Original Article

Comparison of the routine Papanicolaou staining technique with the rapid, economic, acetic acid, Papanicolaou (REAP) technique

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ABSTRACT

Background: *The universal stain for oral cytological screening for precancer and cancer of oral cavity is Papanicolaou stain which has been used in different laboratories with many modifications.*

Objectives: *To assess the superiority of staining of smears by REAP technique compared to PAP technique.*

Material and Methods: *100 smears were collected from 50 patients. One set of smears were stained with conventional PAP technique and the other set with the REAP technique. In the REAP technique, the ethanol bath in the pre Orange G6, post Orange G6 and post EA50 stages is replaced by 1% acetic acid; Tap water is used instead of Scott's tap water and hematoxylin is pre heated in water bath to 60°C for rapid penetration. Methanol is used for final dehydration. The two sets of smears are observed by two independent observers and assessed for the optimal and suboptimal nuclear and cytoplasmic staining. The results are compared and analyzed statistically.*

Results: *Good cytoplasmic transparency and optimal nuclear details were seen in REAP stained smears compared to the conventional PAP smears. The cost involved in REAP method was lesser compared to conventional PAP technique. REAP technique took 3 minutes for completion compared to PAP technique that involves a minimum of 20 minutes.*

Conclusion: *REAP technique produces better stained smears that are cost effective and involve minimal time for mass screening of oral cancer as compared to conventional PAP smears.*

Keywords: *PAP stain, REAP stain, papanicolaou smear, oral cancer screening, mass screening, cytological screening*

Introduction

Routine Papanicolaou (PAP) staining is a commonly employed chairside cytological procedure in the diagnosis of suspicious oral smears as it yields a polychromatic, transparent staining reaction with crisp nuclear/cytological features. There are certain limitations of routine PAP staining i.e. ethanol used as a dehydrating agent in large amounts is costly and requires purchase license, color preservation is not long standing and takes 20 min, relatively a long period for a chairside procedure. Various modifications of PAP staining such as Ultra Fast and Rapid PAP are present. [1] These two techniques take 90 seconds for complete staining thus overcoming the time limitation of routine PAP but the issues of ethanol and color preservation still remain. [2] The REAP technique was Introduced by SB Dighe (2005) and reported to be: better than routine PAP, excellent nuclear and cytoplasmic staining with better color intensity, cost-effective acetic acid replaces costly ethanol, long term color preservation and quicker procedure compared to routine PAP. [1]

The main aim was to assess the superiority of staining of smears by REAP technique compared to routine PAP technique based on the following parameters:

- Intensity of nuclear and cytoplasmic staining

- Time taken for staining
- Cost effectiveness
- Long term color preservation

Material and Methods

Two smears each were collected from 50 volunteers with a clinically normal oral mucosa and free from tobacco-related habits. One set of smears (50 smears) were stained with routine PAP staining technique. The second set of smears (50 smears) were stained with the REAP technique.

Modifications in REAP compared to PAP-

a) Ethanol bath in the pre Orange G6, post Orange G6 and post EA36 stages is replaced by one percent acetic acid
b) Tap water is used instead of Scott's tap water
c) Haematoxylin is pre-heated in the water bath to 60°C for rapid penetration
d) Methanol is used for final dehydration

Staining protocol of REAP technique. [1]

1% acetic acid	10 dips
Harris's Haematoxylin (preheated 60° C)	10 dips
Tap water	10 dips
1% acetic acid	10 dips
OG-6	10 dips
1%acetic acid	10 dips
EA-50	10 dips
1% acetic acid	10 dips
Methanol	10 dips
Xylene	10 dips

Blotting was done after each step.
Mount by D.P.X.

Total time for staining in the present method is 3 minutes (c.f. Conventional PAP stain which is 20 minutes). In Papanicolaou stain, fixed smears are passed through a series of descending grade of ethyl alcohol before nuclear staining. These ethyl alcohol grades are replaced by single 1% acetic acid step. Harris's haematoxylin is used in both methods for nuclear staining but the time is 1 minute in conventional PAP stain and in REAP it is reduced to 10 dips as the stain is preheated to 60°C. Heating of haematoxylin is done in waterbath to 60°C before staining for rapid penetration, acid differentiation is done to remove the excess staining but this step is absent in REAP staining protocol. [1]

In case of standard PAP technique, the bluing agent is Scott's tap water which is replaced by ordinary tap water in REAP stain where the time is also reduced. Before staining with Orange G6 the two changes of dehydrating ethyl alcohol grades are replaced by 1% acetic acid in REAP. The cytoplasmic stains (OG6 and EA50) are same in both methods except the timespent. In both, staining time is reduced from 3 minutes to a few seconds. [1, 2, 3] Two changes of 95% ethyl alcohol with standard PAP stain after OG6 are replaced by 1% acetic acid (10dips). In standard PAP stain final dehydration is done by two changes of absolute alcohol. In REAP the smears are washed in 1% acetic acid & final dehydration by methanol (10 dips). With PAP stain clearing is done by one change of alcohol-xylene followed by 2 changes of xylene (10 dips each). [1, 2, 3] 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 comparison and bias. REAP smears are compared with conventional PAP smears using following various parameters. [1, 2, 3] The smears stained by PAP and REAP were observed by two independent observers and the following parameters were assessed -

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

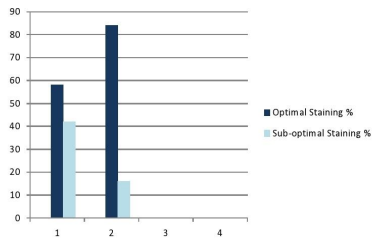
The average of the time taken for routine PAP and REAP staining for each respective smear was compared. The effective cost of PAP and REAP staining procedures was calculated and compared. The preservation of color intensity of the smears stained by PAP and REAP are being compared over a one-year observation period, with periodic checks at quarterly intervals. Chi-square test was conducted to assess the statistical difference in staining intensity between PAP and REAP.

Results

Table I (Graph 1) compares the cytoplasmic staining quality of the REAP and PAP smears. (Fig 1a, b) The differentiation and transparency of the cytoplasm of REAP were optimal in 84% smears. In 16% smears the cytoplasmic stain penetration was suboptimal, especially in areas of overlapping cell clusters. The nuclear details and the chromatin pattern were compared between REAP and PAP smears (Table I) (Graph 2) which were clear and crisp in 92% REAP smears. In only 8% of cases the nuclear staining was suboptimal i.e. the nuclear staining was not crisp and this was due to air drying artifacts. Chi-square values shows statistical difference between PAP and REAP i.e. Cytoplasmic staining- 23.261 and Nuclear staining- 32.210 (p-value <0.001). Thus the results were statistically significant.

Table I: Staining techniques PAP and REAP

Staining technique	Optimal staining %		Sub- optimal staining %	
	Cytoplasmic	Nuclear	Cytoplasmic	Nuclear
PAP	58	62	42	38
REAP	84	92	16	8



Graph 1: Showing optimal and suboptimal cytoplasmic staining

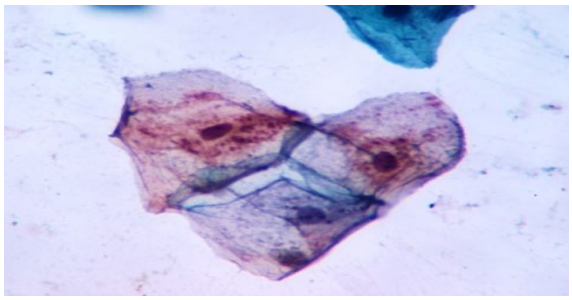


Fig. 1a REAP stained smear showing optimal differentiation and transparency of the cytoplasm with crisp and clear nuclear details and chromatin pattern

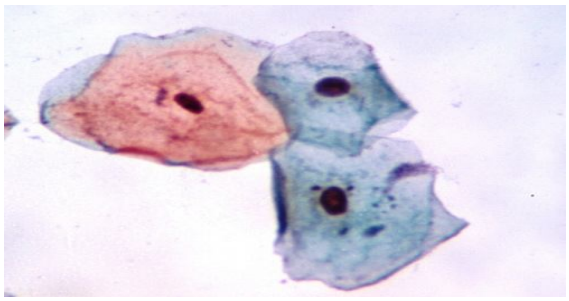


Fig. 1b PAP stained smear showing suboptimal and diffused cytoplasmic and nuclear staining

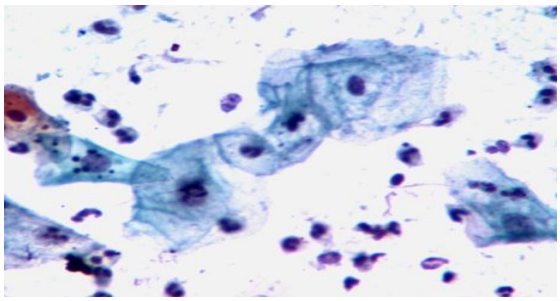


Fig2a. PAP stained smear shows mature basal and intermediate cell with inflammatory cells in the background

There was no difference in the staining reaction of nonepithelial cells, such as white and red blood cells in either staining technique (fig 2a,b). 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 II). Thus REAP is a fast technique and the staining time is 3-4 minutes as compared to 18- 20 minutes with PAP stain.

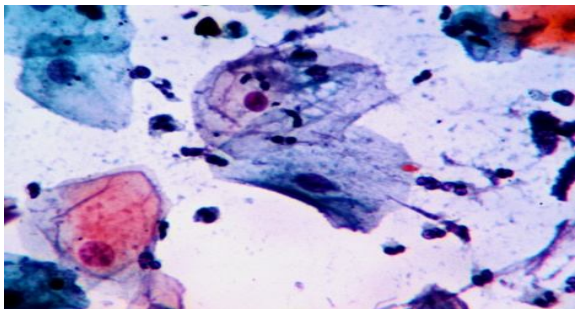
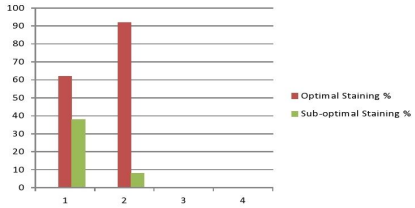


Fig2b.REAP stained smear shows mature basal and intermediate cell with inflammatory cells in the background



Graph 2: Showing optimal and suboptimal nuclear staining

Table II: Comparison of cost effectiveness (INR) of both the stains

PAP Staining		REAP Staining	
Reagent	Cost	Reagent	Cost
Ethanol Absolute 500 ml (SDFCL Mumbai)	660	Methanol AR 500 ml (SDFCL Mumbai)	154
For 20 bottles (Our study)	13200	For 5 bottles (Our study)	770
		Glacial Acetic acid 500 ml (SDFCL Mumbai)	356
		For 2 bottles (Our study)	712
INR 13,200		INR 1,482	

Discussion

After its introduction by George Papanicolaou, Papanicolaou stain underwent various modifications. Over the years cytopathologist have felt the need for a rapid PAP stain that is as fast as Diff- Quik and provides cytomorphological features as exquisite as does PAP stain. REAP technique is the recent modification of standard PAP technique. It is defined as Rapid Economic Acetic acid Papanicolaous stain. As the name implies, the technique is rapid, economical, acetic acid is used as a dehyderant and colour preservation.These qualities had made this technique superior than that of standard PAP.[4,5]

The cytoplasmic transparency and nuclear details are statistically superior in the REAP stained smears compared to the smears stained by routine PAP. An insight into the chemistry of the staining techniques reveals a probable explanation for the better results obtained by REAP over PAP.[4,5]

In REAP pre heated (at 60^0C) 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.[4,5]

.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 occur between acetic acid and ethanol (from OG6 and EA36). This reaction leads to formation of ethyl acetate and water (which was removed).[4,5]

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 staining intensity. So the cytoplasmic staining is comparable to PAP. [6,7]

In PAP staining the color 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 color 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.[8,9,10]

Nuclear stain in REAP technique was preheated at 60^0C, 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 (3-4 min) than standard PAP staining technique (18-20 min). [9,10]

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.

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