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Original Research Article

The merits of rapid, economic acetic acid, papanicolaou stain (REAP) over papanicolaou stain (PAP) technique in cervical cytology

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ABSTRACT

Background: Carcinoma cervix is the most common preventable cancer in women. The universal stain used in screening of pre-cancer and cancer cervix is papanicolaou stain. The objective of this study was to carry out to assess the quality of staining, cost effectiveness, duration of staining and preservation of staining of PAP stain in comparison with REAP stain.
Methods: 200 smears were collected from 100 patients; one set of smears were stained with conventional PAP technique and other set with REAP technique. The ethanol bath in PAP technique is replaced with 1% acetic acid, tap water is used instead of scotts tap water in REAP technique. Methanol is used only for final dehydration. The two sets were assessed for optimal and suboptimal nuclear and cytoplasmic staining and the results were analysed.
Results: Good cytoplasmic transparency and optimal nuclear details were seen in REAP stained smears when compared to the conventional PAP stain. The cost involved in REAP method was lesser when compared to PAP technique. REAP method took 7 minutes whereas PAP method involved 30 minutes.
Conclusions: REAP technique produces better staining, cost effective and simple, with minimum use of alcohol. Involves minimal time for mass cervical cancer screening as compared to conventional PAP smears.

Keywords: Cancer cervix screening, Cervical smears, PAP stain, REAP stain

INTRODUCTION

Conventional papanicolaou stain (PAP) staining is commonly employed and is very reliable technique used for cervical cancer screening programmes. The reason cytologic screening is so effective in preventing cervical cancer is that majority of cases are preceeded by long standing precancerous lesion, which may exist for several years and shed abnormal cells.[1-3]

Papanicolaou stain is a polychromatic, transparent stain gives crisp nuclear and cytological details. PAP staining technique was developed by George Papanicolaou in 1942 and was subsequently modified by him in 1954 and 1960. But there are certain limitations of routine PAP staining i.e. ethanol is used as dehydrating agent in large amounts is costly and requires purchase licence, and is a lengthy procedure.

The rapid, economic acetic acid (REAP) technique was introduced by Dighe SB and was proved to be better than conventional PAP stain. REAP stain provides excellent nuclear and cytoplasmic details with better colour intensity, cost effective as acetic acids replaces costly ethanol, quicker procedure as the staining is complete in 7 minutes and long term colour preservation when compared to routine pap staining. Hence, REAP was proved to be cheap and rapid.

The objective of this study was to assess the superiority of staining of smears by REAP technique when compared to routine PAP technique based on following parameters:

- Intensity of nuclear and cytoplasmic staining
- Time taken for staining
- Cost effectiveness
- Long term colour preservation.

METHODS

A set of two cervical cytology smears were obtained form 100 patients from Department of Gynaecology and Obstetrics, Bhaskar Medical College and General Hospital, Yenkapally, Hyderabad, India. One set was stained by conventional PAP method and the other set by REAP technique at Department of Pathology, Bhaskar Medical College.

Modifications in REAP compared to PAP are as follows

- Ethanol bath in pre-orange G6, post orange G6 and post EA36 stages is replaced by 1% acetic acid
- Tap water is used instead of scotts tap water
- Haematoxylin is preheated in water bath to 60ºC for rapid penetration
- Methanol is used only for final dehydration.

Conventional PAP technique

- Transfer slides directly from alcohol - ether fixative without drying to 80% alcohol and bring down through 70% and 50% alcohol to distilled water
- Stain in Harris hematoxylin for 4 minutes
- Rinse briefly in distilled water
- Dip in 0.25% HCl in 50% ethanol about six times (20-60 sec)
- Place in scotts tap water for 6 minutes
- Rinse in distilled water and run through 50%, 70%, 80% and 95% alcohol
- Stain in OG-6 for 2 minutes
- Rinse in two changes of 95 % alcohol
- Stain in EA36/EA 50 for 2 minutes
- Rinse in three changes of 95% alcohol. Dehydrate in absolute alcohol, followed by equal parts absolute alcohol and xylol, clear in xylol and mount.

Mount in D.P.X.

All the alcohol steps in conventional PAP were replaced by 1 % acetic acid in REAP

Total time for staining by REAP technique is 7 min. whereas it took 30 minutes. for PAP technique in PAP stain alcohol fixed smears are passed through a series of descending grade of ethyl alcohol before nuclear staining. These grades of ethyl alcohol are replaced by single 1% acetic acid step. Harris haemotoxylin is used in PAP stain where as in REAP it is reduced to 10 dips the stain is preheated to 60 ºC for rapid penetration.

Acid differentiation is done to remove excess staining, but this step is absent in REAP staining. The blueing agent used is scotts tap water in PAP staining is replaced by ordinary tap water in REAP stain. Before staining with orange G6 the changes of dehydrate ethyl alcohol grades are replaced by 1% acetic acid in REAP. The cytoplasmic stains (OG6 and EA50) are same in both methods except the time spent. In both, staining time is reduced from 3 minutes to a few seconds.[4-6] Two changes of 95% ethyl alcohol with standard PAP stain after OG6 are replaced by 1% acetic acid (10 dips). In standard PAP stain, final dehydration is done by two changes of absolute alcohol. In REAP the smears are washed in 1% acetic acid and final dehydration by methanol (10 dips). With PAP stain clearing is done by one change of xylene (10 dips each).[4-6] In REAP clearing is done by single change of xylene (10 dips). All the REAP and PAP stained smears were screened by senior pathologists of our department and screened separately without any bias.

RESULTS

A total of 100 paired smears stained by conventional Papanicolaou and REAP stain were examined. The minimum age of patient screened was 20 years and maximum 60 years. The majority were in the age group of 31-40 years. The mean age was 40 years (Table 3). REAP stained smears were compared with conventional PAP stained smears.

The following parameters were assessed

- Optimal and suboptimal nuclear staining
- Optimal and suboptimal cytoplasmic staining

The average time taken for routine PAP and REAP staining for each respective smears was compared. The effective cost of PAP and REAP staining procedures was calculated and compared. The preservation of colour intensity of the smears stained by PAP and REAP are being compared over one year observation period with periodic checks at quarterly intervals.

Table 2 compares the cytoplasmic staining quality of the REAP and PAP smears. The differentiation and transparency of the cytoplasm of REAP were optimal in 86% smears. In 14% smears the cytoplasmic stain penetration was sub-optimal, especially in areas of overlapping cell clusters. The nuclear details and the chromatin pattern were compared between PAP and REAP smears (Table 2) which were clear and crisp in 1% REAP smears. In only 9% of cases the nuclear staining was suboptimal i.e. the nuclear staining was not crisp and this was due to air drying artefacts.

There were no differences in the staining reaction of nonepithelial cells, such as white and red blood cells in

either staining technique. The staining quality of all the REAP smears remained well preserved (without any fading) for more than 1 year and the cost per smear stained with REAP was lesser than the cost of PAP smear (Table 2). Thus, REAP is a fast technique and the staining time is 7 minutes as compared to 30 minutes with PAP stain.

Table 1: Staining technique PAP and REAP.

Staining technique	Optimal staining %		Sub-optimal staining %	
	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear
PAP	52	60	48	40
REAP	86	91	14	9

Table 2: Comparison of cost involved in both the stains.

PAP Staining		REAP Staining	
Reagent	**Cost**	**Reagent**	**Cost**
Ethanol Absolute 500 ml (S.D. fine chemical, Mumbai)	660	Methanol AR 500ml (SD fine chemicals, Mumbai)	154
For 20 bottles (our study)	13,200	For 5 bottles (For our study)	770
		Glacial acetic acid 500ml (SDFCL, Mumbai)	356
		For 2 bottles (our study)	712
INR 13,200		INR 1,482	

REAP technique is the recent modification of standard PAP technique. It is defined as rapid economic acetic acid Papanicoaous stain. As the name implies, the technique is rapid, economical, acetic acid is used as dehydrant and colour preservation. These qualities had made this technique superior than that of standard PAP.

The cytoplasmic transparency and nuclear details are statistically superior in the REAP stained smears compared to the smears stained by routine PAP. In REAP pre-heated (at 60^{o}C) haematoxylin was used, acid differentiation step was discarded and 1% acetic acid was used as the dehydrating agent in place of ethanol. 1% acetic acid acts as a nuclear fixative and it also intensify the staining intensity therefore the nuclear staining in case of REAP was better than PAP. During cytoplasmic staining in case of PAP, both the stains i.e. OG6 and EA36 are alcohol based (ethanol) stain. So, after the cytoplasmic staining was done the smears were dehydrated in ethanol and some of the cytoplasmic stain diffuses into the dehydrating medium. Thus, the cytoplasmic staining intensity reduces. But in case of REAP 1% acetic acid was used as dehydrating agent, thus a chemical reaction occurs between acetic acid and ethanol (from OG6 and EA36). Ethyl acetate is a low molecular weight ester soluble in water. Since most of the water is removed from the cell during the reaction, the ester complexes with the cytoplasmic stains and is deposited in the cells, subsequently preserving stain intensity. So, the cytoplasmic staining is comparable to PAP. In PAP staining the colour preservation was not long standing. During dehydration procedure, some ethanol enters the cell and the smear was mounted in DPX. As the time passes by there was dissolution of stain in ethanol (both cytoplasmic and nuclear stain).

There was percolation of stains into the mounting medium i.e. DPX, so there is no long standing colour preservation. But in case of REAP, ethyl acetate preserves the cytoplasmic staining, acetic acid also acts as a nuclear stain fixative, preserving the nuclear staining, acid differentiation step was absent, cytoplasmic stains are 4 times more concentrated than the routine PAP stain and acetic acid, used as a dehydrate, helps in rapid staining therefore REAP staining technique was time saving (5-7 minutes) than standard PAP staining technique (25-30 minutes). REAP staining is a better technique compared to routine PAP staining in producing smears with excellent cytoplasmic and nuclear staining intensity. This fact is reiterated by the low cost and lesser time associated with the REAP staining technique.

The staining quality was compared for both the stains taking into criteria the cell and cytoplasmic border, cytoplasmic staining, nuclear borders and chromatic staining (Table 1). 4% of the smears had indistinct cell borders and 96% had distinct cell borders in the conventional PAP method. In REAP (2%) of the smears had indistinct cell borders and 98% had distinct cell borders. In conventional PAP 3% showed unsatisfactory cytoplasmic staining and 97% showed satisfactory cytoplasmic staining whereas REAP showed 2% of unsatisfactory cytoplasmic staining and 99% showed satisfactory cytoplasmic staining. 4% of smears had indistinct nuclear borders and 96 had distinct nuclear borders, in smears stained by PAP. Whereas REAP has

showed 3% and 97% respectively. In PAP chromatin staining was hazy in 5% and distinct in 95% in REAP chromatin staining was hazy in 3% and distinct in 97%. Out of 100 paired slides stained by PAP and REAP, PAP showed, 4 cases of suboptimal cytoplasmic and nuclear staining whereas REAP showed 2 cases of suboptimal cytoplasmic staining and 3 cases of suboptimal nuclear staining (Table 3).

DISCUSSION

The present studies included 100 paired slides stained by conventional PAP and REAP methods. The quality off staining was assessed by taking into consideration cell borders cytoplasmic staining, nuclear borders and chromatin staining and were categorized an optimal and suboptimal. In case of Biswas et al, studied 159 cases, showed cytoplasmic and nuclear suboptimal staining of 21 cases stained by REAP method whereas conventional PAP method had shown a suboptimal cytoplasmic and nuclear staining of 29 cases.[6] Both the above studies concluded that REAP stain showed better results than conventional PAP method (Table 4).

Table 3: Age distribution of the cases; N=100.

Age (Years)	%
20-30	32
31-40	54
41-50	09
51-60	05
Total	100

Dighe et al, in their study of 200 cases showed that REAP stained smears had suboptimal cytoplasmic and nuclear staining of 19 and 8, respectively.[7] Whereas PAP stain did not show any suboptimal cytoplasmic or nuclear staining. The present study of 100 cases revealed that REAP stained smears showed suboptimal cytoplasmic staining of 6 cases and suboptimal staining of 4 cases. Conventional PAP showed suboptimal cytoplasmic and nuclear staining in 2 cases.

Stain preservation was excellent for 2 years in the study of Dighe et al and present study whereas in the study done by Biswas et al, it showed good preservation for 6 months only.[6,7] Gachie et al, did not include this parameter in their study (Table 5).[8] Biswas et al, study REAP proved cheaper to conventional PAP by four times.[6] Turnaround time for REAP was only 7 minutes. whereas conventional PAP took 30 minutes, Dighe et al, also showed similar results in case of turnaround time and cost effectiveness (Table 5).[7]

Maximum numbers of cases are in age group 31-40 years (54 %).

Table 4: Comparison of Cell/Cytoplasmic borders of REAP and conventional PAP.

Parameter	REAP %	Conventional PAP %
Cell/Cytoplasmic borders		
Distinct	98	95
Indistinct	2	5
Cytoplasmic staining		
Satisfactory	97	96
Unsatisfactory	3	4
Nuclear-border		
Distinct	96	97
Indistinct	4	3
Chromatin staining		
Distinct	97	95
Hazy	3	5

In case of Gachie et al, turnaround time was same as the above two studies for both the stains but the cost for REAP stain was reduced by 6 times. Present study showed reduction of cost for REAP stain by 4 times and turnaround time was reduced by 5 times (Table 5).[8]

Izhar et al, in their study concluded that REAP stain cannot be used for routine staining in a tertiary care hospital for research purpose due to poor preservation of staining quality after 6 months.[9] The background of smears stained by REAP was extremely clear without any debris when compared with PAP stained slides. Nuclear details were assessed based on the nature of chromatin, vesicularity, membrane integrity, cytoplasmic details were evaluated based on transparency and nature of cell membrane.

Quality of nuclear border staining was same for both PAP and REAP, whereas quality of cell border, cytoplasmic staining and chromatin staining of REAP was slightly more than PAP.

Table 5: Comparison of cycloplasmic stain, nuclear stain, smear preservation, turnaround time and cost per smear of PAP and REAP.

Procedure	Cycloplasmic stain		Nuclear Stain		Smear preservation (2 years)	Turnaround time	Cost per smear
PAP	98	2	96	4	Excellent	30 minutes	Rs. 40
REAP	94	6	98	2	Excellent	7 minutes	Rs.10

Quality of REAP staining was as good as PAP whereas turnaround time and cost per smear in REAP were considerable reduced. Preservation was excellent in both cases for two years.

Table 6: Comparison of quality of staining and smear preservation of PAP and REAP with other authors; N = 100.

Authors		Cytoplasmic stain		Nuclear stain		Smear preservation
		Optimal	Sub-optimal	optimal	Sub-optimal	
Dighe et al N = (200)	PAP	200 (100%)	-	200 (100%)	-	Excellent (2 years)
	REAP	181 (90.5%)	19 (9.5%)	192 (96%)	8 (4%)	Excellent (2 years)
Biswas et al N = (110)	PAP	90 (81.8 %)	201 (18.2%)	100 (90.9%)	10 (9.1%)	Excellent (6 months)
	REAP	100 (90.9 %)	10 (9.1%)	105 (95.4%)	5 (4.6%)	Excellent (6 months)
Gachie et al N = (159)	PAP	130 (81.7%)	29 (18.3%)	130 (81.7)	29 (18.3%)	-
	REAP	138 (86.7 %)	21 (13.3%)	138 (86.7%)	21 (13.3%)	-
Present study	PAP	98 (98%)	2 (2%)	98 (98%)	2 (2%)	Excellent (1 year)
	REAP	96 (96%)	4 (4%)	96 (96%)	4 (4%)	Excellent (1 year)

Table 7: comparison of cost and time of PAP and REAP with other authors; N = 100.

Authors		Time (minutes)	Cost per slide (Rs.)
Dighe et al N = (200)	PAP	20	240
	REAP	3	60
Biswas et al N = (110)	PAP	20	100
	REAP	3	25
Gachie et al N = (159)	PAP	20±0.5	123
	REAP	3±0.5	19
Present study N = 100	PAP	30	40
	REAP	7	10

- Quality of staining - Biswas et al, Gachie et al, REAP fared better than PAP as in Dighe et al and present study REAP fared slightly better than PAP
- Smear preservation - present study and Dighe et al, showed excellent preservation for 2 years
- Biswas et al, showed excellent preservation for 6 months, Gachie et al, did not include this parameter.

Present study showed excellent preservation for one year.

- Dighe et al, Biswas et al, Gachie et al, time taken for PAP and REAP was 20 minutes and 3 minutes respectively and cost per slide for REAP reduced by 4 to 6 times
- Present study the time taken for PAP and REAP was 30 and 7 minutes respectively. Cost per slide was reduced by 4 times.

CONCLUSION

For a stain to be utilized in a mass screening programme in addition to good staining characteristics the technique must be easy, rapid and economical. Need for minimal turnaround time (TAT) for assessing pap smears has encouraged innovations in staining techniques that require lesser staining time with unequivocal morphology. Good cytoplasmic transparency and optimal nuclear details were seen in REAP stained smears when compared to the conventional PAP stain.

The cost involved in REAP method was lesser when compared to PAP technique. REAP method took 7 minutes whereas PAP method involved 30 minutes. REAP technique produces better staining, cost effective and simple, with minimum use of alcohol. It involves minimal time for mass cervical cancer screening as compared to conventional PAP smears.

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