Original Article

Rehydration of Air-Dried Smears with Normal Saline: An Alternative for Conventional Wet Fixation Method in Cervical Cytological Study

Sachin Shivaji Kapse, Surekha U. Arakeri, Divya P. Yerranguntla

Department of Pathology, B.L.D.E University's Shri. B. M. Patil Medical College Hospital and Research Centre, Bijapur, Karnataka, India

Abstract

Context: Papanicolaou (Pap) smear is an effective exfoliative cytological investigation done for early recognition of cervical cancer. It also plays role in diagnosis of inflammatory lesions of cervix. **Aims:** The aim of this study is (1) to compare the cytomorphological features in conventional Pap smear (C-PAPS) and rehydrated air-dried Pap smear (RADPS) and (2) to evaluate the efficacy of RADPS in cytodiagnosis of cervical lesion by comparing with cytomorphological features of conventional wet-fixed Pap smear. **Subjects and Methods:** Paired cervical smears were prepared for 247 patients. One was labeled as C-PAPS and another was labeled as RADPS. Comparison of both smears was done for various cytomorphological parameters. **Results:** Out of 247 smears, 2.4% RADPS and 7.3% C-PAPS were reported as unsatisfactory. Red blood cell (RBC) background was present in 2% of RADPS and 42% of C-PAPS. Cytolysis and air-drying artifact were observed more in C-PAPS amounting to 2% and 4% in RADPS and 11% and 15% in C-PAPS. Cytoplasmic staining (97% RADPS vs. 94% C-PAPS) was superior in RADPS. Cell border, nuclear border, and chromatin of squamous and endocervical cells were better appreciated on RADPS compared to C-PAPS, and also statistically significant difference was observed. **Conclusion:** Rehydrated air-dried technique can be satisfactory alternative for conventional wet fixation method which can be followed routinely or in conjugation with C-PAPS, especially in cervical screening programs.

Keywords: Cervical cytology, Pap smears, rehydrated air-dried smear, wet-fixed smear

Introduction

In developed countries like United States, incidence of cervical cancer has plunged significantly due to routine screening with Pap smear. In developing countries like India, incidence of cervical cancer is more than a one-quarter burden of its global burden. The incidence is as high as 7.9/100,000 population and accounts for 67,477 number of deaths per year among women aged between 30 and 69 years. In India, 5-year survival rate was reported as 46% which was much lower than other Asian countries like China, South Korea, Singapore, and Thailand.[1,2]

Cervical screening by Papanicolaou (Pap) smear study has proven as simple, noninvasive, less expensive as well as excellent screening method to curb the morbidity and mortality associated with cervical carcinoma.[1-3]

Pap stain is an accurate stain for valuation of chromatin in cervical cytology and ensures optimal resemblance to corresponding cells nuclei in histopathology section.[2] Various fixatives are used in exfoliative cytology. Out of which, 95% ethanol is the commonly used fixative.[3] Hence, the conventional method for fixation of Pap smear is to fix the Pap smear immediately in 95% ethyl alcohol after preparing the smear.[1] Delay in fixation can lead to air-drying artifacts and poor fixation which can lead to unsatisfactory staining and difficulty in diagnosis. In addition, faulty technique of fixation leads to loss of material. These patients need repeat smears, adding more workload for clinical and laboratory workers. Moreover, some of the patients may be lost, for follow-up as a result of nonconformity.[1,4]

Address for correspondence: Dr. Sachin Shivaji Kapse, Department of Pathology, B.L.D.E University's Shri. B. M. Patil Medical College Hospital and Research Centre, BLDE Campus, Sholapur Road, Vijayapura, Bijapur - 586 103, Karnataka, India.
E-mail: sachinkapse004@gmail.com

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To overcome these problems, few studies were carried out on rehydration of air-dried cervical smear where they found that dipping of air-dried Pap smears in normal saline for 30 s leads to lysis of red blood cells effectively and retains squamous and glandular cells.[5-8] In most of the studies, the quality of rehydrated air-dried (RAD) smears was either equal or superior to conventional Pap smears (C-PAPS). Thus, RAD technique was suggested as a potential alternative to wet fixation for mass screening of cervical cytology.[1,3-8]

Subjects and Methods

A prospective study was carried out on Pap smears taken from all women coming for routine check-up or with some clinical problem in obstetrics and gynecology outpatient department. Paired cervical smears were prepared for 247 patients.

One slide was immediately fixed in 95% ethanol for 30 min and was labeled as C-PAPS. Another slide which was labeled as rehydrated air-dried Pap smear (RADPS) was air-dried for 30 min; rehydrated with normal saline for 30 s, and immediately added to jar containing fixative 95% ethanol for 30 min. Both smears were stained with routine Pap stain. Both the smears were screened, assessed, and graded for various cytomorphological parameters. The smears were reported as per The 2014 Bethesda System [Table 1].

For continuous variables, the summary statistics of mean and standard deviation were used. For categorical data, percentages were used. Chi-square (χ^2)/Fisher exact test was employed to determine the significance of differences between two groups for categorical data. Data were analyzed using SPSS software v. 17.0 by SPSS Inc. Chicago, USA. Results were considered significant if the *P* value was <0.05.

Table 1: Comparison of general cytomorphological features in conventional pap smear and rehydrated air-dried pap smear (*n*=247)

Cytomorphological features	C-PAPS, *n* (%)	RADPS, *n* (%)	*P*
Cellularity			
Low	46 (19)	24 (10)	0.0015*
Intermediate	82 (33)	68 (28)	
High	119 (48)	155 (62)	
Cytolysis			
Present	27 (11)	6 (2)	0.0002*
Air-drying artifact			
Present	36 (15)	11 (4)	0.0001*
Red blood cell background			
Present	104 (42)	4 (2)	0.015*
Cell border			
Distinct	212 (86)	239 (97)	<0.0001*
Cytoplasmic staining			
Unsatisfactory	14 (6)	7 (3)	0.1797
Satisfactory	233 (94)	240 (97)	
Nuclear border			
Squamous cells			
Distinct	220 (89)	238 (96)	0.0019*
Endocervical cells			
Distinct	67 (91)	70 (97)	0.0933
Nuclear chromatin			
Squamous cells			
Crisp	216 (87)	241 (98)	0.0001*
Endocervical cells			
Crisp	60 (81)	68 (94)	0.0141*

*Significant difference at 5% level of significance (*P*<0.05).
C-PAPS: Conventional Pap smear; RADPS: Rehydrated air-dried Pap smear

Results

Age of patients in the study group ranged from 20 to 80 years with the youngest patient aged 20 years and the oldest 80 years with a mean age of 36.8 years. Majority of the patients were in the age group of 31–40 years. The most common clinical presentation was white discharge per vagina. Out of 247 cases, adequate samples were obtained in 229 (92.7%) cases of C-PAPS and 241 (97.6%) cases of RADPS. Out of 247 RAD cervical smears studied, 234 (95%) were reported as nonneoplastic/negative for intraepithelial lesion or malignancy (NILM).

In C-PAPS, cytological diagnosis was not possible in 18 cases; however in RADPS, only 6 cases' diagnosis was unsatisfactory. By both techniques, common lesion diagnosed on cervical cytology was inflammatory smear (162 cases) followed by normal study (37 cases), bacterial vaginosis (8 cases), atrophic smear (4 cases), candidal infestation (2 cases), trichomonas vaginalis (2 cases), and estrogenic effect (1 case). Two cases of atrophic smear and bacterial vaginosis were diagnosed as unsatisfactory on C-PAPS.

Diagnosis of High-grade squamous intraepithelial lesion (HSIL), Atypical squamous cells of undetermined significance, Atypical glandular cells - not otherwise specified, Atypical squamous cells -cannot exclude HSIL, and Low-grade squamous intraepithelial lesion (LSIL) were rendered in 4, 3, 2, 2, and 1 cases, respectively, by both C-PAPS and RADPS. One case of squamous cell carcinoma (SCC) diagnosed on RADPS was reported as HSIL on C-PAPS.

In present study, histopathological correlation was available in three cases. Out of three cases, one was diagnosed as LSIL and another was diagnosed as ASC-H on C-PAPS and on RADPS. On cervical biopsy, both cases of LSIL and ASC-H were diagnosed as chronic nonspecific inflammation. In one case, discordance was observed between C-PAPS and RADPS. On C-PAPS, it was diagnosed as HSIL, and on RADPS, it was diagnosed as SCC [Figure 1a and b]. On histopathological study of this case, it was diagnosed as large cell non-keratinizing SCC.

Cellularity was high in most of the RADPSs as compared to C-PAPS. Cytolysis was more in C-PAPS compared to RADPS. Air-drying artifacts were more in C-PAPS compared to RADPS. Red blood cell background was absent in most of the RADPS [Figure 2a and b].

Cell borders were more distinctly seen in RADPS. Cytoplasmic staining was satisfactory in more number of cases of RADPSs. Nuclear borders of squamous and endocervical cells were more distinct in RADPS and also crisp nuclear chromatin was found more in RADPSs as compared to C-PAPSs [Table 1 and Figure 3a, b].

Discussion

In developing countries like in Asian and African continent, the morbidity and mortality caused by cervical cancer is more, especially in the rural settings. At the time of presentation, most cases (85%) present in advanced and late stages.[1,2] Screening programs in the resource poor settings as well as increasing the accuracy of Pap smear reporting can help to curb down the incidence of cervical cancer.[4]

In most of the rural health settings like primary health center, where paramedical staff play a pivotal role in Pap smear preparation, hence they should have proper knowledge and should also know the importance of proper fixation methods. Most of the times, Pap smears are not collected because of lack of proper facility for preservation.[4,6]

Various studies were done to find out whether RAD technique method can replace conventional wet fixation technique in Pap smears. These authors used several rehydrating agents like hypotonic solutions, normal saline, tap water, and aqueous glycerin. Normal saline was considered as best rehydrating fluid, as it was simplest, cheapest, and easily available in the laboratories.[4,9-13] In our study, we used normal saline as a rehydrating agent.

Out of 247 paired Pap smears, 97% of RADPSs and 92% of C-PAPS were found to be satisfactory for evaluation. Only 6 cases (2.4%) of RADPSs were found to be unsatisfactory; however, in C-PAPSs, 18 cases (7.3%) were unsatisfactory. These findings were similar to observation found in studies conducted by Rupinder *et al*.[4] and Sivaraman and Iyengar[7] The possible explanation for more satisfactory material and more cellularity in RADPS was that air-drying leads to better adhesion of cells to the slide. In addition, there was loss of material in wet fixation from thick smear while immersing in fixative.[14,15]

Overall, cellularity was high in 62% cases in RADPS; however in only 48% cases, high cellularity was noted in C-PAPS. Gupta *et al*.[6] in their study had similar findings. RADPS had less fixation artifact *per se* and less obscuring of cells by RBCs and inflammatory cells. As the air-dried smears were made at leisure, a thin and uniform preparation of RADPS was possible compared to C-PAPS wherein smears are hurriedly prepared as they are supposed to fix immediately.[14-16]

In the present study, the optimum time for air drying for RADPSs was 30–120 min to avoid air-drying artifact. Maximum duration mentioned in various studies was up to 4 days. However, these authors also mentioned that air-drying artifact, cytolysis, and contamination by organisms was more if smears were kept for longer duration.[2,7]

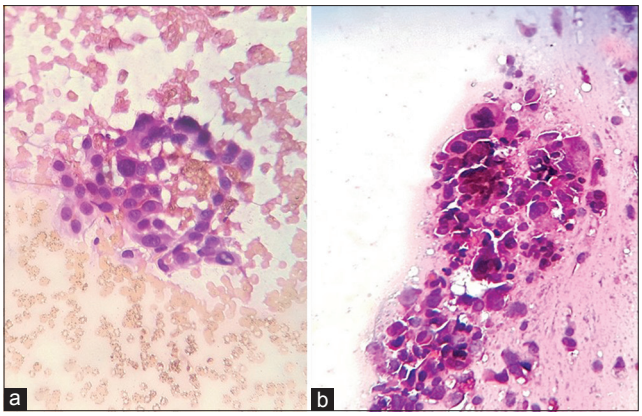

Figure 1: a) Photomicrograph of conventional Pap smear diagnosed as HSIL showing red blood cells in the background (Papanicolaou stain, ×400) and (b) photomicrograph of rehydrated air-dried Pap smear of same case (a) diagnosed as squamous cell carcinoma showing lysis of red blood cells (Papanicolaou stain, ×400)

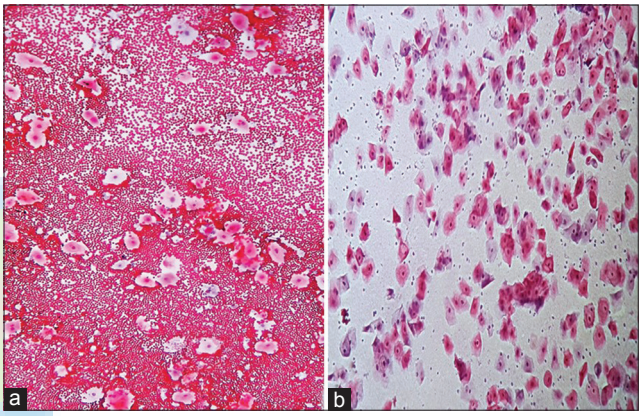

Figure 2: (a) Photomicrograph showing red blood cells obscuring visualization of cells in conventional Pap smear (Papanicolaou stain, ×400) and (b) photomicrograph showing clean background in rehydrated air-dried Pap smear of same case (a) (Papanicolaou stain, ×400)

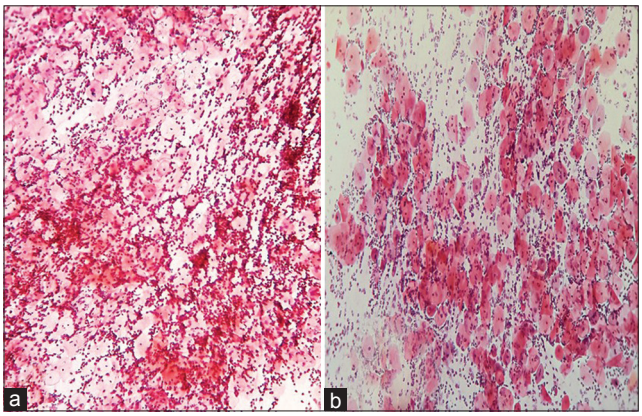

Figure 3: (a) Photomicrograph of conventional Pap smear showing inflammatory cells obscuring squamous cell morphology (Papanicolaou stain, ×400) and (b) photomicrograph of rehydrated air-dried Pap smear of same case (a) showing monolayering of squamous cell (Papanicolaou stain, ×400)

Air-drying artifact was seen in 11% and 15% of RADPS and C-PAPS, respectively, while in study conducted by

Jaiwong *et al.*,[5] no statistical difference was found between two methods.

In the present study, cytolysis was more amounting to 11% in C-PAPS whereas it was 2% in RADPS. A study conducted by Zare-Mirzaie and Abolhasani[17] showed more cytolysis in C-PAPS amounting to 27.4% and in RADPS it was 19.7%. These findings are correlating with our study findings with high percentage of cases showing cytolysis in C-PAPS. However, contrasting result that is more cytolysis in RADPS was seen in study conducted by Gupta *et al.*[6] and Jaiwong *et al.*[5] wherein cytolysis was observed in 17.8% and 47.67% in RADPS and 15.7% and 34.88% in C-PAPS, respectively; however, the difference was statistically insignificant.

In the present study, smears were rehydrated for 30 s to lyse the RBCs and avoid air-drying artifacts. Hemolysis was evident grossly as pinkish appearance of normal saline after immersing heavily blood-stained smears in normal saline due to lysis of RBCs. In the present study, red cell background was seen in only 2% of RADPS as compared to 42% of C-PAPS. Similar findings were observed in study conducted by various authors.[5-7,17] These authors in their study noticed lysis of majority of the background RBCs with only few intact RBCs.[6,7,12,18] Mechanism of RBC lysis was explained in a study conducted by Gill.[18] As per these authors, clean background due to RBC lysis accounted for more number of satisfactory specimens in RAD technique. The advantage of cleaner background was that the infectious agents were distinctly identifiable and also easy to pick up on the RADPS. In addition, it was easy to diagnose precursors, pre-neoplastic, and neoplastic conditions.[4-7,10,12]

Distinct cell border was seen in 97% RADPS and 86% of C-PAPS. This might be due to more number of air-drying artifacts and cytolysis in C-PAPS.

Moreover, in our study, we found that size of squamous cells was increased which has been documented in various studies.[4-7,10,12] Therefore, there was ease in diagnosing epithelial as well as glandular cell abnormalities on RADPS.

Cytoplasmic staining was found to be superior in RADPS as compared to conventional C-PAPS. Unsatisfactory staining of smears was 6% in C-PAPS as compared to RADPS where only in 3% cases unsatisfactory staining was noted. In study conducted by Sivaraman and Iyengar,[7] Gupta *et al.*,[6] Jaiwong *et al.*,[5] and Zare-Mirzaie and Abolhasani,[17] satisfactory cytoplasmic staining was in 59.5%, 79%, 100%, and 62.4% C-PAPS smear and 60.6%, 87.8%, 100%, and 65.8% in RADPS. Factors favoring better cytoplasmic staining in RADPS were better penetration as well as fixation of smears due to lysis of RBCs and less obscuring by inflammatory cells leading to thin and uniform smears.[6-8,12]

Nuclear border of squamous and endocervical cells were more distinctly visible in RADPS. Out of 247 smears, indistinct nuclear border in squamous cell was seen in 11% of C-PAPS, 4% of RADPS, and in endocervical cells in 9% and 2% of C-PAPS and RADPS, respectively. Similar results were seen in study conducted by Jaiwong *et al.*[5] Study conducted by Zare-Mirzaie and Abolhasani[17] showed no statically significant difference in distinctness of nuclear border of squamous cell. While Gupta *et al.*[6] in their study showed C-PAPS had more distinct squamous cell nuclear border as compared to RADPS, difference was not significant statistically.

Crispness of nuclear chromatin of squamous and endocervical cells was more evident in RADPS compared to C-PAPS. Nuclear chromatin of squamous cell and endocervical cell was crisp in 87% and 80% of C-PAPS, which was less compared to 98% and 99% of RADPS. Jaiwong *et al.*[5] in their study observed that in C-PAPS, crisp nuclear chromatin of squamous and endocervical cell was seen in 96.5% and 84%, respectively, and in RADPS, it was 87.8% and 76.5%. Hazy nuclear chromatin of squamous as well as endocervical cell was more evident in C-PAPS and less in RADPS. Possible explanation for this is more cytolysis and air-drying artifact on C-PAPS. The air drying had added advantage as there was increase in nuclear size, flatter and depth of focus on nuclei is shallower, which give better cytomorphology and advantage in taking photograph.[12,18,19] Hence, it was easy to diagnose precursors, preneoplastic, and neoplastic conditions better in RADPS as compared to C-PAPS.

Various authors mentioned that there are only few disadvantages of RAD such as air-drying artifacts, cytolysis, and contamination by organisms if smears are kept for longer period for air drying. Further studies should be conducted for standardizing the maximum time for which air drying can be done as well as effect of environmental factors on Pap smear. Another disadvantage is due to over hydration (>30 s) which can cause artifactual pseudo-nucleomegaly. This can be prevented by restricting duration of rehydration for 30 s.[4,7,12] In our study, we found that RAD technique is better substitute for traditional C-PAP method if air drying and rehydration timings are maintained.

Various authors mentioned that RAD smears show increase in cell size, prominent intracytoplasmic inclusions, and greater cellularity as compared to CPS and thus help in better cytomorphological assessment and interpretation of cervical smear.[4-8,17,18,20-23]

Air-dried rehydration technique was also tried on nongynecologic smears with Fine needle aspiration cytology (FNAC), exfoliative cytology, and effusion cytology as mentioned above,[15,17,18] also with different staining methods like H&E,[9] Giemsa,[16] and IHC.[24,25]

Conventional wet fixation method has been popularly followed as a part of cultured in curriculum and is being routinely used worldwide in health-care settings. Few limitations of this method have been neglected such as air-drying artifacts, unsatisfactory for evaluation due to loss of cellularity while fixation, overlapping of cells, RBCs, and inflammatory cells obscuring the diagnostic cells. Such limitations cannot be underestimated as it can prove costly if the precursors, pre-neoplastic, and neoplastic lesion are missed.[4,6,7,26,27]

There are numerous advantages of RAD technique as shown by various studies conducted by different authors such as reduced number of unsatisfactory smears, lysis of RBC leading to clearer background, cellularity was maintained as there was no loss of material while fixation, and reduction in air-drying artifacts. RAD method was a preferred technique by paramedics/technicians as it was less tedious/cumbersome. There was ease in making diagnosis as there is less obscuring by RBCs and inflammatory cells.[4,7,17,19,20,28]

Limitation in this study was that split smears are prepared; it can lead to variation in material collected. In addition, artifactual nucleomegaly was observed due to over-hydration that may cause errors in interpretation.[4,6,17,20]

Conclusion

Conventional wet fixation method is most commonly followed method and is considered as best method for cervical smears study; it is being routinely used worldwide in health-care settings. However, it has certain limitations. The problems faced with this method are availability of ethanol, more number of air-drying artifacts, loss of cellularity, overlapping of cells, hemorrhagic and inflammatory background, and cytolysis which lead to more number of unsatisfactory smears, due to which the sensitivity of the conventional Pap technique is low.

In order to overcome this problem, RAD technique can be a satisfactory alternative. In resource poor settings and in remote areas, this technique can be successfully applied as it is simple and less cumbersome. In addition, RAD can be practiced routinely or in conjugation with C-PAP in tertiary care setup. This might help in early detection of cervical cancer and thus helps to bring down the burden of morbidity and mortality associated with cervical cancer.

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Conflicts of interest

There are no conflicts of interest.

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