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A Study of the Modified Liquid Based Exfoliative Cytology: An advanced cytodiagnostic tool.

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Running title: Liquid Based Exfoliative Cytology

Clinical significance: Modified Liquid Based Cytology offers a cost effective and highquality smear that enhances the sensitivity and quality of smear.

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ABSTRACT

Oral exfoliative cytology is a cost effective and probably the best approach for the diagnosis and initial assessment of the oral lesions. Modified Liquid Based Cytology offers high quality smears compared to the conventional brush cytology thereby improving the sensitivity and importance of the smear, with limited available resources.

Objective of the study: To **e**valuate the sensitivity and specificity of modified LiquidBased Cytology and to compare its efficiency with the Conventional brush cytology of oral cancer cases, thereby helping in early detection of those lesions,.

Materials and Methods: In this prospective comparative study, smears were obtainedfrom the oral squamous cell carcinoma (OSCC) lesions with the use of a cytobrush; after obtaining a prior written consent from the patient. Two smears each, using the Conventional technique and the modified LBC techniques, were prepared. Samples obtained for the modified LBC sample, immersed in a prepared reagent; was centrifuged; after which smears were made from the cell pellet formed. The smears obtained by both the techniques were stained with the Papanicolaou (PAP) and Giemsa stains. They were further analyzed with respect to the cell staining, background clarity, uniformity in cell distribution and cellular overlapping together with the altered cellular & nuclear details.

Results: The modified LBC cytology showed a statistically significant result, over theConventional cytology, based on various cytological criteria.

Conclusion: The results indicate a better performance of the modified LBC as comparedto the Conventional cytology; hence it could be implemented as a technique for an accurate and early diagnosis of oral cancer cases as compared to the other conventional screening techniques.

Keywords: Exfoliative cytology; Giemsa; Liquid Based Cytology; PAP; (LBC); oral squamous cell carcinoma; PAP.

INTRODUCTION

Oral cancer is the sixth most common cancer globally. It comprises the top three among the cancers in India. Rate of oral cancer in India is high, amounting to about 20/ 100,000 population and comprises of about 30% of all cancers in the country. [1] The 5-yearsurvival rate is approximately 50%, and is the lowest, as compared to other major cancers. This increased mortality rate can be attributed to the

advanced stage of the disease on initial identification, and other varied reasons for delayed biopsy. On the contrary, the prognosis for oral cancer is exceptionally good with 5-year survival rates of up to 90%, if it is detected early.[2]

Exfoliative cytology (EC) is a screening diagnostic test used for early diagnosis of oral diseases, such as squamous cell carcinoma, pemphigus, potentially malignant disorders, candidiasis, and salivary gland lesions among others. This diagnostic technique, though not comparable to the oral biopsy, which is a "gold standard" for oral diagnosis, is a relatively simple and rapid technique, that is cost effective and non-interventional and does not require anaesthesia. This procedure is well-received by patients, and enables the professionals to carry out an unhindered diagnosis and monitor the follow-up after providing the necessary treatment.[3]

In the earlier days, the scraping of the surface of the lesion using a wooden spatula provided the sample for cytology. Later the brush biopsy technique or the Conventional cytology method replaced the spatula, where the Oral CDx brush, was used to obtain samples. It was understood that, though these procedures were beneficial and provided the necessary results, they were not preferred, due to inaccuracy of the results such as high false-positive values, etc. [2]

Mehrotra et al in a review of 22 articles observed that the sensitivity and specificity of Conventional exfoliative cytology, in carcinoma suspected lesions, ranged between 76.8%–100%, and 88.9%–100%, respectively. [4]

On the other hand, Nanove stated that the sensitivity for the modified LBC technique was about 95.1% and the specificity about 99%. [4]

The Liquid-Based Cytology method provided high cellularity on slides as a thin and homogeneous layer on a clear background, and enabled the clear and precise identification of abnormal cells. The use of this technique has tremendously minimized the number of false negative and unsatisfactory results, and increased the sensitivity and specificity as compared to the Conventional cytology. Other factors like the standardisation of cell transfer,decreased obscuring factors, and incomplete usage of cells from the sample removed, in the case of the Conventional technique, are the other factors in favour of MLBC.[5]The inter-operator variation will not occur in the MLBC as the entire processing is controlled by the laboratory.[5]

Liquid based cytology was initially developed for cervical uterine cancer screening. [6]

However, this technique requires high-end laboratory equipments and well-trained staff to handle, process, and analyze the samples properly. [4]

MATERIALS AND METHODS

Fifty patients who had clinically suspected oral malignant lesion (frank carcinoma) and histopathologically diagnosed oral squamous cell carcinoma, were selected from among the patients who visited the Outpatient department of various dental institutions in Bengaluru, Karnataka, INDIA and other private

oncodiagnosticcenters. Prior ethical committee sanction for the study was obtained. Patients who had proliferative, traumatic or immune mediated epithelial lesions (e.g., papilloma, aphthous ulcer, lichen planus, traumatic ulcer, frictional keratosis etc) were rejected. No dry tap samples were included in the study and the samples lost during processing were also discarded.[11]

A structured proforma was prepared, mentioning the salient features, details regarding adverse habits of each patient, and a detailed clinical examination of the lesion was done. Procedure was explained to the patient and only those who were willing to sign the informed consent form were included. Lesional area was carefully handled during the cytology procedure such that there was no excessive bleeding and discomfort. Acomplete transepithelial biopsy was obtained by using the commercially available cytodiagnostic brush (Swan manufacture PAP kit disposable brush) by applying moderate pressure. The brush was rotated over the lesion, about 5 times, in a clockwise direction, until pinpoint bleeding was seen. This ensured that the sample comprised of all the epithelial layers, including the basal and superficial layers. The material from the brush was spread on the middle third of two clean dried glass slides; and one was fixed immediately using 100% ethyl alcohol for staining with PAP stain, and other slide was left to air dry for Giemsa stain.

For the Modified LBC, the brush head was detached from the handle, after taking the brush cytology sample; so that it could be accommodated in the test tube containing a sample of: 20 milliliter of 95% ethanol+ 6 milliliter of glacial acetic acid+ 74 milliliter of normal saline (Merck Darmstat, Germany): and this reagent was centrifuged at 3000 rotations per minute(rpm), for 10 minutes. The 100 cubic millimetre sediment was obtained; this was again centrifuged at 1000 rpm for 5 minutes using the vortex centrifugal machine (Rami Manufacturer, Vasai, Mumbai). The formed supernatant: 10 cubic millimeter was poured off and replaced by 1% acid alcohol (1 microliter glacial acetic acid + 99 microlitre isopropyl alcohol) for 30 min. Following this, the supernatant was discarded leaving behind only a few drops, which was shaken vigorously with 1% acid alcohol (1-microliter glacial acetic acid + 99 microliter isopropyl alcohol). A few drops of this sample mixture, was placed on the middle third of two clean glass slides, which was then spread, by using a sterilized cervical wooden spatula (Swan Manufacture PAP kit- cervical wooden spatula). Both the slides were allowed to air dry and were subsequently stained with PAP and Giemsa stains.

Cytology Assessment criteria and score[i]

A. Cellularity

1. No cells.
2. Scanty cells.

3. Adequate cells.
4. Abundant cells (≥5,000 cells).

B. Staining Quality

0. Lack of staining of cells.
1. Weak to moderate staining of cells with staining artefact.
2. Strong staining of cells with little or no artifact.

C. Clear background

0. Abundant debris present (inadequate for diagnosis).
1. Debris present, but can make diagnosis.
2. Clear background.

D. Uniform distribution

0. Cells restricted to only one area of the slide
1. Few areas with cells
2. Cells distributed evenly throughout slide.

E. Cellular overlapping

0. Cells present only in clumps.
1. Only few areas with clumping seen.
2. Minimal overlapping of cells.

F. Cellular and Nuclear Detail

0. Abnormal cytoplasmic fragments with irregular nuclear membrane and with or without clear nucleoli.
1. Abnormally configured keratotic cell**s** with long cytoplasmic processes, bright pink cytoplasm and intensely hyperchromatic irregular nucleus.
2. Pleomorphic cells with nuclei demonstrating chromatin clearing,

prominent irregular nucleoli and clear nuclear outlines. [7]

RESULT

All the Means were calculated using the statistical package for social sciences (SPSS) version 10.8 data software. Results showed that both techniques had different Mean ranges; with Student„t" test at P≤0.01 significant level. The result**s** of the study were tabulated. They were found to be statistically significant**.**

Table 1: Statistical comparison between Conventional PAP smear and Modified Liquid Based Cytology (LBC) PAP smear

Cytological assessment criteria	**Conventional PAP stain-Mean SD**	**Liquid Based cytological PAP Mean SD**	**T test**	**P value**
Cellularity	1.88(0.62)	2.5(0.61)	4.99	0.00
Staining	1.44(0.64)	2 (0)	7.18	0.00
Clear background	0.76(0.71)	1.82(0.43)	8.93	0.00
Uniform cell distribution	1.04(0.40)	1.66(0.47)	7.01	0.00
Cellular overlapping	0.78(0.54)	1.7(0.50)	8.75	0.00
Cell nuclear detail	1.12(0.65)	1.92(0.27)	7.93	0.00

Student t test (for independent mean value), *P≤0.01 significant SD (Standard deviation)

Results show that the Mean values obtained by Modified Liquid Based Cytology method are significantly better than the Conventional Cytology method when stained with PAP stain. At a P≤0.01 significant value and using the student„t" test all the cytological criteria are statistically significant.

FIGURE 1

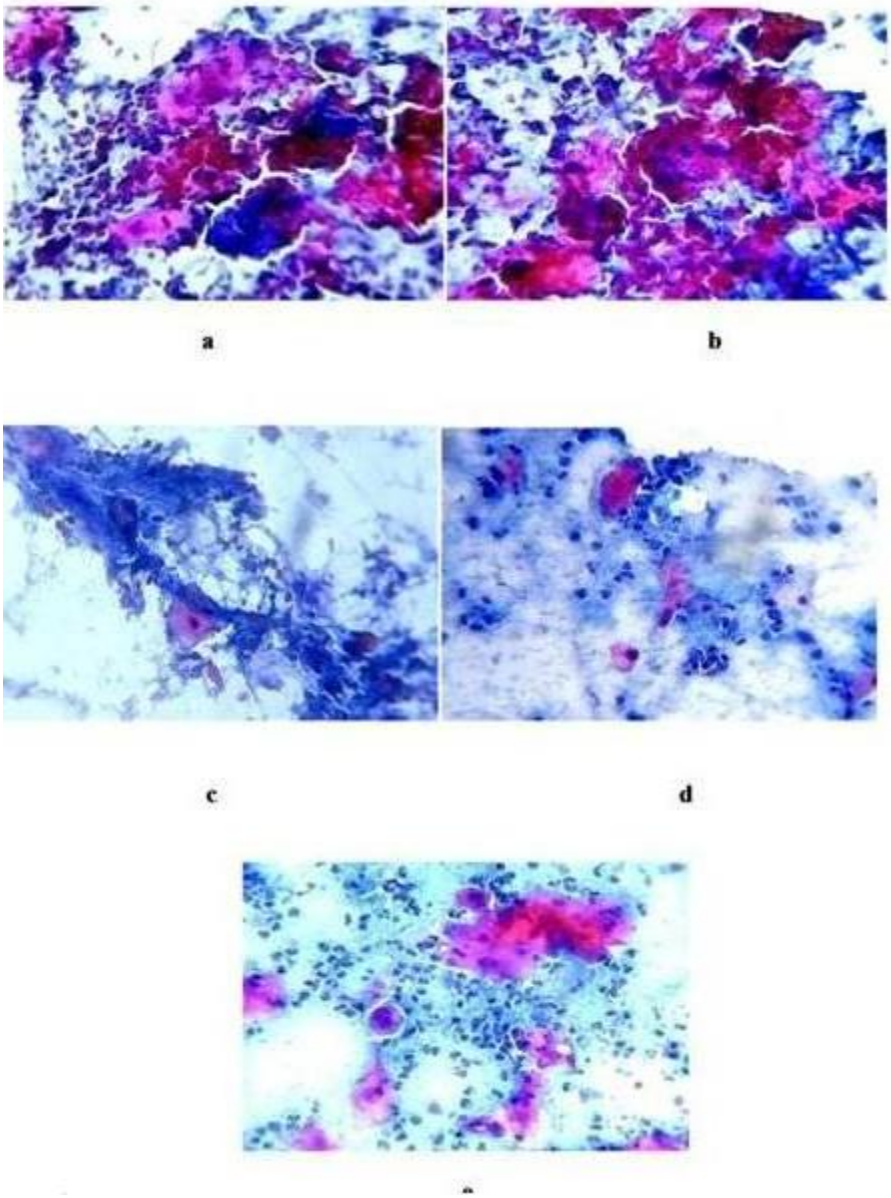


Figure 1: (a) Conventional PAP stain shows improper cellular outline (b) debrinous background which hindered the diagnosis (c) cellular overlapping and (d) improper staining (e) Improper nuclear detail and clumping.

Figure 2: a) Liquid based cytology with PAP stain shows better cellular outline b) Almost clear background c) Minimal cellular overlapping d) Optimised staining e) Proper nuclear details f) Fragments of dysplastic epithelium

FIGURE 2

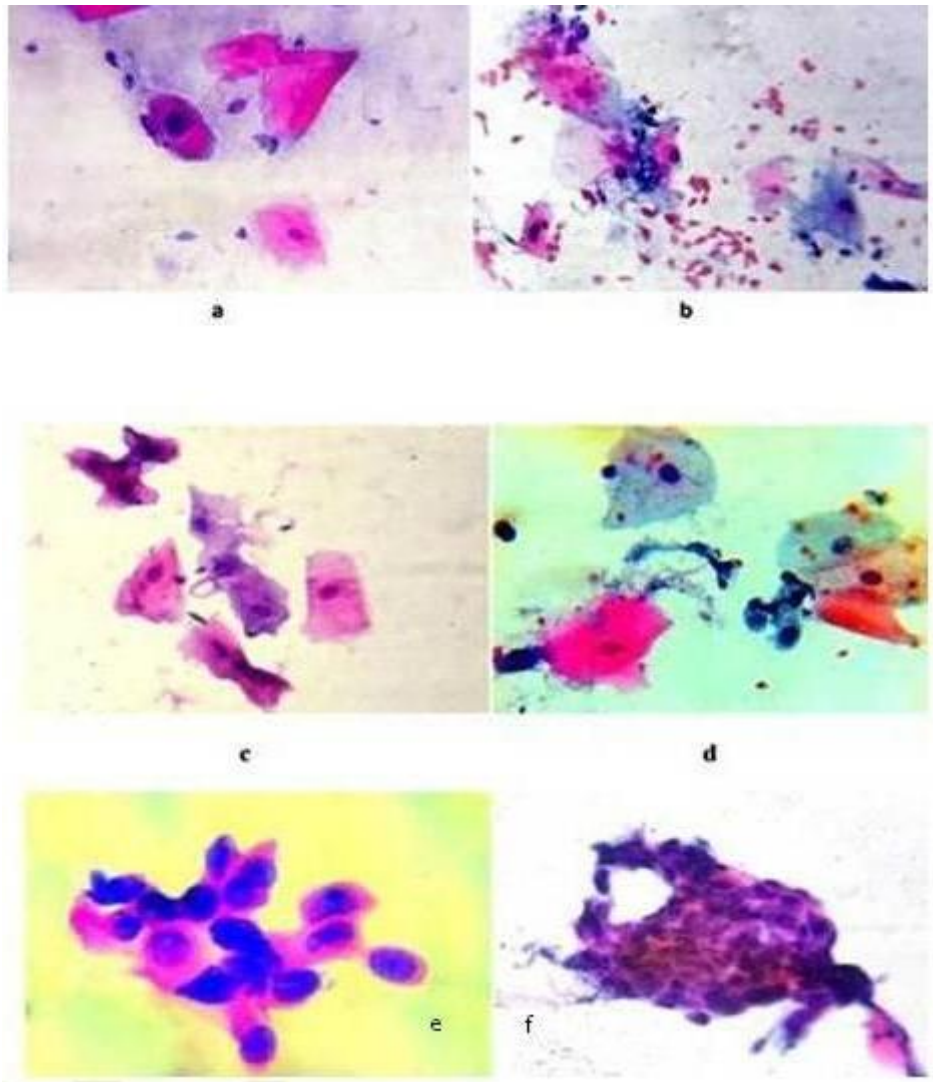


Table 2:

Statistical comparison between the Giemsa staining of Modified Liquid Based Cytology method and Conventional Cytology method

Cytological assessment criteria	Conventional PAP stain-Mean SD	Liquid Based cytological PAP Mean SD	T test	P value
Cellularity	1.88(0.65)	2.26(0.82)	3.78	0.00

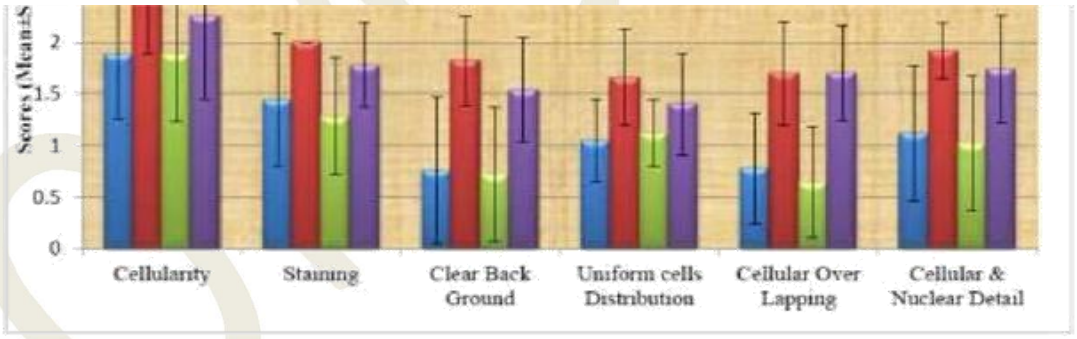
Staining	1.28(0.57)	1.78 (0.41)	4.98	0.00
Clear background	0.72(0.65)	1.54(0.50)	3.68	0.00
Uniform cell distribution	1.12(0.32)	1.4(0.49)	3.33	0.00
Cellular overlapping	0.64(0.54)	1.7(0.46)	8.74	0.00
Cell nuclear detail	1.02(0.65)	1.74(0.52)	6.05	0.00

Student t test (for independent mean value), *P≤0.01 significant SD (Standard deviation)

Results show that the Mean values obtained by Modified Liquid Based Cytology method are elevated as compared to the Conventional cytology method when stained with Giemsa stain. At a P≤0.01 significant value and using the student's" test all the cytological criteria are statistically significant.

Figure 3: Graph depicting the cellular variations in the PAP and Giemsa stained sections of slides prepared by the modified LBC technique and the Conventional technique

FIGURE 3



Graph 1: Statistical comparison between Conventional cytology with Modified LBC

Figure 4: a) Conventional Giemsa stained smear shows irregular cell outline and b) Debrinous background that hindered diagnosis c) Cellular overlapping and d) Improper staining and air drying artefact e) Improper nuclear detail f) Improper cellular detail

FIGURE 4

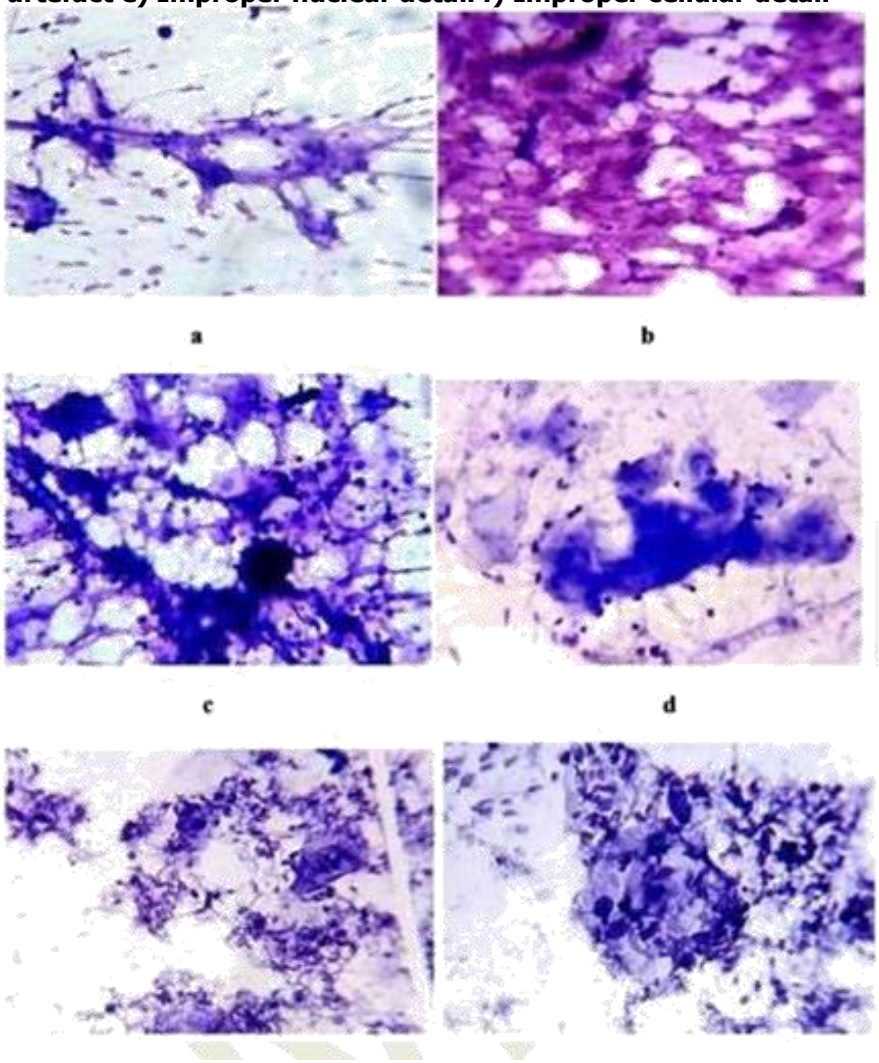
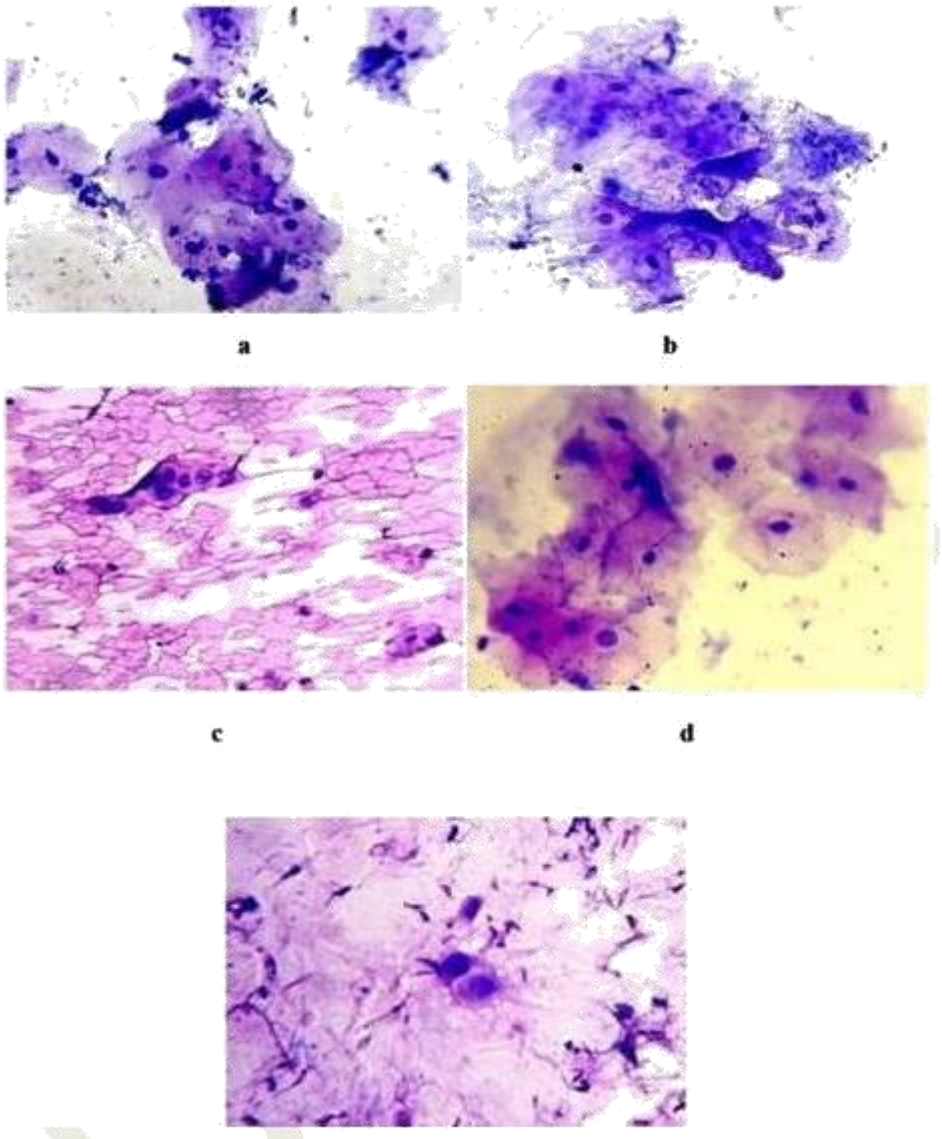


Figure 5: a) Liquid based cytology with Giemsa stain shows better cellular outline b) Almost clear background c) Minimal cellular overlapping d) Optimised staining e) Proper nuclear details

FIGURE 5



DISCUSSION

Since 1990s, after the modified LBC method was first introduced; various comparative studies have been done to indicate its advantage over the Conventional exfoliative cytology. Results obtained from the Liquid Based preparations of the cervical samples,for example, have shown that this technique have demonstrated.[8]

- ☐ Reduced set of problems relating to sampling error, poor transfer and fixation of the cellular sample and a significant reduction in false negative

- ☐ Immediate fixation together with improved nuclear and cytoplasmic details
- ☐ All the materials can be taken and seen microscopically
- ☐ Background appears more clear and devoid of the blood and mucus, so that epithelial cells of interest are visible and counted rapidly
- ☐ LBC slides can also be used for automated analysis

However, the technique has drawbacks too. They include:

- ☐ Randomisation of cells causes smear patterns to be altered
- ☐ Abnormal cells are dispersed
- ☐ If less in quantity the LBC preparations can be difficult to screen and interpret
- ☐ Epithelial cells appear individually and may be slightly smaller than seen on conventional smears.
- ☐ Cells are single rather than in sheets. They are smaller, and showed spindling. Chromatin detail was attenuated.
- ☐ The nucleoli were prominent and intranuclear inclusions were difficult to see.
- ☐ There was a decrease in the extracellular debris and, the number of small mononuclear cells, myoepithelial cell or the red blood cells; in the background matrix were absent.
- ☐ LBC was more expensive than conventional test [9] and
- ☐ A pathologist needed to be familiar with artifacts to prevent misrepresentations.[10]

Our study also showed a statistically significant result in relation to the smears prepared by the LBC method; and stained with PAP and Giemsa stains; in relation to various cellular parameters like adequate cellularity, staining, clarity of background, uniform distribution of cells, cellular overlapping, cellular & nuclear details; as compared to the Conventional technique ($P \leq 0.01$ significant level).

Nevertheless, in spite of the many advantages offered by the modified LBC, the conventional method for preparation of exfoliative cytology sample is still the standard practice engaged in routine laboratories across countries around the world.

CONCLUSION

In conclusion, however, one must take into consideration not only the expenses and the diagnostic performances, but also the eventual objective of technical advancements provided by LBC. The LBC provides repetitive results as well as provides a conserved material for performing additional laboratory techniques such as fluorescence in situ hybridization (FISH), immunocytochemistry, and other types of molecular analysis.

Abbreviations:

1. **MLBC---------- Modified Liquid based cytology**
2. **Cc---------- - Conventional cytology**
3. **OSCC---------- Oral squamous cell carcinoma**
4. **PAP----------Papanicolaou**
5. **Rpm rotations per minute**

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Conflict of interest: The authors have no conflict of interest with regard to the study

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