Modified ultrafast Papanicolaou staining technique: A comparative study

ABSTRACT

Introduction: Ultrafast Papanicolaou stain (UFP) was introduced as a hybrid of Romanowsky and Papanicolaou (PAP) stain. It enhances the quality and reduces the time. In the present study, a modified staining technique was adapted where Gill’s Hematoxylin was replaced by Harris Hematoxylin.

Aims: The aim of the study was to assess the use of the modified ultrafast Papanicolaou (MUFP) stain for fine needle aspiration cytology (FNAC) of head and neck swellings in comparison with the routine PAP stain, hematoxylin and eosin (H and E), and Giemsa.

Materials and Methods: Forty FNACs of head and neck swellings were collected. FNAC procedure was performed by standard method; two smears were fixed in 95% propanol and stained with PAP and H and E. Two smears were air dried, 1 was stained with Giemsa, and 1 was rehydrated with normal saline, fixed in alcoholic formalin, and stained with MUFP. Four parameters were considered and scored (background, cell morphology, nuclear staining, and overall staining pattern).

Results: The quality of MUFP smears were better when compared to routine PAP, H and E, and Giemsa, and was statistically significant by Wilcoxon matched pair test.

Conclusions: MUFP stain in comparison to routine PAP, H and E, and Giemsa provides an excellent and suitable alterative in cytological staining for the study of various organs.

Key words: Fine needle aspiration cytology, giemsa, hematoxylin and eosin, modified ultrafast papanicolaou stain, papanicolaou stain

Introduction

Fine needle aspiration cytology (FNAC) is an accurate, low cost, and a rapid diagnostic test. However, the speed of turnaround of test results varies among different institutions and in different clinical situations. The speed of reporting of FNAC can be improved by rapid assessment of smears or on-site cytopathology. Quick diagnosis of FNAC plays an important role in efficient medical practice. In an outpatient setting, a quick report of aspiration cytology allows the clinician to discuss further lines of action or management options with the patient during the very first visit, which is of great benefit for both the physician and the patient.[1-4]

Papanicolaou (PAP) staining was first described by Papanicolaou in 1943 and widely used as a screening test despite being time consuming and requiring a large amount of alcohol.[5] The need for minimal turnaround...
time for assessing fine needle aspiration (FNA) smears has encouraged innovations in staining techniques that require lesser staining time with unequivocal cell morphology.[6] The May Grunwald and Giemsa (MGG) stain, Diff Quik stain, and toluidine blue stain have been used traditionally for the rapid assessment of FNAC smears. However, many cytopathologists prefer the traditional, transparent crisp nuclear features offered by wet fixed smears stained by conventional PAP stain, to the opacity of nuclei resulting from air dried smears stained by Romanowsky stains.[6,7,8] PAP stain has undergone various modifications in different laboratories. The original modifications of PAP stain (1943) were published by Dr. Papanicolaou in 1954 and 1960.[9,10] The modified Papanicolaou method was intended to reduce the staining time and the cost of staining without compromising the quality or the cytodiagnosis of the smear.[11]

Ultrafast Papanicolaou (UFP) stain was introduced by Yang and Alvarez in 1995.[12] Kamal et al.[6] modified this technique (replaced Richard-Allan Hematoxylin with Gill’s Hematoxylin) because not all reagents used in UFP are readily available.[6,12,18] UFP stain is a hybrid of air dried Romanowsky preparation and wet fixed PAP preparation. It incorporates principles of air drying of cells, followed by rehydration in normal saline and fixation in alcoholic formalin. Air drying is to make the cells appear larger and thus increase the resolution for analysing cellular details. Normal saline is to rehydrate the cells so that transparency is regained in addition to the hemolysis of the background blood, and alcoholic formalin (pH 5) is to bring out the vibrant colours in the cells and the nucleoli, which stains red. All this is achieved in just 90 seconds. The entire procedure is fast enough to permit immediate microscopic assessment of the fine needle aspiration material. The only limitation of UFP stain, which was originally described by Yang and Alvarez, is that the staining solutions are commercial preparations. The Richard-Allan hematoxylin and cytostain used by them are manufactured by Richard-Allan, Inc. (Richland, Michigan, USA) and are therefore not available universally.[7] In MUFP, Gill’s hematoxylin, modified EA, and isopropyl alcohol were used instead of Richard-Allan hematoxylin, Richard-Allan cytostain, and 95% ethyl alcohol, respectively. In the present study, Harris hematoxylin which is readily available replaced Gill’s hematoxylin.

The objective of this prospective study was to compare and assess the quality of MUFP in fine needle aspiration smears of head and neck swellings with routine PAP stain, H and E, and Giemsa stain, as well as to assess the alternative use of Harris hematoxylin in place of Gill’s hematoxylin.

Materials and Methods

This prospective study was conducted among 40 patients with head and neck swellings in the cytopathology laboratory after obtaining ethical clearance from the ethical committee and patients consent. Fine needle aspiration was carried out from head and neck swellings of the patients referred from different clinical departments for diagnostic purpose. The FNAC of patients with thyroid (17), lymph nodes (16), salivary glands (4), and others (palatal 2, neck 1) were collected along with clinical details. The FNAC procedure was performed by standard method. Four smears were made on clean glass slides, two were fixed in 95% propanol for 15 minutes, and then stained with PAP and H and E stain, respectively. Two smears were air dried, one was stained by Giemsa and one was rehydrated with normal saline for 30 seconds and fixed in alcoholic formalin for 10 seconds and stained by MUFP stain.

Stain preparation

Stain preparation was done according to Choudary et al.[12] which included alcoholic formalin (300 mL) + Isopropyl alcohol (2053 mL) + Distilled water (647 mL) = 3000 mL. EA-36: [(Modified with omission of Orange- G) – Light green SF (450 mL) + Eosin yellow (450 mL) + Bismarck brown (100 mL) + Phosphotungstic acid (2 g) + Saturated aqueous lithium carbonate 10 drops], and Harris hematoxylin [Hematoxylin crystals (1g) + Alcohol 95% (10ML) + Potassium/Ammonium alum (20 g) + Distilled water (200 mL) + Mercuric oxide (added at the end) 0.5 g].

Staining procedure

Total staining procedure was carried out in 130 s, following the steps which included 6 slow dips under tap water, stained with Harris hematoxylin for 30 s, 6 slow dips under tap water, 6 dips in 95% Isopropyl alcohol, stained with EA-36 for 15 s.

Table 1: Assessment of staining

| Score  | Background                          | Cell morphology                      | Nuclear characteristics | Overall staining |
|--------|------------------------------------|--------------------------------------|-------------------------|-----------------|
| 1      | Hemorrhagic                        | Not preserved                        | Dull                    | Bad             |
| 2      | Clean                              | Moderately preserved                 | Crisp                   | Moderately good |
| 3      | Good                               | Well preserved                       |                         | Good            |

Table 1: Assessment of staining
6 dips in 95% isopropyl alcohol, 6 dips in 100% Isopropyl alcohol, 10 slow dips in xylene, and finally DPX Mounted with cover slip for examination.

In MUFP, Gill’s hematoxylin, modified EA, and isopropyl alcohol were used instead of Richard-Allan hematoxylin, Richard-Allan cytostain and 95% ethyl alcohol, respectively.

In the present study, Harris hematoxylin which is readily available, replaced Gill’s hematoxylin. The quality of the stains (MUFP, PAP, H and E, Giemsa) were assessed by considering four parameters – the background of smears, cell morphology, nuclear characteristics of the cells in the smear, and overall staining [Table 1]. Maximum score

Table 2: Comparison of MUFP with PAP, H and E, and Giemsa procedure with respect to staining pattern scores in head and neck swellings (thyroid, lymph node, salivary gland, and others) by Wilcoxon-matched pair test

| Swellings    | Procedures | Mean | SD  | Median | Mean diff | t    | Z     | P     |
|--------------|------------|------|-----|--------|-----------|------|-------|-------|
| Thyroid      | MUFP       | 2.22 | 0.54| 2.00   | −0.93     | 19.00| 6.0365| 0.00001*|
|              | PAP        | 1.29 | 0.49| 1.00   |            |      |       |       |
|              | MUFP       | 2.22 | 0.54| 2.00   | −0.8       | 20.50| 5.8888| 0.00001*|
|              | H and E    | 1.40 | 0.49| 1.00   |            |      |       |       |
|              | MUFP       | 2.22 | 0.54| 2.00   | −0.76      | 20.50| 5.7514| 0.00001*|
|              | Giemsa     | 1.46 | 0.50| 1.00   |            |      |       |       |
| Lymphnode    | MUFP       | 2.06 | 0.69| 2.00   | 0.67       | 90.00| 4.9803| 0.00001*|
|              | PAP        | 1.39 | 0.49| 1.00   |            |      |       |       |
|              | MUFP       | 2.06 | 0.69| 2.00   | 0.61       | 68.00| 4.7264| 0.00001*|
|              | H and E    | 1.45 | 0.50| 1.00   |            |      |       |       |
|              | MUFP       | 2.06 | 0.69| 2.00   | 0.45       | 55.00| 4.0456| 0.00001*|
|              | Giemsa     | 1.61 | 0.58| 2.00   |            |      |       |       |
| Salivary gland| MUFP      | 2.00 | 0.74| 2.00   | 0.83       | 0.00 | 2.5205| 0.0117*|
|              | PAP        | 1.17 | 0.39| 1.00   |            |      |       |       |
|              | MUFP       | 2.00 | 0.74| 2.00   |            |      |       |       |
|              | H and E    | 1.33 | 0.49| 1.00   | 0.67       | 0.00 | 2.3664| 0.0180*|
|              | MUFP       | 2.00 | 0.74| 2.00   |            |      |       |       |
|              | Giemsa     | 1.40 | 0.51| 2.00   | 0.57       | 0.00 | 2.1903| 0.0410*|
| Others       | MUFP       | 1.81 | 0.83| 2.00   | 0.75       | 3.50 | 2.4463| 0.0144*|
|              | PAP        | 1.06 | 0.25| 1.00   |            |      |       |       |
|              | MUFP       | 1.81 | 0.83| 2.00   | 0.75       | 3.50 | 2.4857| 0.0193*|
|              | H and E    | 1.10 | 0.27| 1.00   |            |      |       |       |
|              | MUFP       | 1.81 | 0.83| 2.00   | 0.50       | 4.00 | 2.6531| 0.0289*|
|              | Giemsa     | 1.25 | 0.35| 1.00   |            |      |       |       |

*P<0.05

Figure 1: (a) Clean background well preserved cell morphology, crisp nuclear characteristics, overall good staining pattern (MUFP stain ×40); (b) Hemorrhagic background well preserved cell morphology, crisp nuclear characteristics, moderately good overall staining pattern (Pap stain, ×40)

Figure 2: (a) Hemorrhagic background, moderately preserved cell morphology, crisp nuclear characteristics, moderately good overall staining pattern (H and E stain ×40); (b) Hemorrhagic background not preserved cell morphology, dull nuclear characteristics, and bad overall staining (Giemsa stain ×40)
possible for a single case, taking into account all the four parameters was 10. The scores obtained for MUFP were compared with that of routine PAP, H and E, and Giemsa stain, and the data was subjected to statistical analysis and results discussed [Table 2].

Results

The quality of MUFP smears [Figure 1a] was better when compared to routine PAP [Figure 1b], H and E [Figure 2a], and Giemsa [Figure 2b] in FNAC of head and neck swellings and was statistically significant by Wilcoxon matched pair test with \( P < 0.05 \) [Table 2]. The smears showed clean background, well-preserved cell morphology with transparent crisp nuclear features and overall good staining pattern. When a comparison of staining pattern was done on the swellings located in different regions, better MUFP staining was observed in thyroid swellings as compared with other swellings.

Discussion

FNAC is an accurate, low cost, and a rapid diagnostic test. However, the speed of test results varies in different clinical situations and in different institutions. The speed of reporting of FNAC can be improved by rapid assessment of smears or on-site cytopathology.[1,7] Quick diagnosis of FNAC plays an important role in efficient medical practice. In an outpatient setting, a quick report of aspiration cytology allows the clinician to discuss further lines of action or management options with the patient at the very first visit, which is of significant benefit for both the physician and the patient.[2-4] Routine PAP staining is a commonly employed cytopathological procedure in the diagnosis of smears as it yields a polychromatic, transparent staining reaction with crisp nuclear/cytological features.[8] Since its inception, PAP stain remains the traditional and preferred stain. The different stains used for air dried smears such as May–Grunwald–Giemsa, Jenner–Giemsa, and Diff–quick fail to offer the transparency for the study of subtle nuclear features, as seen by the PAP stain. The traditional PAP stain involves wet fixation and subsequent staining, together requiring at least 30 min.[12] Giemsa stain was primarily designed for the demonstration of malarial parasites. Later, it was used in histology due to its good quality staining of the chromatin and the nuclear membrane. It is a common Romanowsky stain used in cytology, it helps in studying the cell morphology in air dried smears.[14] H and E stain is the most widely used histological stain, which is based on its comparative simplicity and ability to demonstrate clearly an enormous number of different tissue structures. Hematoxylin components stain the cell nuclei blue-black, with good intranuclear detail and the eosin stains cell cytoplasm and most connective tissue fibers in varying shades in intensities of pink, orange, and red. It is used as a rapid stain in cytology designed for frozen sections.[15]

The rapid PAP stains were developed by Kline, Tao, and Sato with respective staining time of 4 min, 5 min, and 90 s. However, the quality of rapid stains is not as satisfactory as they show suboptimal cell morphology and require wet fixation.[12] To overcome these problems, Yang and Alvarez developed UFP stain which is a hybrid of Romanowsky and conventional PAP stain to reduce the staining time to 90 s.[11] Kamal et al. modified the UFP to overcome the problems of shortage of Richard-Allan Hematoxylin, Richard-Allan cytohistain, and ethyl alcohol reagents in Indian set up. This method has a short staining time of 130 s.[6] In the present study Kamal’s MUFP staining method was used for evaluating the FNAC smears of various organs by replacing Gill’s Hematoxylin with easily available Harris Hematoxylin and compared the results with those of routine PAP, H and E, and Giemsa staining. In the present study, there was statistical significant difference (\( P < 0.05 \)) comparing MUFP stain with routine PAP, H and E, and Giemsa in all the parameters. This was consistent with the study conducted by Shinde et al.,[13] Kamal et al.[7] and Choudary et al.[12] It was further observed that in the thyroid swellings MUFP showed better staining pattern compared to other stains.

Conclusion

MUFP stain in comparison to routine PAP, H and E, and Giemsa provides excellent and suitable alternative in cytological staining. Excellent morphological quality and lesser staining time is the need of the present hour. MUFP stain fulfils the requirements for the cytopathological staining in the study of various organs; it is quick, reliable, and can be done using reagents that are locally available and useful especially in India.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

References

1. Yang GC, Alvarez II. Ultrafast Papanicolaou stain: An alternative preparation for fine needle aspiration cytology. Acta Cytol 1995;39:55-60.
2. Yang GCH, Hoda SA. Combined use of the scratch and smear sampling technique and Ultrafast Papanicolaou stain for intraoperative cytology. Acta Cytol 1997;41:1513-8.

Journal of Cytology / Volume 34 / Issue 3 / July-September 2017
3. Basolo F, BalochZW, Baldanzi A, Miccoli P, LiVolsi VA. Usefulness of Ultrafast Papanicolaou stained scrape preparations in intraoperative management of thyroid lesions. Mod Pathol 1999;12:653-7.

4. Paessler M, LiVolsi VA, Baloch ZW. Role of ultrafast Papanicolaou-stained scrape preparations as an adjunct to frozen sections in the surgical management of thyroid lesions. Endocr Pract 2001;7:89-94.

5. Izar S, Kaur R, Masih K. Efficacy of rapid, economical, acetic acid, Papanicolaou stain in cervical smears as an alternative to conventional Papanicolaou stain. J Cytol 2014;31:154-7.

6. Kamal MM, Bodele A, Munshi MM, Bobhate SK, Kher AV. Efficacy of a Modified Ultra Fast Papanicolaou (UFP) Stain for Breast Aspirates. Indian J Pathol Microbiol 2000;43:417-21.

7. Kamal MM, Kulkarni MM, Wahane RN. Ultrafast Papanicolaou Stain Modified for Developing Countries: Efficacy and Pitfalls. Acta Cytol 2011;55:205-12.

8. Asthana A, Singh AK. Comparison of the routine Papanicolaou staining technique with the rapid, economic, acetic acid, Papanicolaou (REAP) technique. Int J Med Dent Sci 2014;3:484-9.

9. Gill GW. Enviro-Pap: An environmentally friendly, economical, and effective Pap stain. Lab Med Indianapolis 2006;37:105-8.

10. Biswas RR, Paral CC, Dey R, Biswas SC. Rapid economic, acetic acid, Papanicolaou Stain (REAP) - Is it suitable alternative to standard PAP stain? Al Ameen J Med Sci 2008;1:99-103.

11. Gachie RN, Muchiri LW, Ndungua JR. A Comparison of modified and standard papanicolaou staining methods in the assessment of cervical smears at Kenyatta national hospital. East Afr Med J 2011;88:244-50.

12. Choudary P, Sudhamani S, Pandit A, Kiri VM. Comparison of modified ultrafast Papanicolaou stain with the standard rapid Papanicolaou stain in cytology of various organs. J Cytol 2012;29:241-5.

13. Shinde PB, Pandit AA. Application of modified ultrafast Papanicolaou stain in cytology of various organs. Diagn Cytopathol 2006;34:135-9.

14. Garcia JJ. The Giemsa Stain: Its History and Applications. Int J Surg Pathol 2007;15:292-6.

15. Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. 6th ed. Churchill Livingstone: London; 2008. p. 121.