Ahmed et al. in their study on oral lesions using CLBC technique found optimal cellularity and stated that this method gave better diagnostic accuracy when compared to the conventional method. Dwivedi et al. in their study comprising normal mucosa, hyperkeratotic lesions, and ulcerated and atrophic lesions found no statistically significant difference between the two techniques in terms of cellularity of the smears. The authors attributed it to inadequate scraping of the buccal mucosa in ulcerated areas as it caused discomfort to the patients. Additionally, in their study the cells were lost due to errors in sample processing. In our study, we found a statistically significant difference between the two techniques. We found that CLBC technique (66%) offers better results than the conventional technique (33%) in terms of adequate cellularity. In CLBC method, as the sample collected was flushed out in a suspending solution, the number of cells lost due to adherence to toothbrush was minimized. Centrifugation technique, which was implemented in our study, helped us in getting a cell button with adequate concentration of cells.

When clarity of the smear background was evaluated between the two techniques, most of the samples of CLBC (80%) showed clear background as compared to the conventional method (48%) and this is concurrent with the results obtained by Dwivedi et al. This was due to the use of glacial acetic acid in the suspending solution that lyses the red blood cells and centrifugation technique that removes mucin, debris, microbial colonies, and other artifacts present in the background. Ahmed et al. and Hayama et al. who have conducted studies on oral lesions also obtained similar results and reported that the scantiness of background staining in CLBC method enhances sensitivity and quality.

The conventional method does not have a liquid medium for the uniform spreading of cells; scant cells were present in the center and most of the cells accumulated in the periphery. According to Dwivedi et al., the process of resuspending the cell pellet in alcohol and then pouring it over a horizontally placed glass slide led to sedimentation of cells and prevented the uniform distribution of cells in CLBC method, which they followed. Our method offered smears with uniform distribution compared to the conventional technique, which can be attributed to a small amount of sample taken per slide that was evenly spread with the help of a glass slide.

Studies have shown cell elongation to be a significant drawback of the LBC technique. However, our method revealed much less cellular elongation as compared to the conventional technique. Carefully performed centrifugation will not cause any significant distortion in the cellular morphology of exfoliated cells and will not have any adverse effect on diagnostic efficacy of the smear as evident with our smears. Cellular overlapping and cellular elongation were less in CLBC method compared to the conventional method. Cellular overlapping or clumping of cells seen in CLBC technique was only 38% in comparison to the conventional technique, which was 80%. As we have collected samples from apparently normal oral mucosa, blood and mucus contents were very less. But even in the presence of mucus and blood components, the centrifugation method gives an even distribution with less clumping and elongation of cells.

Very few slides showed the presence of mucus and inflammatory cells in both the techniques as the samples were collected from apparently normal mucosa. No statistically significant result was obtained.

Davey et al. and Dwivedi et al. who had conducted similar studies reported that there was no evidence that LBC reduced the proportion of unsatisfactory slides in comparison to the conventional technique. But in our study, we found a statistically significant difference between the two techniques and proved CLBC to be better than the conventional method. A modification in the CLBC method, which we incorporated rendered better results than previous studies.
Conclusion

CLBC technique can offer better smears using materials already available in the laboratory setup; it is cost-effective and hence, can be implemented in laboratories with limited resources. As this method is relatively technique-sensitive, improvement in this front can yield better results.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

References

1. Park PK. Textbook of Preventive and Social Medicine. 18th ed. Jabalpur: M/S Banarsidas Bhanot; 2005. p. 302-5.
2. Jain A, Maheshwari V, Alam K, Mehdi G, Sharma SC. Apoptosis in premalignant and malignant squamous cell lesions of the oral cavity: A light microscopic study. Indian J Pathol Microbiol 2009;52:164-6.
3. Ilter E, Midi A, Haliloglu B, Celik A, Yener AN, Ulu I, et al. Comparison of conventional and liquid-based cytology: Do the diagnostic benefits outweigh the financial aspect? Turk J Med Sci 2012;42(Suppl 1):1200-6.
4. Dwivedi N, Agarwal A, Raj V, Kashyap B, Chandra S. Comparison of centrifuged liquid based cytology method with conventional brush cytology in oral lesions. Eur J Gen Dent 2012;1:192-6.
5. Kujan O, Desai M, Sargent A, Bailey A, Turner A, Sloan P. Potential applications of oral brush cytology with liquid-based technology: Results from a cohort of normal oral mucosa. Oral Oncol 2006;42:810-8.
6. Mehrotra R, Gupta A, Singh M, Ibrahim R. Application of cytology and molecular biology in diagnosing premalignant or malignant oral lesions. Mol Cancer 2006;5:11.
7. Babshet M, Nandimath K, Pervatikar SK, Naikmasur VG. Efficacy of oral brush cytology in the evaluation of the oral premalignant and malignant lesions. J Cytol 2011;28:165-72.
8. Mulki S, Shetty P, Pai P. Oral rinse as a simpler approach to exfoliative cytology: A comparative study. J Clin Diagn Res 2013;7:3036-8.
9. Merhotra R, Singh MK, Pandya S, Singh M. The use of an oral brush biopsy without computer-assisted analysis in the evaluation of oral lesions: A study of 94 patients. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;106:246-53.
10. Ogden GR, Cowpe JG, Green M. Cytobrush and wooden spatula for oral exfoliative cytology: A comparison. Acta Cyto 1992;36:706-10.
11. Ahmed HG, Edris AM, Mohmed EA, Hussein MO. Value of centrifuged liquid-based cytology by Papanicolaou and May-Grunwald in oral epithelial cells. Rare Tumors 2009;1:e12.
12. Burd EM. Human Papillomavirus and cervical cancer. Clin Microbiol Rev 2003;16:1-17.
13. Koss LG, Melamed MR. Koss' Diagnostic Cytology and it’s Histopathologic Bases. 5th ed. Lippincott Williams & Wilkins; 2006. p. 738-76.
14. Hayama FH, Motta AC, Silva Ade P, Migliari DA. Liquid-based preparations versus conventional cytology: Specimen adequacy and diagnostic agreement in oral lesions. Med Oral Patol Oral Cir Bucal 2005;10:115-22.
15. Davey E, Barratt A, Irwig L, Chan SF, Macaskill P, Mannes P. Effect of study design and quality on unsatisfactory rates, cytology classifications, and accuracy in liquid-based versus conventional cervical cytology: A systemic review. Lancet 2006;367:122-32.

“QUICK RESPONSE CODE” LINK FOR FULL TEXT ARTICLES

The journal issue has a unique new feature for reaching to the journal’s website without typing a single letter. Each article on its first page has a “Quick Response Code”. Using any mobile or other hand-held device with camera and GPRS/other internet source, one can reach to the full text of that particular article on the journal’s website. Start a QR-code reading software (see list of free applications from http://tinyurl.com/yahh2tc) and point the camera to the QR-code printed in the journal. It will automatically take you to the HTML full text of that article. One can also use a desktop or laptop with web camera for similar functionality. See http://tinyurl.com/2bw7fn3 or http://tinyurl.com/3ysr3me for the free applications.