

# Cytomorphologic Analysis of Wet and Spray Fixation Methods in Oral Exfoliative Cytology

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## KEYWORDS

Cellular, exfoliative cytology, fixative, nuclear, impurities, Papanicolaou stain.

## ABSTRACT

For a long time, oral exfoliative cytology (OEC) has been implemented as an effective preliminary diagnostic tool for pathological lesions and various methods for fixation of the cytology specimens have been studied. The present study was undertaken to compare the efficacy between the wet and spray type of fixation methods for Papanicolaou (PAP) stained oral cytosmears. The study comprised of 45 healthy subjects in the age group of 20-25 yrs. For each subject, two smears were collected from the buccal mucosa and subjected to wet and spray fixation methods respectively. Both the smears were stained using a commercial Rapid Pap Kit. Smears were observed microscopically and evaluated for cytomorphological features involving uniformity of staining, cellular morphology, nuclear morphology, cellular staining, nuclear staining and presence of impurities. Comparisons were made between the two methods of fixation and statistically analysed using McNemar non-parametric test. Cells were evenly distributed in wet-fixed smears (n=38, 95%) compared to spray fixed smears (n=19, 47.5%). Wet-fixed smears showed lesser impurities (n=13, 32.5%) than spray fixed smears (n=27, 67.5%). However, other parameters such as cytological and nuclear morphology, staining of cytoplasm and nucleus were found to be not significant when compared between the two methods of fixation ( $p < 0.05$ ). The study shows that wet-fixed smears have better cellular distribution and relatively fewer impurities when compared to the spray fixed smears. The method of wet-fixed smears may be used as an alternative to spray fixed smears. A larger sample size may be required for further validation.

## INTRODUCTION

Oral exfoliative cytology is a simple, non-invasive chair side procedure done to collect oral cells with the help of wooden spatula, stainless steel spatula, cytobrush etc. Exfoliative cytology plays an imperative role in the diagnosis of potentially malignant disorders and may also reflect the various events occurring in the body as revealed by their cytomorphological and nucleomorphological variations [1].

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The fixation of cytological smears using ethanol is considered as the gold standard. Undoubtedly, diagnostic accuracy and reliability of cytosmears depend greatly on the quality of collection, fixation, staining and interpretation. Inadequacy in any of these steps will adversely affect the quality and efficiency of diagnostic cytology [2].

Different methods of fixation include air drying, wet fixation, spray fixation and lysing fixation for blood samples. The process of submerging freshly prepared smears immediately in a liquid fixative is called wet fixation. This is the ideal method for fixation and may involve the use of 95% ethyl alcohol, propanol or isopropanol [3]. Spray fixatives usually consist of commercial aerosols composed of an alcohol base, which fixes the cells and wax-like substance forming a thin protective coating over

the cells. These fixatives are widely used in situations where smears are to be couriered to a distant cytology laboratory for evaluation [4].

Numerous studies regarding the use of various types of fixatives in oral cytology have been reported [2,5]. Currently, many laboratories have been using Rapid pap staining commercial kit for routine smears which comprise of a spray type of fixative. However, studies comparing the methods of fixation in oral cytology are very few. Therefore, the present study was undertaken to evaluate and compare the efficacy of fixation methods in PAP stained oral cytosmears using wet and spray fixation methods. The present study was thus designed to evaluate the background, cellular morphology, nuclear details and the overall staining which form the basis for an effective cytosmear screening.

## MATERIALS AND METHODS

The study design was approved by the Institutional Research Ethical Committee of KVG Dental College (Ref no.: IECKVGDCH/SS09/2016-17).

Forty-five voluntary dental students from KVG Dental College and Hospital in the age group of 20-25 years were selected for the study. The smears were performed by a single operator in several batches to avoid bias. For each subject, two smears were collected from the buccal mucosa using the scraping method with a sterile stainless steel spatula. One of the smears was wet-fixed in 96% ethyl alcohol by immersion [equal parts of diethyl ether and ethanol] while the other smear was spray fixed using the commercial aerosol provided with PAP stain kit according to the manufacturer's instruction.

All slides were fixed for a minimum of 10 minutes. They were then allowed to dry and processed according to standard methods of staining using the commercial PAP stain kit. The smears were then examined blindly by two cytopathologists independently. During histological examination, only slides containing more than 40 cells are considered as acceptable and following this, the slides were analysed and scored using the parameters as listed in Table 1.

The results obtained from smears prepared by both methods were analysed using SPSS version 21. Binomial non-parametric McNemar test was used for the statistical analysis of the binomial data and a p-value of <0.05 was considered significant.

**Table 1.** Evaluation of criteria used for scoring

Parameters	Criteria for scoring	
	1.(Acceptable)	0.(Unacceptable)
<b>Uniformity of staining</b>	Uniformly stained throughout the individual cell	Staining seen as different shades
<b>Cellular morphology</b>	N:C ratio appropriate with no overlapping of cell borders; Absence of folds	Overlapping, folded and disintegrated cells
<b>Nuclear morphology</b>	Distinct nuclear border	Indistinct border and folded
<b>Cellular staining</b>	Intact cytoplasmic membrane, transparent cytoplasm and uniform staining	Disintegrated cytoplasmic membrane, granular cytoplasm, patchy staining or out of focus
<b>Nuclear staining</b>	Smooth and clear nuclear membrane	Obliterated, disintegrated and nucleus is out of focus
<b>Impurities</b>	Present	Absent

## RESULTS

From a total of 45 spray fixed smears, five smears were found to be unacceptable. The remaining 40 smears from both the groups showed acceptable nuclear staining and was not statistically significant between both the groups (Table 2).

It was observed that the cells were evenly distributed in wet-fixed smears (n=38, 95%) compared to spray fixed smears (n=19,47.5%). The presence of impurities such as mucous plugs, debris and microbial colonies were greater in spray fixed smears (n=27, 67.5%) as compared to wet-fixed smears (n=13, 32.5%).

Comparison of the following parameters; cellular morphology, nuclear morphology and cytoplasmic staining between the wet and spray fixed smears were found to be not statistically significant.

**Table 2.** Descriptive analysis of the parameters in wet and spray fixed smears

		Uniformity (n=40)	Cell morphology (n=40)	Nuclear morphology (n=40)	Cytoplasmic stain (n=40)	Nuclear stain (n=40)	Impurities (n=40)
Wet	Present	38 (95%)	32 (80%)	40 (100%)	30 (75%)	40 (100%)	13 (32.5%)
	Absent	02 (5%)	08 (20%)	0 (0%)	10 (25%)	00 (0%)	27 (67.5%)
Spray	Present	19 (47.5%)	28 (70%)	38 (95%)	30 (75%)	40 (100%)	27 (67.5%)
	Absent	21 (52.5%)	12 (30%)	02 (5%)	10 (25%)	00 (0%)	13 (32.5%)

## DISCUSSION

In an attempt to decrease the morbidity and mortality that are often associated with certain diseases such as oral cancer, the importance of early diagnosis is often emphasised. Oral exfoliative cytology has been established as a preliminary diagnostic tool for early detection of oral cancer and is widely used as the method is fast and cost effective procedure. Even with the advent of newer diagnostic tool such as the Oral CDx® brush biopsy, the PAP staining method remains as an effective diagnostic tool especially in a resource-limited setting.

PAP staining procedure has been revolutionised since its advent. From the cost of the reagents to the decreased turnaround time and quality of the slides, Rapid PAP has shown maximum advantages compared to conventional PAP staining. In addition, the PAP staining procedures have been modified to save reagent consumption and minimise processing time while improving the staining quality of the slides [6].

The standard PAP stain involves polychromatic and transparent staining of the nuclear and cytoplasmic materials and is the basis on which cytological diagnosis is made [7]. The staining of both the nuclear and cytoplasmic constituents are crucial in the study of OEC and any drawbacks during the smear preparation, fixation or staining can adversely affect the process of slide interpretation. There have been attempts to replace alcohol as a fixative with other natural products such as honey or other natural sweeteners and it was reported that results equivalent to that of using ethanol have been achieved [2,5]. However, the use of natural products is often limited due to their short shelf life and complications associated with the preparation of these substances.

The present study was carried out to compare the effectiveness of the fixation methods using

commercially available fixatives. When using the spray fixed smear method, five out of the 45 smears were found to be unacceptable while none of the smears were considered as unacceptable when processed using the spray fixed method. The possibility of the cells being displaced during the spray fixative method could have contributed to the failure of the five smears.

In a quantitative study, exfoliated cells were analysed using wet, spray and dry type of fixation. Using a semi-automated image analysis, it was reported that there was no significant statistical difference in the mean values of the nuclear and cytoplasmic areas for all the methods that was employed in the study [8].

Similar results were also observed in this study where the following parameters; differences in both the wet and spray fixed methods in terms of cellular and nuclear morphology, cytoplasmic and nuclear staining were also found to be not significant in both the wet and spray fixed methods. On the other hand, cellular distribution for the wet-fixed smears was observed to be statistically significant. This can be attributed to the technique involved in the spray fixed smear method where spraying of the fixative was carried out at a certain angle. Ideally, slide positioning and the distance and angulation between the slide and the fixative during the spraying process should be maintained throughout the process of slide preparation. However, this factor is often overlooked during mass screening and when assembling smears in large numbers. In comparison, the wet fixation method is not technique sensitive and therefore allows for better cellular distribution as evident in our study. Thus, the wet fixation technique would be more suitable for mass screening and would be advantageous in avoiding the technical error during interpretation or analysis of the smears.

It was observed that the presence of impurities was more dominant in spray fixed smears (n=27, 67.5%), when compared to the wet fixation method. Exposure towards dust and other impurities during the drying process of the spray fixed smears before undergoing the staining process could have contributed to the presence of impurities. On the other hand, the impurities were not present in wet fixation as the smears were immediately immersed in the fixative solution. Hence, the presence of impurities was found to be minimal in wet fixation (n=13, 32.5%) ( $p=.003$ ,  $p>1$ ). An earlier study by Sahay et al. has shown that delayed fixation of more than 1 hour and 30 minutes can result in degenerative changes of the cytospin while delayed fixation did not have any effect on the preservation or the quality of the staining at the light microscopic level [9]. In conclusion, wet-fixed smears have better cellular distribution and relatively fewer chances of incorporating impurities when compared to the spray fixed smears.

## CONCLUSIONS

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The method of wet-fixed smears may be used as an alternative to spray fixed smears for Papanicolaou (PAP) stained oral cytospins.

The results of the present study suggest that the method of wet-fixed smears may be used as an alternative to spray fixed smears for Papanicolaou (PAP) stained oral cytospins. However, a larger sample size will be required for further validation of these findings.

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## DECLARATION OF INTEREST

The author reports no conflicts of interest. The author alone is responsible with the content of this article.

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