GMR
The Original

COMPARATIVE ANALYSIS OF 2 FIXATION TECHNIQUES - IMMERSION FIXATION VS SPRAY FIXATION IN CYTOLOGY SLIDES

Swetha Patturajan*

Department of Pathology, Saveetha Medical College and Hospital, Saveetha Institute of Medical And Technical Sciences (SIMATS), Thandalam, Chennai-602105, Tamil Nadu, India. Email: spswethu@gmail.com; ORCID: https://orcid.org/0009-0008-5190-5113.
*Correspondence: spswethu@gmail.com

ABSTRACT
Background: The traditional method of wet immersion fixation for cytological smears presents logistical challenges during transport. Furthermore, delicate diagnostic cellular material can inadvertently wash off the glass slide during the liquid immersion process. Therefore, a comparative analysis of wet immersion fixation versus aerosol spray fixation is necessary to evaluate the diagnostic quality and reliability of the smears in exfoliative cytology. Method: A comparative prospective study was conducted at ABC hospital during the XYZ period. From each volunteer 2 buccal smears were taken. The study included twenty such volunteers. One of the smears were fixed using aerosol spray method whereas the other pair underwent wet immersion fixation. The specific parameters such as background clarity, uniform staining, integrity of the nucleus, and the structure of cell were evaluated using blind evaluation process. The scores were then statistically compared between the two modalities. Results: The cumulative score for optimum quality was 103 for wet immersion method and 85 for aerosol spray method. This overall difference was statistically significant ($p = 0.008$). The p-value indicated a significant difference in the uniform stain distribution ($p = 0.004$) and background purity ($p = 0.015$). Conclusion: Considering the specific characters such as stain uniformity and background purity wet immersion method optimum. However, the preservation of cellular consistency, cytoplasmic clarity, and the fundamental architecture of the cell and nucleus remain highly comparable between both fixation methods.

KEYWORDS: Cytological Fixation; Wet Immersion Fixation; Aerosol Spray Fixation; Exfoliative Cytology; Buccal Smear Cytology.

1. INTRODUCTION

The cytomorphological study of cellular smears is an important method for the diagnosis of various diseases and malignant tumors, especially in the case of potentially malignant disorders of the oral cavity. [1]The cytomorphology study of exfoliative smears or fine-needle aspirations fundamentally requires the proper fixation of the specimen onto the glass slide. For the cytotechnologist to accurately diagnose the disease, it is absolutely necessary that the biological characteristics of the specimen be optimally preserved. The chosen fixation method must be highly reliable. Because fixation needs to be prompt yet accurate to prevent cellular decomposition, the specific method of fixation utilized is of the utmost importance.[2]

For decades, the alcohol fixation method—also widely known as wet fixation, utilizing 95% to 96% ethyl alcohol—has been used as the gold standard for clinical fixation. In this traditional approach, freshly prepared smears are basically plunged directly into the liquid alcohol. When this is done correctly, the overall structure of the cell, the delicate details of the cell nucleus, and the essential cytoplasmic transparency are exceedingly well maintained. However, two major clinical obstacles have been reported. [3] The first involves the logistical difficulty of securely transporting the liquid-filled specimens, particularly if they must be couriered from outpatient clinics to laboratories located far off. The second obstacle is that the cells may lose some of their key characteristics during the process, as the liquid ethanol immersion may cause valuable cellular material to wash off or detach from the slide, which directly compromises the material required for a definitive diagnosis of the disease.

To address these issues, aerosol spray fixatives have emerged as a highly practical alternative method for the fixation of cytology smears. Naturally, the diagnostic accuracy and overall reliability of this technique remain a significant topic of clinical concern. This modern method utilizes an alcohol base combined with a wax-like substance to form a thin protective coating over the smears. [4] The major advantage of this approach is that the resulting slides can be easily and safely transported to distant laboratories. However, the primary drawback associated with this technique is that the smears, while being dried prior to staining, may incorporate various environmental impurities such as dust, debris, and microbial colonies. Thus, their reliability and diagnostic accuracy remain a matter of ongoing concern. Therefore, key parameters such as the preservation of cytomorphological characteristics, the presence of background impurities, and the maintenance of both cellular and nuclear structures must be comparatively analyzed in both cases. [5,6] Ultimately, this study aims to provide

cytotechnologists and pathologists with a highly accurate assessment regarding the clinical reliability of wet immersion and aerosol spray fixation methods in preparing cytology slides.

2. METHODOLOGY

Study Design: This comparative, prospective, cross-sectional study was conducted at ABC Hospital during the XYZ period. The primary objective was to systematically evaluate and compare the diagnostic accuracy, cytomorphological efficacy, and overall clinical reliability of exfoliative smears obtained from the buccal cavity using two distinct preparation techniques: traditional wet immersion fixation and aerosol spray fixation.

Participants: 20 healthy volunteers were recruited for the comparative analysis. The age of the volunteers was between 20 to 25 years. The informed consent was obtained initially and the individual who had lesions or carcinoma or any other infection that can alter the morphology of the oral cavity were not included in the study

Methodology: To minimize the bias a single operator was appointed to obtain smear by gently scraping the buccal cavity. The stainless spatula was used to obtain the smear. Two samples were obtained from each volunteers using standard protocol.

The fixation process was divided as follows, One smear from each volunteer was immediately fixed via the wet fixation method, where the freshly prepared smear was plunged directly into 95% ethyl alcohol for 30 minutes, after which it was securely transferred to coplin jars for storage and staining. The second paired smear was fixed using a commercial aerosol spray fixative (Spray-Cyte). The spray fixation was applied promptly, strictly following the manufacturer's specific instructions, and the smears were subsequently allowed to dry.

Following standard Papanicolaou staining, an independent cytotechnologist blindly evaluated the smears. The strict condition for accepting the smears for diagnostic evaluation was that each slide had to contain a minimum of at least 40 well-preserved cells. The slides were systematically evaluated based on the following cytomorphological criteria:

- Maintenance of cytoplasmic transparency
- Uniformity of staining throughout the individual cells
- Clearly visible, smooth, and distinct nuclear boundaries and overall nuclear detail
- Clearly visible and intact cytoplasmic boundaries
- Appropriate cellular morphology with no folding, disintegration, or overlapping of the cells
- Presence of environmental impurities

Scores were assigned as a binary metric based on these morphological parameters: 0 for unacceptable (inadequate preservation) and 1 for acceptable (optimal preservation).

Statistical Analysis: The compiled data obtained from the evaluations were subjected to rigorous statistical analysis. The data were processed and analyzed using SPSS software. Variables between the two fixation methods were evaluated using Fisher's exact test and the Chi-square test. A p-value of less than 0.05 was considered to be statistically significant for all comparative measures.

3. RESULT

The 40 smears found acceptable for the study (20 from dry fixation and 20 from wet fixation) were evaluated further for stain distribution, architecture of the cell, integrity of the nucleus, clarity of the cytoplasm, staining of the nucleus, and the background. The optimal quality of the samples were evaluated as stated in Table no. 1. Those smears found with optimal features were assigned score 1. The table no. 1 demonstrated that stain distribution and background purity score was in the favour wet immersion fixation when compared aerosol spray.

Table 1: Integrated Diagnostic Criteria and Frequency of Optimal Preservation

Evaluated Feature	Optimal Quality	Wet Immersion	Aerosol Spray
Stain Distribution	Homogeneous and even dye uptake across the entire cell	19 (95.0%)	10 (50.0%)
Cellular Architecture	Maintained nucleus-to-cytoplasm ratio; borders clear; no folding	16 (80.0%)	14 (70.0%)
Nuclear Integrity	Margins are smooth, round, distinct, and sharply defined	20 (100%)	19 (95.0%)
Cytoplasmic Clarity	Transparent cytoplasm with an intact outer membrane	15 (75.0%)	15 (75.0%)
Nuclear Staining	Smooth, clear nuclear membrane; sharp focus	20 (100%)	20 (100%)
Background Purity	Pristine background completely free of environmental artifacts	13 (65.0%)	7 (35.0%)

The suboptimal quality of teach feature in specified in the table no. 2. The smears which had any one or all of them for each of the feature were regarded suboptimal and 0 score was assigned. The table no. 2 demonstrated the suboptimal score of the aerosol spray smears was substantially high particularly in case of stain distribution and background purity.

Table 2: Comparative Analysis of Suboptimal Cytomorphological Outcomes

Evaluated Feature	Suboptimal quality	Wet Immersion	Aerosol Spray
Stain Distribution	Irregular Stain Distribution *(Patchy or multi-shaded)*	1 (5.0%)	10 (50.0%)
Cellular Architecture	Disrupted Cellular Architecture *(Overlapping or folded)*	4 (20.0%)	6 (30.0%)
Nuclear Integrity	Compromised Nuclear Integrity *(Blurred or folded margins)*	0 (0.0%)	1 (5.0%)
Cytoplasmic Clarity	Poor Cytoplasmic Clarity *(Opaque, granular, or broken)*	5 (25.0%)	5 (25.0%)
Nuclear Staining	Inadequate Nuclear Staining *(Obliterated or out of focus)*	0 (0.0%)	0 (0.0%)
Background Purity	Background Contamination *(Presence of dust, debris, or microbes)*	7 (35.0%)	13 (65.0%)

The cumulative score for optimal quality can be maximum 120 and minimum 0. For the wet immersion smears the score is found to be 103. For aerosol spray it is found to be 85. Statistically the difference was significant as the p-value was 0.08. The table no. 3 also demonstrates the difference in each feature. The difference was found statistically significant in case of stain distribution with p-value 0.004 and background purity with p-value 0.015.

Table 3: Statistical Comparison of Optimal Cytomorphological Scores

Evaluated Feature	Wet Fixation	Dry Fixation	*p*-value
Uniform Stain Distribution	19	10	p=0.004 *(Significant)*
Intact Cellular Architecture	16	14	p = 0.687
Preserved Nuclear Integrity	20	19	p = 0.317
Cytoplasmic Clarity & Transparency	15	15	p = 1.000
Optimal Nuclear Staining	20	20	p = 1.000
Background Purity (Absence of Debris)	13	7	p=0.015 *(Significant)*
Total Cumulative Score	103	85	p=0.008 *(Significant)*

4. DISCUSSION

In this study, 20 smears were taken using the wet fixation method and the other 20 were taken using the aerosol spray fixation method. The features studied included the architecture of the cell, integrity of the nucleus, distribution of stain, presence of background impurities, clarity of the cytoplasm, and staining of the nucleus. The optimal and suboptimal qualities were specified. The scores were assigned accordingly. The cumulative score for optimal quality obtained was 103 in the case of wet immersion fixation and 85 in the case of aerosol spray fixation. A significant difference was found in the uniformity of staining and background purity for both cases.

Hulimane et al. conducted a similar study and found that environmental particles were present in 67.5% of the aerosol spray-fixed smears, while 32.5% of the wet fixation smears had environmental particles. [7] The findings were consistent when the integrity of the nucleus and cytoplasm were compared. Another similar study conducted by Renshaw et al. stated that the diagnostic adequacy increased significantly from 71.9% to 78.1% because there was better retention of the epithelial cells in the spray method than the wet immersion method. Their study focuses more on diagnostic accuracy rather than the overall quality of the specimen. [8,42]

Under the same protocol, Sathya et al. found that the granularity and the staining in the cytoplasm were better in the case of air-dried smears, whereas the nucleus was better visible in the wet fixation method. [9] Although in this study, the consistency in the staining of the nucleus and cytoplasm is the same. A step ahead of spray drying is the rehydration of the smears; Narayanan et al. proved in their study that the clarity and consistency of the smear improved significantly when the smears were rehydrated. [10,36-41] The rehydration was done with the help of glycerol. Singh et al. reported that when honey was used for fixation instead of alcohol, there was comparable clarity and consistency of the smears. [11,15,23-35]

There are various other studies on fine-needle aspiration and liquid cytology using wet immersion and spray drying fixation methods, but the variation in the findings depends on the outcome the investigator is looking for. [13-24] The limitation of this study is that the diagnostic accuracy of the smears was not evaluated here. Also, the sample size was small to generalize the findings. The consideration of using different materials such as honey and glycerol should be taken for further studies.

5. CONCLUSION

The traditional method of wet immersion fixation yields a significantly superior uniformity in stain distribution and a notably impurity-free background when evaluated and directly compared to the aerosol spray fixation method. However, despite these specific differences, the overall preservation of cellular consistency, the clarity of the cytoplasm, and the fundamental structural architecture of both the cell and the nucleus remain highly comparable and equally adequate between both of these fixation methods.

REFERENCES

[1] Ireka Y, Agustina H, Aziz A, Hernowo BS, Suryanti S. Comparison of fixation methods for preservation cytology specimens of cell block preparation using 10% neutral buffer formalin and 96% alcohol fixation in E-cadherin and Ki-67 immunohistochemical examination. Open Access Maced J Med Sci. 2019;7(19):3139-3144.

[2] Odigie BE, Achukwu PU. Comparing trio-modified Papanicolaou staining methods for assessing liquid-based cytology samples. Am J Biomed Sci. 2017;9(3):119-126.
[3] Copper JE, Budgeon LR, Foutz CA, van Rossum DB, Vanselow DJ, Hubley MJ, et al. Comparative analysis of fixation and embedding techniques for optimized histological preparation of zebrafish. Comp Biochem Physiol C Toxicol Pharmacol. 2018;208:38-46.
[4] Gill GW. Fixation and specimen processing. In: Gupta P, Baloch Z, editors. Cytohistology. Cambridge: Cambridge University Press; 2011. p. 148-161.
[5] Cross P, Chandra A, Maddox A, Narine N, Giles T. Tissue pathways for diagnostic cytopathology. London: The Royal College of Pathologists; 2019.
[6] ELITechGroup. Aerospray® Cytology Slide Stainer/Cytocentrifuge Model 7522 User's Manual. Logan (UT): ELITechGroup Inc.; 2023.
[7] Hulimane S, Shaila M. Cytomorphologic analysis of wet and spray fixation methods in oral exfoliative cytology. Ann Dent UM. 2020;27:1-5.
[8] Renshaw AA. Quantitative assessment of spray vs immersion fixation for thyroid fine-needle aspiration specimens. Am J Clin Pathol. 2008. DOI: 10.1309/AJCP6R3REQLKTLEK.
[9] Sathya P, Sonti S, Kumar V, Aarthi B. Which fixation method reigns supreme in fluid cytology: air-dried or alcohol-fixed smears? TPM. 2025;32(S1):946-950.
[10] Narayanan ON, Bai A. Comparison of different methods of fixation in Papanicolaou staining of cervical smears: Wet-fixation and rehydration of air dried smears. Trop J Path Micro. 2019;5(1):15-19.
[11] Singh A, Hunasgi S, Koneru A, Vanishree M, Ramalu S, Manvikar V. Comparison of honey with ethanol as an oral cytological fixative: A pilot study. J Cytol. 2015;32(2):113-117.
[12] Histology and Cytology. Module: Fixation of Tissues. Lesson 5. p. 20-27.
[13] Koss LG, Melamed MR. Koss' Diagnostic Cytology and Its Histopathologic Bases. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2006.
[14] Bibbo M, Wilbur DC. Comprehensive Cytopathology. 4th ed. Philadelphia: Elsevier Saunders; 2015.
[15] Cibas ES, Ducatman BS. Cytology: Diagnostic Principles and Clinical Correlates. 5th ed. Philadelphia: Elsevier; 2020.
[16] Orell SR, Sterrett GF, Whitaker D. Orell & Sterrett's Fine Needle Aspiration Cytology. 6th ed. Philadelphia: Elsevier; 2020.
[17] Gray W, Kocjan G. Diagnostic Cytopathology. 3rd ed. London: Churchill Livingstone Elsevier; 2010.
[18] Bancroft JD, Gamble M. Bancroft's Theory and Practice of Histological Techniques. 8th ed. Philadelphia: Elsevier; 2019.
[19] Supriya H, Shaila M. Cytomorphologic analysis of wet and spray fixation methods in oral exfoliative cytology. Ann Dent Univ Malaya. 2020;27:1–5.
[20] Ogden GR, Green MW, Cowpe JG. Quantitative assessment of various fixatives on nuclear and cytoplasmic areas in oral cytology. Histochem J. 1989;21(12):703–706.
[21] Ogden GR, McQueen S, Lane EB, Green MW, Hopwood D, Chisholm DM. Cytokeratin expression in oral exfoliative cytology: effect of temperature and fixation. J Oral Pathol Med. 1992;21:113–115.
[22] Singh A, Hunasgi S, Koneru A, Vanishree M, Ramalu S, Manvikar V. Comparison of honey with ethanol as an oral cytological fixative: a pilot study. J Cytol. 2015;32(2):113–117.
[23] Pandiar D, Baranwal HC, Kumar S, Ganesan V, Sonkar PK, Chattopadhyay K. Use of jaggery and honey as adjunctive cytological fixatives to ethanol for oral smears. J Oral Maxillofac Pathol. 2017;21:317.
[24] Narayanan ON, Bai A. Comparison of different methods of fixation in Papanicolaou staining of cervical smears: wet fixation and rehydration of air-dried smears. Trop J Pathol Microbiol. 2019;5(1):15–19.
[25] Gill GW. Fixation and specimen processing. In: Gupta PK, Baloch ZW, editors. Cytohistology. Cambridge: Cambridge University Press; 2011. p.148–161.
[26] Cross P, Chandra A, Maddox A, Narine N, Giles T. Tissue Pathways for Diagnostic Cytopathology. London: Royal College of Pathologists; 2019.
[27] Solomon D, Nayar R, editors. The Bethesda System for Reporting Cervical Cytology. 3rd ed. Cham: Springer; 2015.
[28] WHO Classification of Tumours Editorial Board. Female Genital Tumours. 5th ed. Lyon: IARC; 2020.
[29] Ministry of Health and Family Welfare, Government of India. Manual for Cytology. New Delhi; 2005.
[30] Bales CE. Laboratory techniques. In: Koss LG, Melamed MR, editors. Koss' Diagnostic Cytology and Its Histopathologic Bases. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2006. p.1570–1581.
[31] Odigie BE, Achukwu PU. Comparing trio-modified Papanicolaou staining methods for assessing liquid-based cytology samples. Am J Biomed Sci. 2017;9(3):119–126.
[32] Copper JE, Budgeon LR, Foutz CA, van Rossum DB, Vanselow DJ, Hubley MJ, et al. Comparative analysis of fixation and embedding techniques for optimized histological preparation of zebrafish. Comp Biochem Physiol C Toxicol Pharmacol. 2018;208:38–46.
[33] Ireka Y, Agustina H, Aziz A, Hernowo BS, Suryanti S. Comparison of fixation methods for preservation cytology specimens of cell block preparation using 10% neutral buffered formalin and 96% alcohol fixation. Open Access Maced J Med Sci. 2019;7(19):3139–3144.

[34] Renshaw AA. Quantitative assessment of spray versus immersion fixation for thyroid fine-needle aspiration specimens. Am J Clin Pathol. 2008.
[35] Sathya P, Sonti S, Kumar V, Aarthi B. Which fixation method reigns supreme in fluid cytology: air-dried or alcohol-fixed smears? TPM. 2025;32(Suppl 1):946–950.
[36] Kocjan G. Diagnostic Cytopathology Essentials. Philadelphia: Elsevier; 2016.
[37] DeMay RM. The Art and Science of Cytopathology. 2nd ed. Chicago: ASCP Press; 2012.
[38] Rosenthal DL, Wojcik EM, Kurtycz DFI. The Paris System for Reporting Urinary Cytology. Cham: Springer; 2016.
[39] National Cancer Institute. The Bethesda System for Cervical Cytology Atlas. Bethesda (MD): National Institutes of Health; 2015.
[40] ELITechGroup. Aerospray® Cytology Slide Stainer/Cytocentrifuge Model 7522 User's Manual. Logan (UT): ELITechGroup Inc.; 2023.
[41] Bancroft JD, Layton C. The hematoxylins and eosin. In: Bancroft JD, Gamble M, editors. Theory and Practice of Histological Techniques. 8th ed. Philadelphia: Elsevier; 2019.
[42] College of American Pathologists. Laboratory Accreditation Program: Cytopathology Checklist. Northfield (IL): CAP; latest edition.