"COMPARISON OF CYTO-MORPHOLOGICAL FEATURES IN CONVENTIONAL PAPANICOLAOU STAINING METHOD (CPAP) WITH REHYDRATED AIR DRIED PAP SMEARS (RADPS) AND RAPID ECONOMICAL ACETIC ACID PAP (REAP) STAINING METHOD IN CERVICAL SMEARS: AN OBSERVATIONAL STUDY"

By

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Submitted to the

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IN
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Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"COMPARISON OF CYTOMORPHOLOGICAL FEATURES IN CONVENTIONAL PAPANICOLAOU STAINING METHOD WITH REHYDRATED AIR DRIED PAP SMEARS (RADPS) AND RAPID ECONOMICAL ACETIC ACID PAP (REAP) STAINING METHOD IN CERVICAL SMEARS: AN OBSERVATIONAL STUDY."**, is ethical and justifiable. The proposed research project has been cleared by the JNMC Institutional Ethics Committee.

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J.N.Medical College, Belagavi.

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List of Abbreviations:

ADA- Air-drying Artifacts

ASC-H- Atypia of Squamous Cells cannot exclude HSIL

ASCUS- Atypia of Squamous Cells of Undetermined Significance

CPAP- Conventional Papanicolaou

DPX-Dibutyl Pthalate Xylene

HPV- Human Papilloma Virus

Hrs- Hours

HSIL- High grade Squamous Intra-epithelial Lesion

LSIL-Low grade Squamous Intra-epithelial Lesion

Mins – Minutes

NILM- Negative for Intra-epithelial Lesion Malignancy

OPD – Out Patient Department

PAP-Papanicolaou

PHC- Primary Health Care

QI- Quality Index

RADPS- Rehydrated Air-dried Papanicolaou Smears

RBC-Red Blood Cells

REAP-Rapid, Economic, Acetic-acid Papanicolaou

Rs- Rupees

SCJ- Squamo-Columnar Junction

SD- Standard Deviation

Sec- Seconds

TBS- The Bethesda System

VS-Versus

ABSTRACT:

TITLE:

Comparison of cyto-morphological features in Conventional Papanicolaou Staining method with Rehydrated Air Dried Pap Smears (RADPS) and Rapid Economical acetic Acid Pap (REAP) staining method in cervical Smears: An observational study

Background:

Cervical cancer is one of the prevalent cancers of women in India. PAP smears are the most commonly used screening test for cancer cervix. The limitations of Conventional PAP (CPAP) lead to the development of other modified techniques (RADPS, REAP). Most of the previous studies have compared either of the modified techniques to conventional.

Our study compared two modified techniques i.e. RADPS & REAP with that of CPAP.

Aims &Objectives:

To compare the cyto-morphological features of cervical smears stained by Conventional Papanicolaou with that of modified Papanicolaou staining methods of RADPS, REAP.

Materials & methods:

A one-year prospective study from October 2022 to October 2023 was done on patients who visited Gynecology out-patient department of KLE DR. PRABHAKAR KORE hospital, Belagavi.After taking Informed consent from 250 patients 3 sets of smears from each patient were collected. One set is stained in the conventional pap method, other two are stained by RADPS & REAP. All 3 were compared &analyzed..

RESULTS:

In our study, RADPS showed high scores in staining (79.6%), preserved cell morphology (76.8%), optimal cytoplasmic details (61.2%), crisp nuclear characteristics (65.6%), distinct nuclear borders (89.6%), and clear background (92.0%). REAP had the least air drying artifacts (68.0%). Statistically significant differences were observed among techniques in most features, except for air drying artifacts in CPAP vs RADPS and REAP vs RADPS, and cell borders in RADPS vs REAP

Conclusion:

In our study, concerning cyto-morphological features, RADPS proved to be a better alternative to CPAP & REAP and were associated with good overall staining, well-preserved cell morphology, optimal cytoplasmic details, distinct nuclear borders & crisp nuclear characteristics with clear background. However, No statistical difference was noted about features like cell borders & air-drying artifacts. About time saving & cost-effective procedures, REAP proved to be better. Both the modified pap procedures were technician-

friendly as they are easy to perform and can be used in developing countries like India where resources are limited & cancer prevalence is high.

Key words:Cyto-morphological features, PAP stain, Conventional, Modified PAP stain.

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INTRODUCTION:

Cervical cancer involving the cervix, the lowermost part of the uterus is the fourth most common cancer affecting women worldwide.[1]In the year 2020, approximately six lakh women were reported with cervical cancer and 3 lakh women died from the disease worldwide.[2]The scenario in India is no different. In India, it is considered the second most common cancer affecting women &accounting for 9.4% of all cancers.[3] As per 2020 statistics, 18.3% (123,907)of new cases of cancer cervix were recorded in India and it is a leading cause of cancer-related deaths in women in low-income countries.[4]

Cervical cancer is related to multiple factors including early marriage, poor genital hygiene, multiple sexual partners, and repeated pregnancy. [5] Early marriage, teenage intercourses,high parity,and cofactors like smoking& long-term usage of contraceptives are associated with increased risk of HPV acquisition among women.[6, 7]

A study by Bhatla et al noted that women from poor socio-economic status were mostly affected by cancer cervix suggestingthat an increase in awareness & improvement of living standards can lead to a decrease in the incidence of the same. [8]The situation is alarming in rural areas of Indiawhere lack of education, poor hygienic conditions,lack of proper screeningfacilities and delayed treatment may contribute further to the progression & spread of carcinoma cervix.[9- 11]The application of some screening programs to identify such cancers at an early stage must be undertaken which is the need of the hourwhich was also highlighted by the implementation of a National screening program for cancer in 2016 in India.[12, 13] Unfortunately, a study done by Gopika al in 2022 that only 1.9% of women underwent screening for cervical cancer stressing upon the fact that the screening programs are effective only when frequently conducted by the government & utilized by the public. [14]These screening tests play a vitalrole in detecting the pre-cancerous changes in the cervical cells so that early intervention can be undertaken to prevent cervical cancer.[15- 17] Incidental early cancer detection during these screeningtests canhelp ensure that adequate diagnostic and treatment services are provided on time. [18,-20]

Screening for cervical cancer includes tests like theHPV (Human papillomavirus) test and the (PAP)Papanicolaoutest which can be applied singly or in combination as a Co-test.[21-24]

According to American Cancer Society (ACS) guidelines 2020, the following has been recommended (Table.1) [25]

Table 1: ACS Guidelines for cervical cancer screening

Age	Test	Interval
21-29	Single pap test	Every yrs.
25-65	HPV alone	Every yrs.
30-65	PAP alone	Every yrs.
	HPV alone	Every yrs.
	PAP and HPV co-test	Every yrs.

Among the two, the Pap test is an effective screening test for cervical cancer which has lowered cancer incidence by 70% in developed countries.[26, 27]Several studies have proved the efficacy of PAP smears in diagnosing cervical lesions.[28-30]As this is a widely used test,research is being done by modifying this staining technique toimprove its accuracy without compromising on the quality so that its application &utilization in urban as well as rural setups can be enhanced and simplified.[31, 32]

2) AIMS & OBJECTIVES:

To compare the cyto-morphological features of cervical smears stained by Conventional Papanicolaou(CPAP) with that of modified Papanicolaou staining methods ofRehydratedAirdriedPapSmear(RADPS),RapidEconomicAceticacidPap(REAP).

3) REVIEW OF LITERATURE:

Gross Anatomy

The Female Genital Tract comprises of Uterus, Cervix, Vagina, Fallopian tubes &Ovaries (Fig.1).[33]

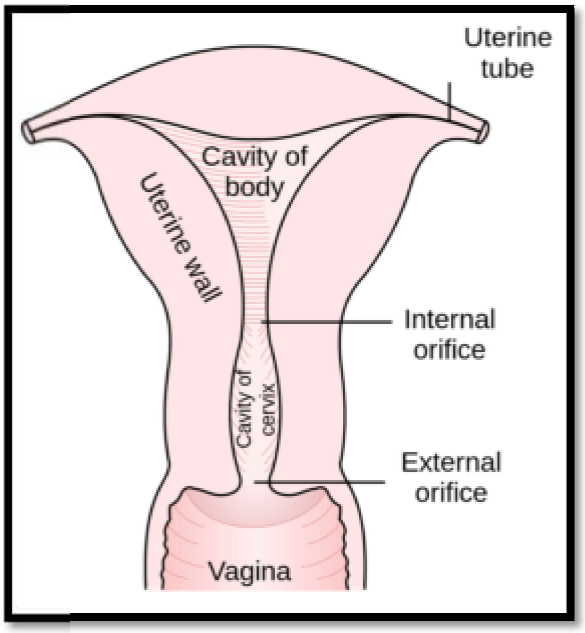


Figure 1: Gross Anatomy of Female Genital Tract

The cervix forms theinferior part of the uterus thatprotrudes into the vagina. The normal length of an adult cervix is 2.5-3cm. The Portio-vaginalis or exocervix has a convex elliptical surface. It is circular in the nulligravida and slit-like in the parous woman. The external os connects the exo& endocervix. The normal endocervical canal measures 8mmin size & is elliptical. The Mackenrodt and the utero-sacral ligament are the main supports for the uterus.[34]

ARTERIAL SUPPLY:

The descending branches of the uterine arteryprovidethe arterial supply to the cervix.

VENOUS & LYMPHATIC DRAINAGE:

The venous blood from the cervix drains into a uterine vein and then into internal iliac veins.

The lymphatic drainage from the anterior& lateral cervix is into lymph nodes along the uterine artery. The Posterior & lateral cervix drains along internal iliac lymph nodes. All these ultimately drain tothe obturator & pre-sacral lymph nodes. [35]

NERVE SUPPLY:

The pain sensation from the cervix is carried to the brain by pelvic splanchnic nerves S2-S3.[36]

NORMAL HISTOLOGY OF CERVIX:

The normal cervix is lined by stratified squamous & columnar epithelium. The endocervix is lined by a single layer of columnar cells with numerous tubular mucous glands. Non-keratinized stratified squamous epithelium lines the ectocervix(Fig.2).

The squamo-columnar junction(SCJ) -the transformation zone: This zone has significance in pre-malignant & malignant lesions of the cervix (Fig.3)[37, 38]

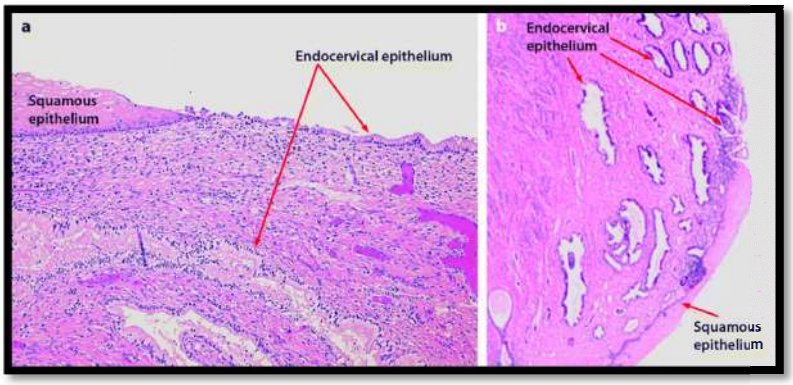


Figure 2:Normal Histology of Ectocervix & Endocervix (H& E stain, 10x&40x)

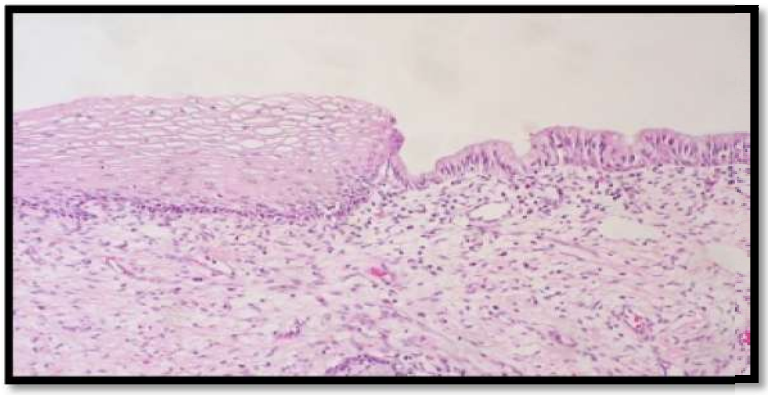


Figure 3: Histology of Squamo-columnar junction (H&E stain, 10x)

Normal Cytology of the Cervix:

Cervical cytology aids in examining the morphology of different cells of the cervix. It helps detect various lesions of the cervix like infections, inflammations, and precancerous, cancerous lesions. The cells observed in cervical cytology are superficial squamous cells, intermediate squamous cells, Basal cells & Parabasal cells.

Superficial Squamous Cells:

They are 25-30µm, large polygonal cells with 4µm, small, pyknotic nuclei. The cytoplasm is eosinophilic because of the chemical affinity of the cytoplasm for acidic dyes such as eosin (Fig.4).

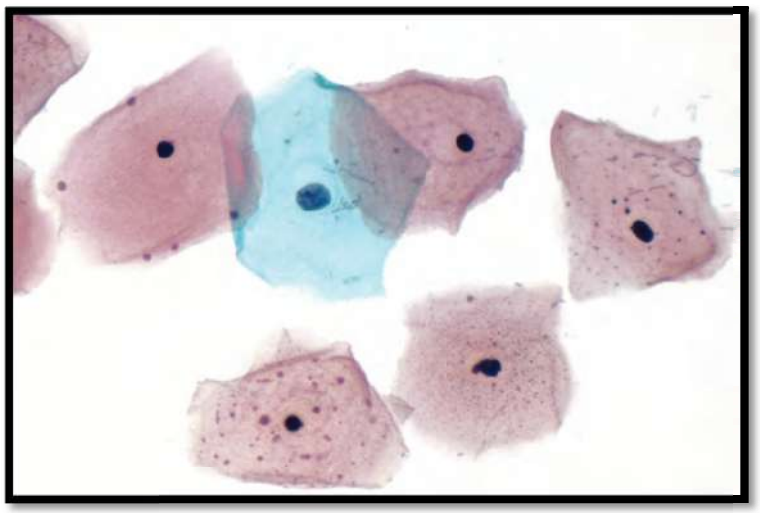


Figure 4:Superficial Squamous cells (Pap stain, 10x)

Intermediate Squamous Cells:

The intermediate squamous cells in the cervix are of the same size as the superficial cells but their cytoplasm is basophilic and opaque in the Pap stain. The nuclei are vesicular, spherical to oval, measuring about 8µmin diameter, with a well-defined nuclear membrane (Fig.5). Navicular cells are a boat-shaped variant of intermediate cells that stain yellow on pap smears. These cells are predominant in conditions like pregnancy, inflammatory conditions & hormonal deficiencies.

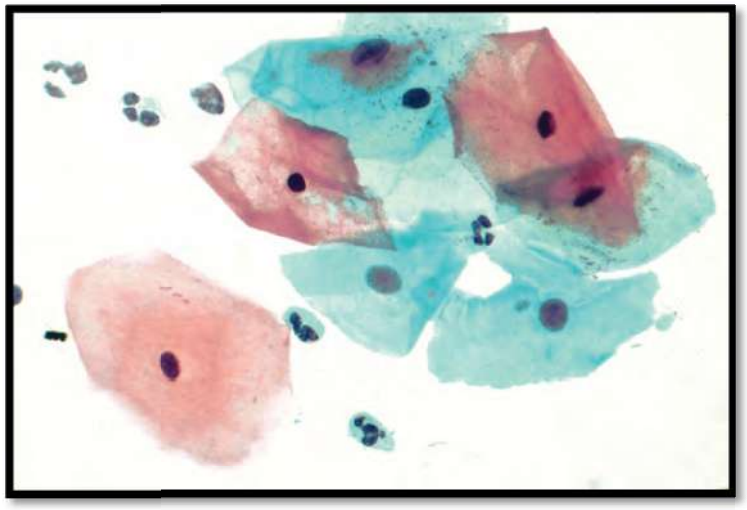


Figure 5:Intermediate squamous cells (Pap stain, 10x)

Parabasal Cells:

The parabasal cells measure 12 to 30µm in diameter and have vesicular nuclei (Fig.6). The presence of parabasal cells indicates an adequate cervical pap smear.

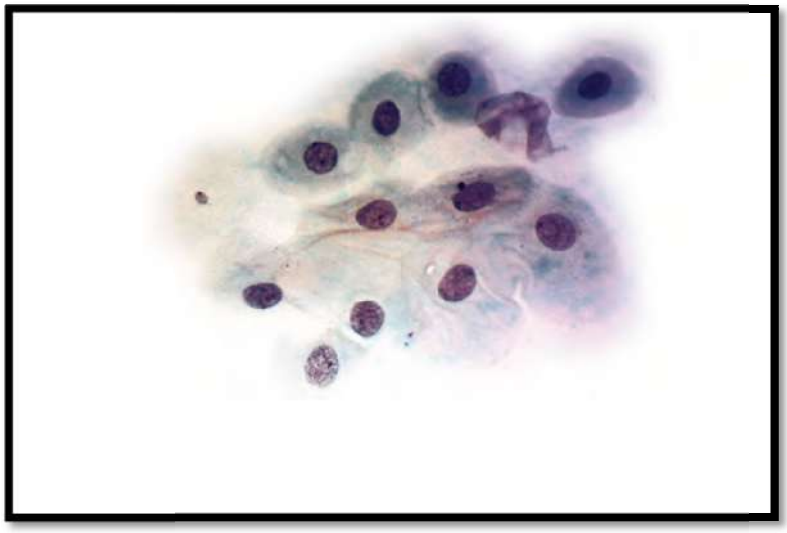


Figure 6: Parabasal cells (Pap stain, 10x)

Basal cells:

These cells are small in size, round to oval in shape. The cytoplasm is scant and basophilic with relatively large nuclei &round nucleoli. The size & configuration of basal squamous cells mimic cancer cells (Fig.7).Their presence is suggestive of some pathological process or vigorous brushing. [39]

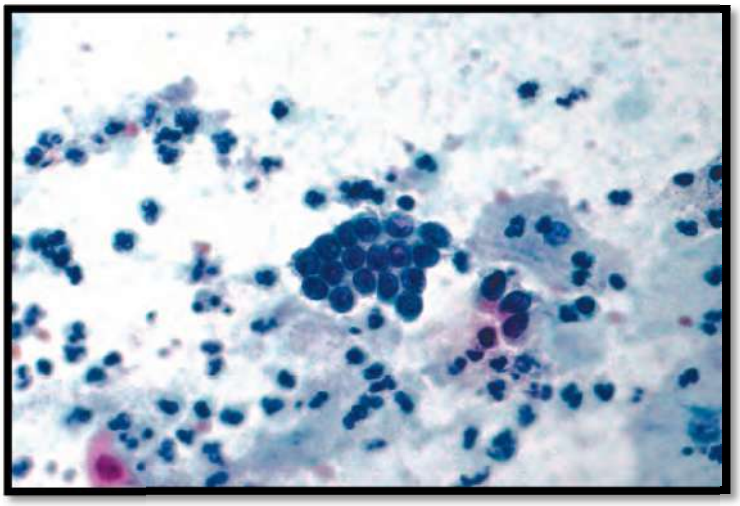


Figure 7: Basal cells (pap stain, 10x)

Cells Originating from the Endocervical Epithelium:

The endocervical cells are collected using a cytobrush. These cells measure approximately 20µm in length and 8 to 12µmin width. The endocervical cells are columnar, seen in sheets, and palisade arrangement, also form tight clusters or plaques, resembling a honeycomb arrangement (Fig.8).

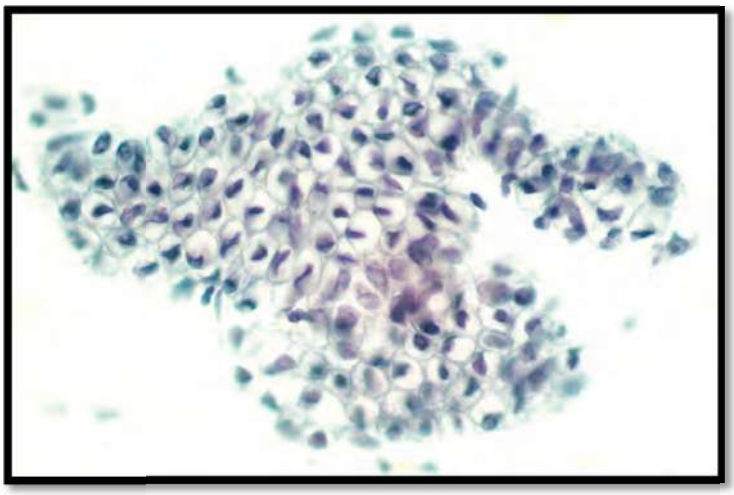


Figure 8: Honeycomb arrangement of endocervical cells (pap stain, 10x)

Ciliated Endocervical cells are seen in brush specimens from proximal segments of the endocervical canal. These cells are ciliated, slightly larger with hyperchromatic nuclei& are supported by a terminal plate. (Fig.9). Few cytopathologists interpret the presence of these cells as tubal metaplasia. [40]

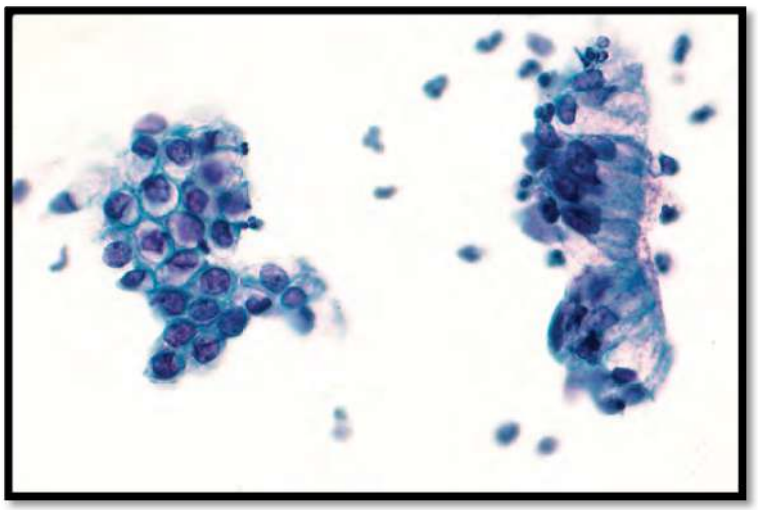


Figure 9: Ciliated endocervical cells (Pap stain, 10x)

HISTORICAL ASPECTS OF PAP SMEAR:

In 1843, Walter Hayle Walsh in London first reported malignant cells in the sputum of a few patients with lung disease.[41, 42] Then, he started collecting cell debris from the vagina&cervix and observed malignant cells under a microscope.[43, 44]Later in 1943, Dr Georgiou Papanikolaoumade significant efforts in proving the efficacy of pap smears in cervical cytopathology.[45, 46] Hepublished hispaper *Diagnosis of Uterine Cancer by the Vaginal Smear*after which he was known as the INVENTOR of PAP SMEAR/PAP TEST.[47]Since then, using pap smears for screening cervical cancer has saved the lives of millions of women. [48-50]

Method of sample collection (Fig.10):

1) A sterile Cuscospeculum is used for clear visualization of the cervix
2) The cells from the ectocervix are scraped using Ayer's spatula
3) A small cyto-brush is used to take a sample from the endocervix.
4) Clean, dry glass slides to prepare smears
5) 95%Alcohol for immediate fixation of smears.[51, 52]

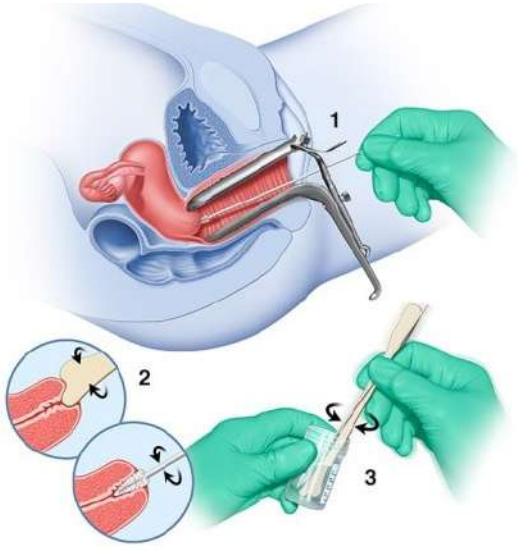


Figure 9: Method of pap smear collection

Table.2: CPAP staining procedure[53]:

CPAP	
95%alcohol fixation	15mins
95% alcohol	10dips
80% alcohol	10dips
70% alcohol	10dips
50% alcohol	10 dips
Tap water	10dips
Harris haematoxylin	2minutes
Scotts tap water	3-5mins
50% alcohol	10dips
70% alcohol	10dips
80% alcohol	10dips
95% alcohol	10dips
Orange G -6	1minute
95% alcohol	10dips
95% alcohol	10dips
Eosin Azure	10 minutes
95% alcohol	10 dips
95% alcohol	10 dips
100% alcohol	10 dips
Xylene	10 dips
DPX mount cover slip	

Despite being cumbersome & costly, Conventional Pap smears have been the widely used screening test.[54-56] Toovercomethese demerits, several modified staining techniques were innovated out of which, Rehydrated Air Dried Pap Smears(RADPS)& Rapid Economical Acetic acid Pap smears(REAP) had a better outcome in lines of cost-effectiveness& less alcohol usage. [57-60]

Table.3: Staining procedure of Modified PAP (RADPS, REAP) and CPAP.

RADPS	REAP	CPAP
Air dried for 30-120minutes	95%alcohol for fixation	95%alcohol for fixation
95% alcohol10dips	1% Acetic acid 10 dips	95% alcohol 10dips
80% alcohol 10dips	-	80% alcohol 10dips
70% alcohol 10dips	--	70% alcohol 10dips
50% alcohol 10 dips	-	50% alcohol 10 dips
Tap water 10dips	-	Tap water 10dips
Harris haematoxylin2minutes	Harris haematoxylin pre-Heated 60degrees 10 dips	Harris haematoxylin2minutes
Scotts tap water 3-5mins	Tap water 10 dips	Scotts tap water 3-5mins
50% alcohol 10dips	-	50% alcohol 10dips
70% alcohol 10dips	-	70% alcohol 10dips
80% alcohol 10dips	1% Acetic acid 10 dips	80% alcohol 10dips
95% alcohol 10dips	-	95% alcohol 10dips
Orange G -6 for 1minute	OG 6 10 dips	Orange G -6 for 1minute
95% alcohol 10 dips	-	95% alcohol 10 dips
95% alcohol 10 dips	1% Acetic acid 10 dips	95% alcohol 10 dips
Eosin Azure 10 minutes	EA 10 dips	Eosin Azure 10 minutes
95% alcohol 40 dips	1% Acetic acid 10 dips	95% alcohol 40 dips
95% alcohol 40 dips	-	95% alcohol 40 dips
100% alcohol 10 dips	Methanol 10 dips	100% alcohol 10 dips
Xylene 10 dips	Xylene 10 dips	Xylene 10 dips
DPX mount cover slip	DPX mount cover slip	DPX mount cover slip

REPORTING CERVICAL CYTOLOGY:

The Bethesda System of Cervical Cytology has aided in maintaining standardized terminology & uniformity in reporting cervical pap smears worldwide.[61-63]

The five components this system has taken for reporting cervical pap smears are (Fig.11):

1) Specimen type

2) Adequacy

3) General category

4) Interpretation

5) Adjunctive testing

Specimen adequacy

- Satisfactory for evaluation
- Satisfactory for evaluation but has limition(s) (e.g. limited by excess blood, drying artefact)
- Unsatisfactory (e.g. lack of cells)

General categorisation

- Within normal limits, benign cellular changes, epithelial cell abnormalities

Descriptive diagnosis

(1) Benign cellular changes
This includes (a) infections or (b) reactive changes not associated with any increased risk of cervical intraepithelial neoplasia or cancer

(2) Epithelial cellular abnormalities
Squamous: ASCUS, LSIL, HSIL, invasive cancer
Glandular: AGUS, AIS, adenocarcinoma

ASCUS = Atypical squamous cells of undetermined significance
LSIL = Low-grade squamous intraepithelial lesion
HSIL = High-grade squamous intraepithelial lesion
AGUS = Atypical glandular cells of undetermined significance
AIS = Adenocarcinoma in situ

Figure 10: The Bethesda System for cervical cytology

Cyto-morphology in different lesions of the cervix is as follows:[64, 65]

NILM (Negative for Intraepithelial lesion or Malignancy):

This category includes:

. Presence of infective organisms (candida, trichomonas-vaginalis, actinomyces)

. Reactive cellular changes

. Non-neoplastic findings with adequate squamous cells

. Atrophic changes.

ASC-US

Atypical parakeratosis.

Atypical atrophic squamous cells or koilocytosis

Atypia due to repair or inadequate sample.

ASC-H

For smears specifically suspicious for HSIL. In this condition, squamous cells are small, immature withirregular membranes, hyperchromatic nuclei & with atypia.

LSIL:

The squamous cells in Low-gradeSquamousIntra-Epithelial Lesions are large and equal to the size of normal superficial/intermediate squamous cells.

The Nuclei show atypia, enlargement with a slight irregular contour. They are hyperchromatic with coarse chromatin. Koilocytes are also seen.

HSIL:

The cells in High-grade Squamous Intraepithelial Lesions are small, parabasal-sized cells arranged singly or in groups(syncytium). These cells show marked nuclear atypia with enlarged, hyperchromatic nuclei& marked coarse chromatin.

Squamous cell carcinoma:

All the features of HSIL along with tumor diathesis, irregularly distributed chromatin, presence of macro-nucleoli, and tadpole/fiber cells in the keratinizing variant.

Adenocarcinoma:

Cells are arranged in a glandular pattern with central Lumina and peripheral nuclei. Cells are pleomorphic and show nuclear crowding with loss of polarity and hyperchromatic nuclei with clumped chromatin.

4) MATERIALS & METHODS:

Source of data:

A one-year prospective study from October 2022 to October 2023 was done on patients who visited the Gynecology outpatient department of KLE DR. PRABHAKAR KORE Hospital, Belagavi.

Study design: Observational study

Inclusion Criteria: 18-75yrs women

Exclusion Criteria:

Menstruating and pregnant

2. Inadequate material on smear

3. Patient not consenting to the procedure

Sample size:250

(G POWER software by considering effect size to be 0.25, beta=0.95, alpha=0.05, 1-beta=power of the test=0.99 & degree of freedom=2, the total sample size will be=248)

Data Analysis: All samples were analyzed using SPSS software version 23.Acomparison among 3 tests was done using the Friedman test.

P value < 0.05 was considered significant.

Ethical clearance:

The present study was approved by Jawaharlal Nehru Medical College's Institutional Ethics Committee on Human Subjects Research.

Methodology:

Informed consent of the patient was taken after briefing about the procedure. Three sets of cervical smears were collected from each patient. Of them, 2 slides were immediately fixed in

95% Ethanol. One slide was allowed to dry & then rehydrated with Normal saline for 30 seconds withinhrs. From the collection of the sample & then stained. The wet fixed slides are stained by CPAP & REAP techniques. The Air-dried smearswere stained by the RADPS technique.

The age, cytological diagnosis, cellularity, Individual Cyto-morphological Features score, Total score, and Quality index in 3 different staining procedures were reported, compared &analysed.

The cyto-morphological features taken for comparison were:

1)Overall staining	Poor	1
	Average	2
	Good	3
2)Cell morphology	Poorly preserved	1
	Moderately preserved	2
	Well preserved	3
3)Cytoplasmic details	Unsatisfactory	1
	Sub optimal	2

	Optimal	3
4)Cell borders	Distinct	1
	Indistinct	2
5)Nuclear characteristics	Smudgy chromatin	1
	Moderately crisp	2
	Crisp	3
6)Nuclear borders	Indistinct	1
	Distinct	2
7)Background	Haemorrhagic	1
	Clear	2
8) Air-drying artefacts	>50%	1
	<50%	2
	0%	3
Total score:		21

Quality Index= Obtained score÷Total score

RESULTS:

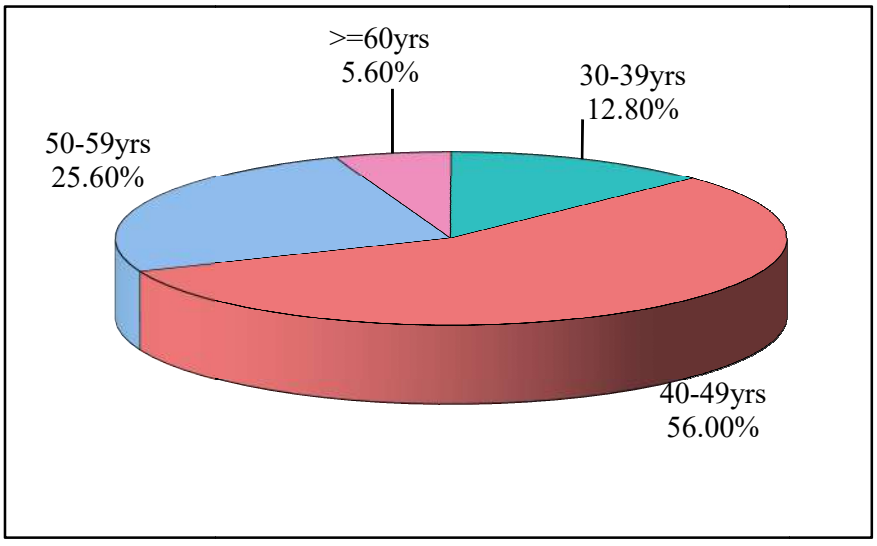
In our study, the sample size was 250. Results were analyzed with status to Age, Diagnosis, Cellularity, Cyto-morphological features, Total score & Quality Index.

Based on age, all 250 patients were divided into 4 groups. In this study, the youngest patient was 30yrs&the oldest was 66yrs. 140(56%) of total cases were in the age group of 40-49 years.

Table 4: Age-wise distribution

Age group	Number	Percentage
30-39yrs	32	12.80
40-49yrs	140	56.00
50-59yrs	64	25.60
>=60yrs	14	5.60
Total	250	100.00
Mean	47.17	
SD	6.84	

Figure 12: Age-wise distribution



- In the present study, the lesions included were:

1)Negative for Intraepithelial Lesion or Malignancy (NILM)

2) Negative for Intraepithelial Lesion or Malignancy (NILM) - Inflammatory smear

3) Negative for Intraepithelial Lesion or Malignancy (NILM)-Atrophy

4) Negative for Intraepithelial Lesion or Malignancy (NILM)-Candidiasis

5) High-grade Squamous Intraepithelial Lesion (HSIL)

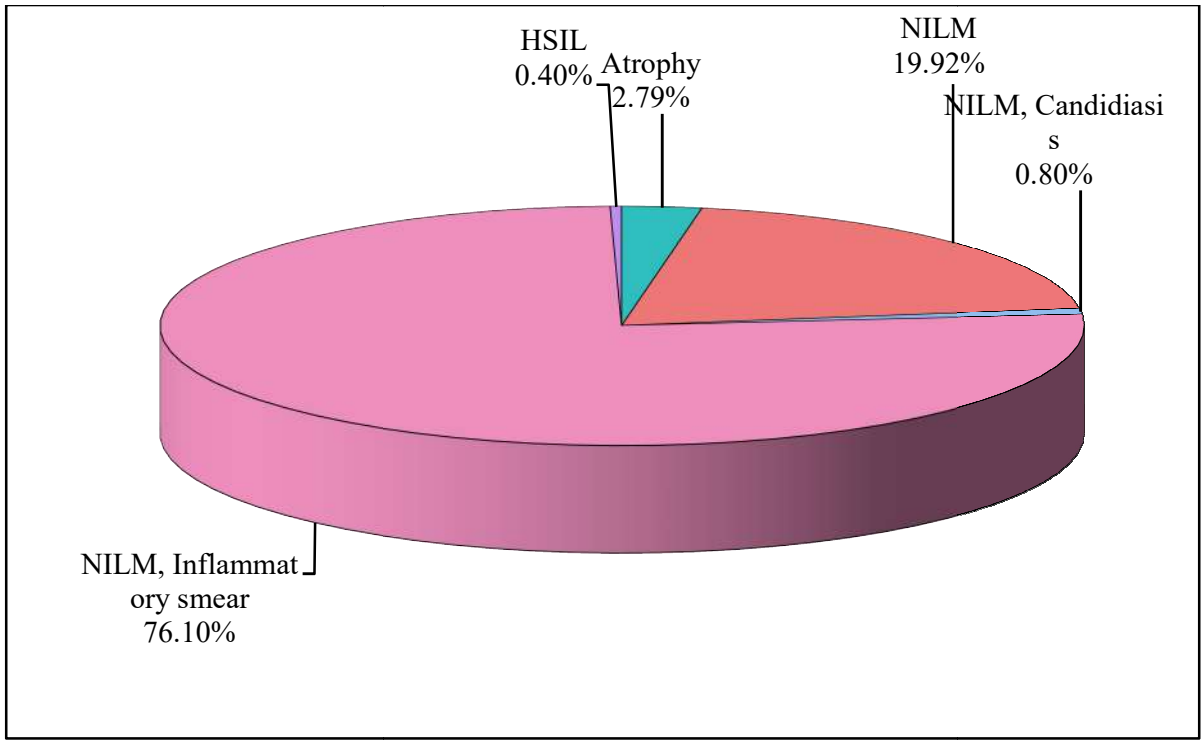
Out of 250 cases, 191(76.40%) were diagnosed as NILM-Inflammatory smears.

1 case (0.4%) was diagnosed as HSIL on cytology which correlated with histopathology examination.

Table 5: Diagnosis wise distribution

Diagnosis	Number	Percentage
Atrophy	7	2.80
NILM	50	19.92
NILM, Candidiasis	2	0.80
NILM, Inflammatory smear	191	76.40
HSIL	1	0.4
Total	250	100.00

Figure 13: Diagnosis-wise distribution



Out of 250 samples,124(49.6%)ofCPAP smears showed high cellularity as compared to RADPS, and REAP stained smears which showed moderate cellularity in 108(43.2%), and 158(63.2%) cases respectively. The p-value of 0.0001 was statistically significant.

Table 6: Comparison of three techniques (CPAP,RADPS,REAP) with the status of Cellularity by Friedman's test

Options	CPAP	%	RADPS	%	REAP	%	Z-value	p-value	
Low	29	11.60	109	43.60	40	16.00	146.9481	0.0001*	
Moderate	97	38.80	108	43.20	158	63.20			
High	124	49.60	33	13.20	52	20.80			
Total	250	100.00	250	100.0	250	100.0			

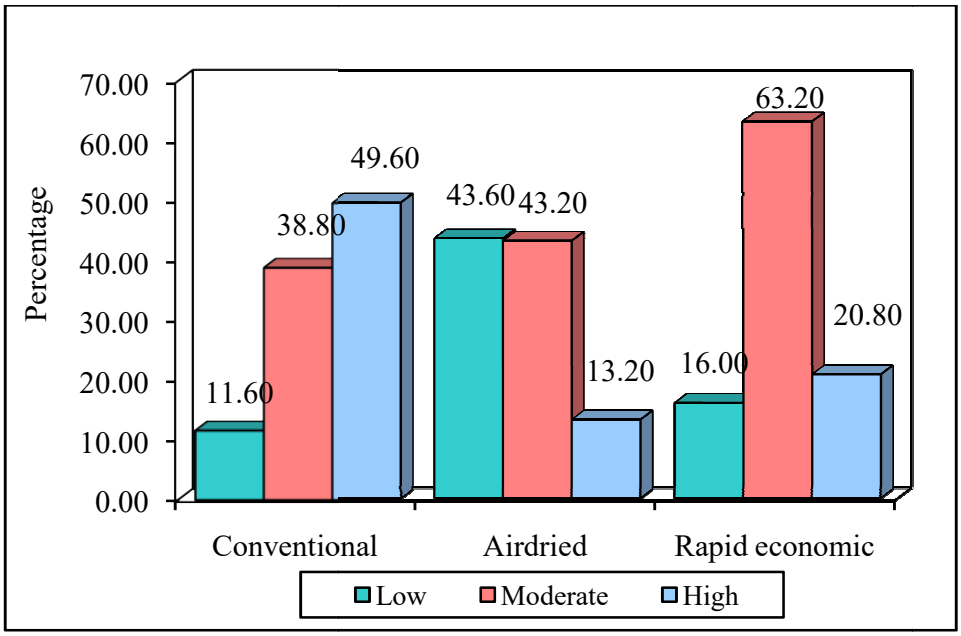
*p<0.05

Table 7: Pair-wise comparison of three techniques (CPAP, RADPS, REAP) with the status of Cellularity by Wilcoxon matched pairs test

Conventional vs Air-dried		Conventional vs Rapid economic		Air-dried vs. rapid economic	
Z-value	p-value	Z-value	p-value	Z-value	p-value
9.0508	0.0001*	6.4162	0.0001*	7.2828	0.0001*

*p<0.05

Figure 14: Comparison of three techniques (CPAP, RADPS, REAP) with the status of Cellularity



- Comparison of three techniques (CPAP, RADPS, REAP) with the status of Cyto-morphological components by Friedman's test

- The Cyto-morphological features included were,
    1) Overall staining
    2) Cell morphology
    3) Cell borders
    4) Cytoplasmic details
    5) Nuclear characteristics
    6) Nuclear borders
    7) Background
    8) Air-drying artifacts

    - Good overall staining - score 3wasseen in 199(79.6%) cases of RADPS.Average staining -score 2 was observed in161(64.4%)CPAP&150(60%)REAP smears.
    - Well preserved cell morphology was seen in 192(76.80%) RADPS,118(47.2%) CPAP & (49) 19.6% REAP smears.
    - Distinct cell borders were found in 236(94.40%) cases of CPAP which was followed by 227(90.80%) cases of RADPS.
    - Optimal cytoplasmic details in 153(61.20%) RADPS cases. Suboptimal details in 218(87%) CPAP &155(62%) REAP smears.
    - Crisp nuclear characteristics in 164(65.60%) cases followed by 103(41.2%) REAP &98(39.2%) CPAP.
    - Distinct nuclear borders were seen in 224(89.60%) RADPS, 138(55.2%) CPAP &94 (37.6%) REAP smears.
    - Clear background in 230(92.0%) cases of RADPS. 139(55.6%) REAP &41(16.40%) CPAP smears.
    - Air Drying Artifacts (ADA) were least in 170(68.0%) of REAP smears& in RADPS,<50%artifacts were seen in98(39.2%)&>50% artifacts were seen in 32(12.8%) smears, 31(12.4%) CPAP smears showed >50% ADA.
    - The p-value of 0.001 was obtained in all the cyto-morphological features and was statistically significant.

Pair-wise comparison of the 3 techniques was statistically significant for all the cyto-morphological components except for

- Air drying artifacts in CPAP vs RADPS (p-value 0.1411) and REAP vs RADPS (p-value 0.0112).
- Cell borders in RADPS vs REAP (p-value 0.1846).

Table 8: Comparison of three techniques (CPAP, RADPS, REAP) with the status of Cyto-morphological components by Friedman's test

Components	Options	CPAP	%	RADPS	%	REAP	%	Z-value	p-value
Overall staining	1	37	14.80	19	7.60	60	24.00	301.3765	0.0001*
	2	161	64.40	32	12.80	150	60.00		
	3	52	20.80	199	79.60	40	16.00		
Cell morphology	1	5	2.0	16	6.00	8	3.20	386.1056	0.0001*
	2	127	50.80	43	17.20	193	77.20		
	3	118	47.20	192	76.80	49	19.60		
Cell borders	1	14	5.60	23	9.20	49	19.60	348.0254	0.0001*
	2	236	94.40	227	90.80	201	80.40		
Cytoplasmic details	1	9	3.60	42	16.0	91	36.40	369.7843	0.0001*
	2	218	87.00	55	22.00	155	62.0		
	3	23	9.20	153	61.20	4	1.60		

Nuclear characteristics	1	2	0.80	33	13.20	7	2.80	278.9653	0.0001*
	2	150	60.00	53	21.20	140	56.00		
	3	98	39.20	164	65.60	103	41.20		
Nuclear Borders	1	112	44.80	26	10.40	156	62.40	233.4701	0.0001*
	2	138	55.20	224	89.60	94	37.60		
Background	1	209	83.60	20	8.00	111	44.40	227.1441	0.0001*
	2	41	16.40	230	92.00	139	55.60		
Air drying artifacts	1	31	12.40	32	12.80	46	18.40	20.4861	0.0001*
	2	77	30.80	98	39.20	34	13.60		
	3	142	56.80	120	48.00	170	68.00		
	Total	250	100.0	250	100.0	250	100.0		

*$p<0.05$

Table 9: Pair-wise comparison of three techniques (CPAP, RADPS, REAP) with the status of Cyto-morphological components by Wilcoxon matched pairs test

Components	CPAP vs RADPS		CPAP vs REAP		REAP vs RADPS	
	Z-value	p-value	Z-value	p-value	Z-value	p-value
Overall staining	11.7742	0.0001*	10.2851	0.0001*	7.3738	0.0001*
Cell morphology	13.5692	0.0001*	9.9544	0.0001*	10.9029	0.0001*
Cell borders	12.0797	0.0001*	11.7065	0.0001*	1.3267	0.1846
Cytoplasmic details	13.3744	0.0001*	7.7768	0.0001*	11.3743	0.0001*
Nuclear characteristic	12.1082	0.0001*	10.1737	0.0001*	7.1061	0.0001*
Nuclear Borders	11.5798	0.0001*	4.2949	0.0001*	9.3333	0.0001*
Background	11.6764	0.0001*	6.5188	0.0001*	7.7091	0.0001*
Air drying artifacts	5.5109	0.0001*	1.4717	0.1411	2.5360	0.0112

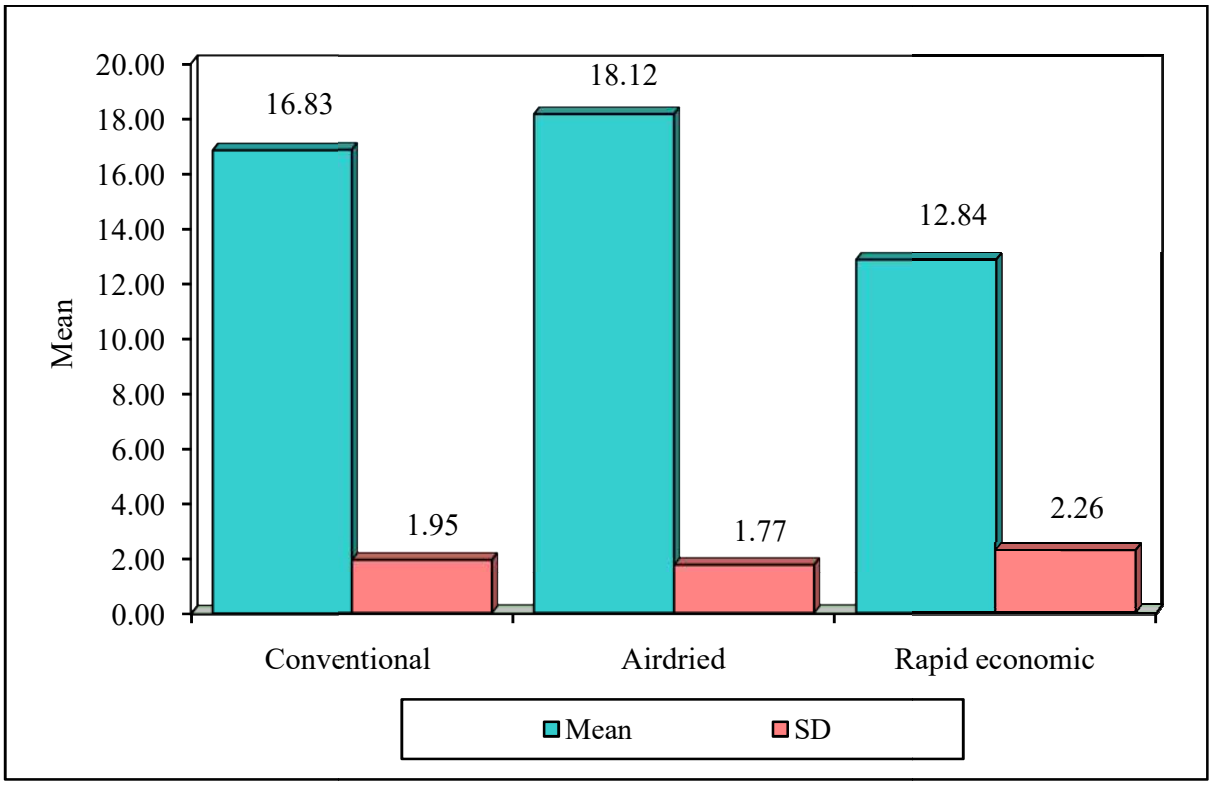
On comparison of three techniques with total Cyto-morphological scores by paired t-test, Total Scores were found to be highest in RADPS smears with the mean being 18.12. The mean total score in CPAP& REAP smears was 16.83 & 12.84 respectively. The p-value was 0.0001, statistically significant.

Table 10: Comparison of three techniques (CPAP, RADPS, REAP) with total Cyto-morphological scores by paired t-test

Techniques	Mean	SD	Mean Diff.	SD Diff.	t-value	p-value
Conventional	16.83	1.95				
Air-dried	18.12	1.77	-1.28	2.77	-7.3248	0.0001*
Conventional	16.83	1.95				
Rapid economic	12.84	2.26	3.99	3.43	18.3753	0.0001*
Airdried	18.12	1.77				
Rapid economic	12.84	2.26	5.28	2.65	31.5034	0.0001*

- *p<0.05

Figure 15: Comparison of three techniques (CPAP, RADPS, REAP) with total Cyto-morphologicalscores:



The Quality Index (QI) was also calculated by applying the formula

QI= Obtained score ÷ Total score

The QI was highest for RADPS with the mean being 0.86, SD 0.08., followed by a CPAP mean of 0.80, SD 0.09. & REAP with mean as 0.61, SD 0.11.

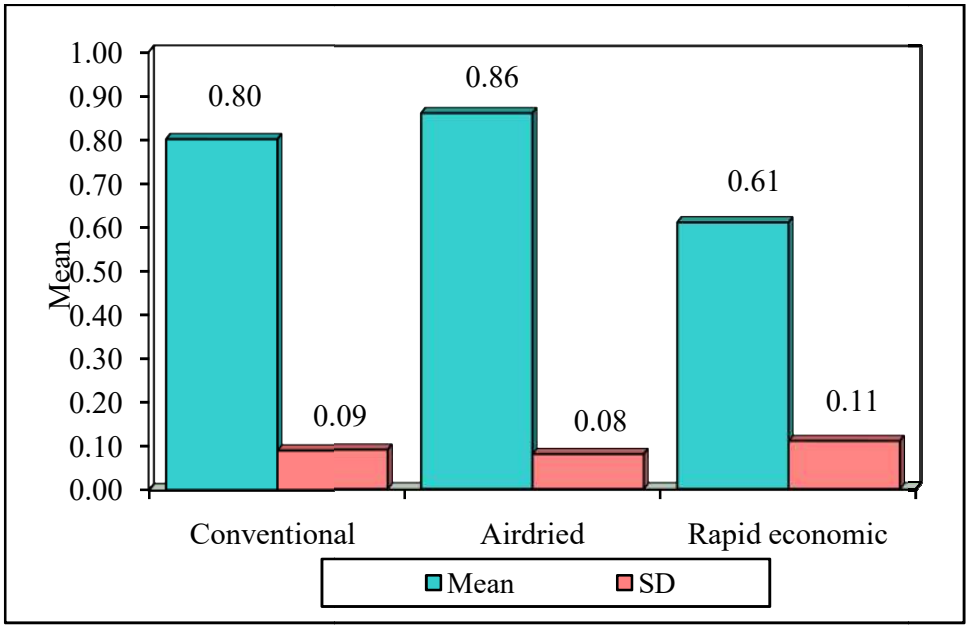
p-value was 0.0001, statistically significant.

Table 11: Comparison of three techniques (CPAP, RADPS, REAP) with Quality Index scores by paired t-test

Techniques	Mean	SD	Mean Diff.	SD Diff.	t-value	p-value
CPAP	0.80	0.09				
RADPS	0.86	0.08	-0.06	0.13	-7.3242	0.0001*
CPAP	0.80	0.09				
REAP	0.61	0.11	0.19	0.16	18.3760	0.0001*
RADPS	0.86	0.08				
REAP	0.61	0.11	0.25	0.13	31.4942	0.0001*

*$p<0.05$

Figure 16: Comparison of three techniques (CPAP, RADPS, REAP) with Quality Index scores



PHOTOGRAPHS:

Photo 1: High cellularity smear (CPAP stain, 40x)

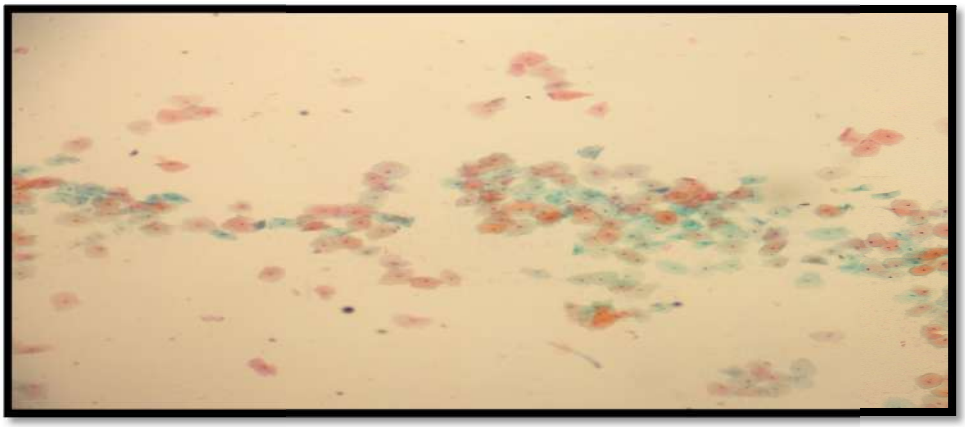


Photo 2: Moderate cellularity (REAP stain, 40x)

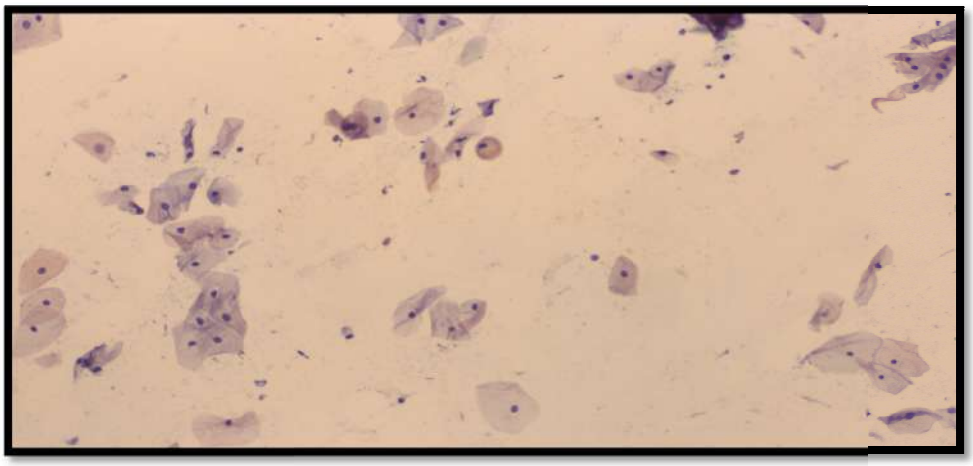


Photo 3: Low cellularity(RADPS stain, 40x)

Photo 4: Poorly preserved cell morphology (CPAP stain, 100x)

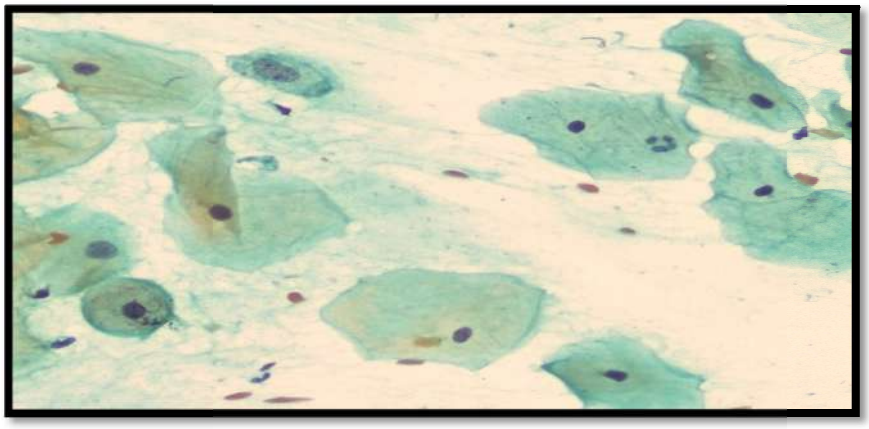


Photo 5: Moderately preserved cell morphology (REAP stain, 100x)

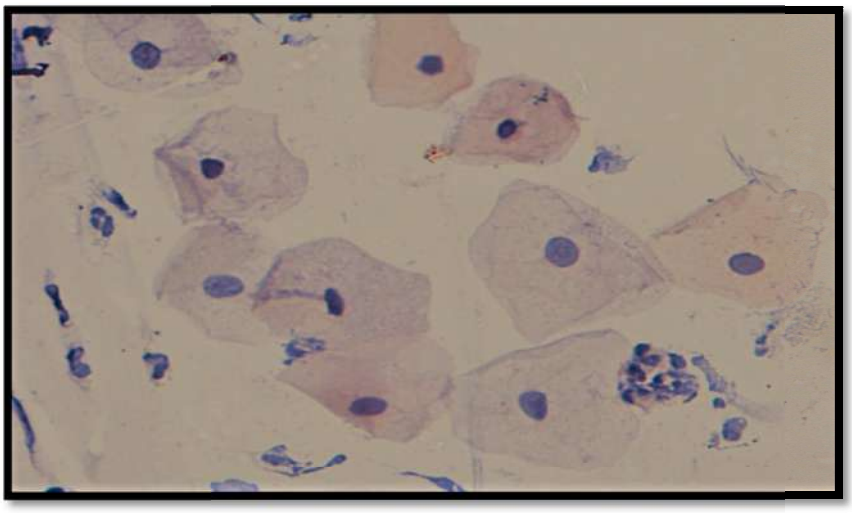


Photo 6: Well preserved cell morphology in RADPS in 100x magnification

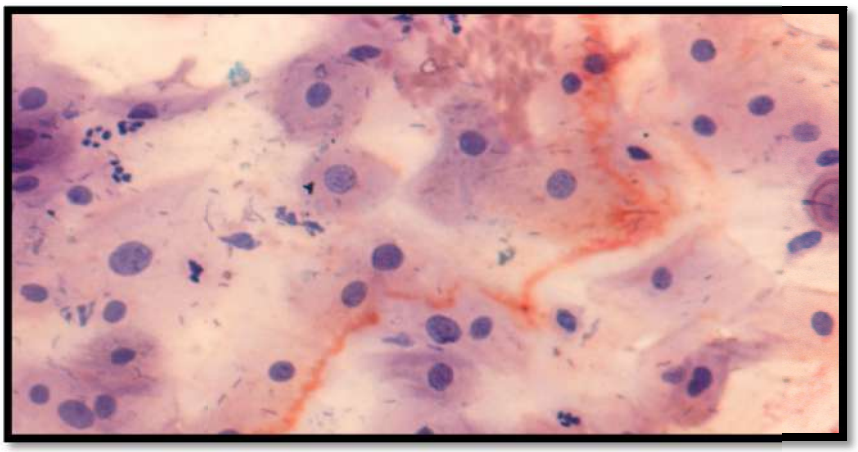


Photo 7: Indistinct cell borders (CPAP stain,100x)

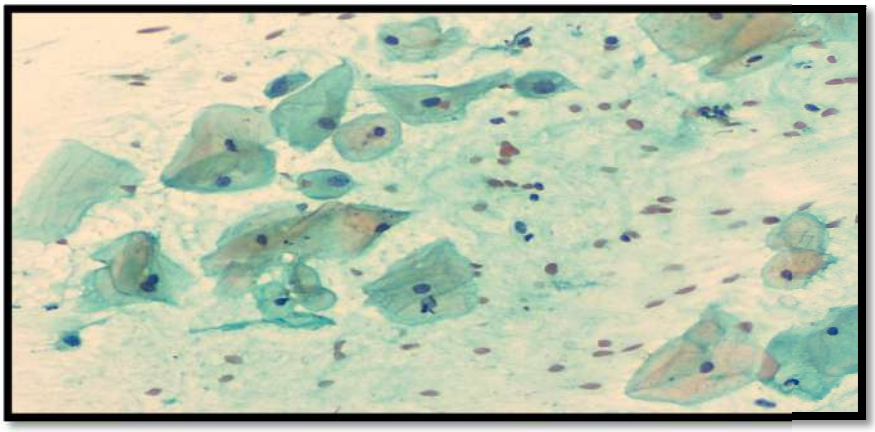


Photo 8: Indistinct cell borders (REAP stain,100x)

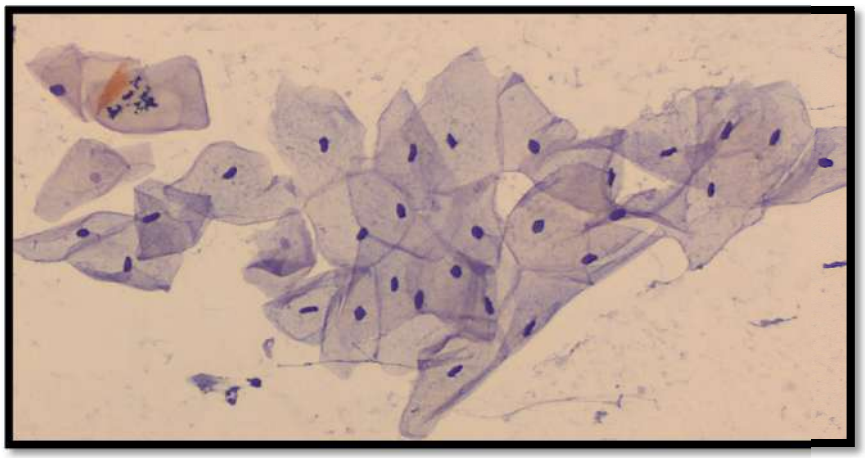


Photo 9: Distinct Cell borders (RADPS stain, 10x)

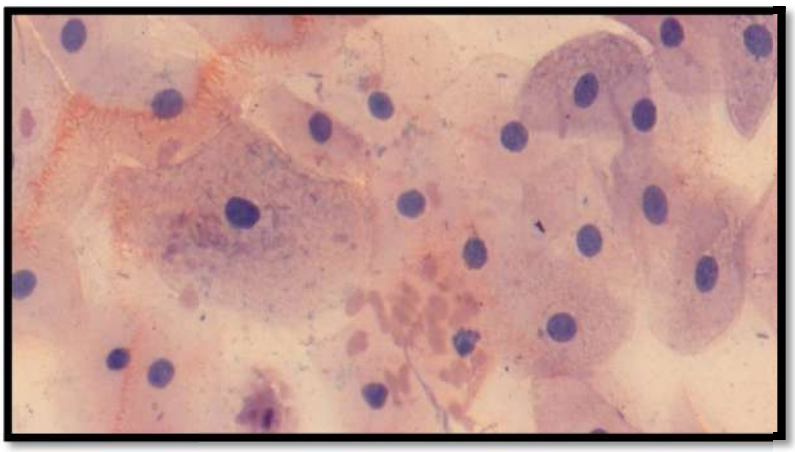


Photo 10: Unsatisfactory Cytoplasmic details (CPAP stain,20x)

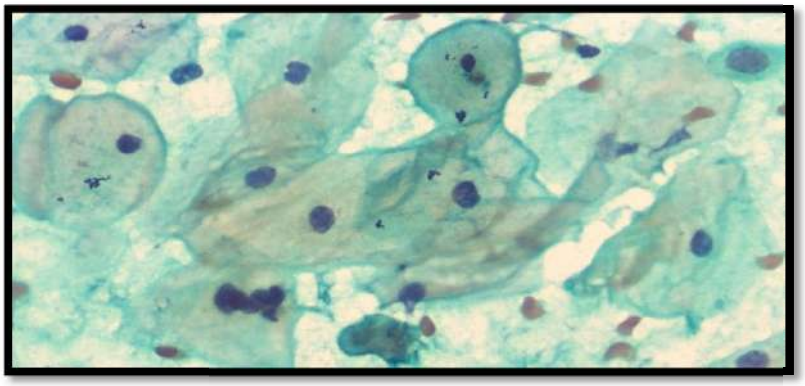


Photo 11: Sub optimal cytoplasmic details (REAP stain, 20x)

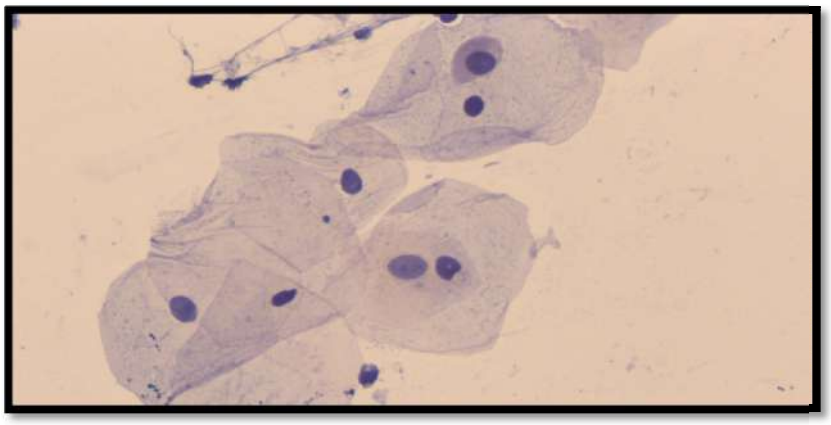


Photo 12: Optimal cytoplasmic details (RADPS stain, 20x)

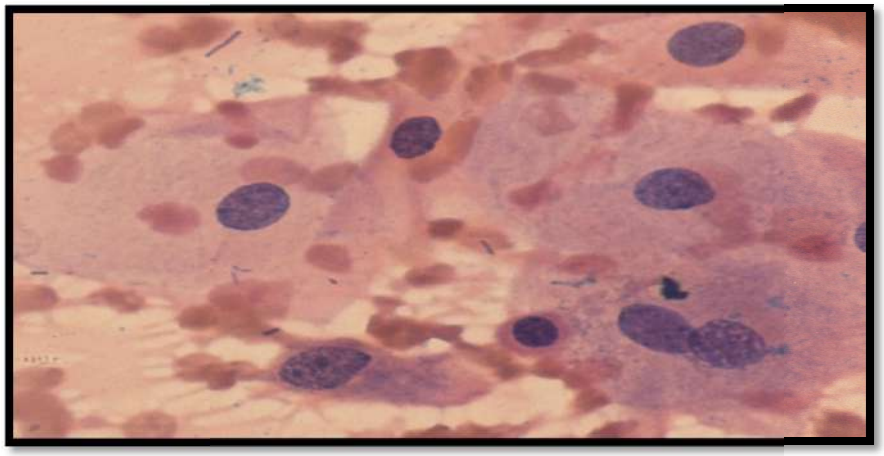


Photo 13: Smudgy Nuclear Characteristics (CPAP stain, 40x)

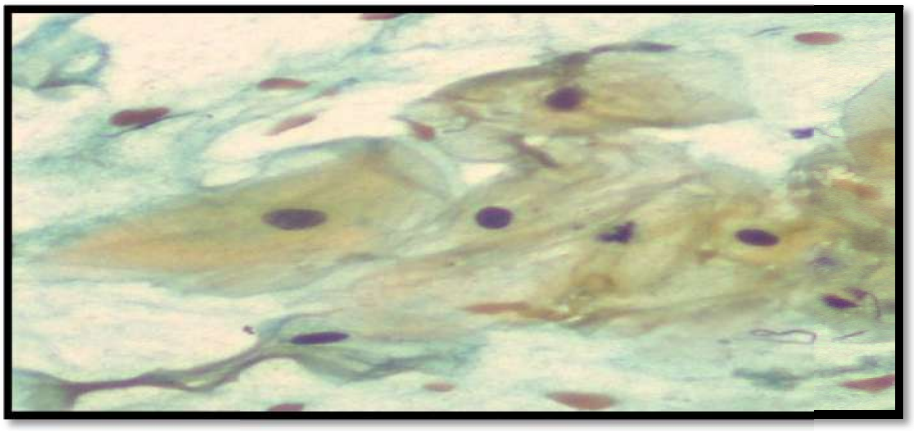


Photo 14: Moderately crisp nuclear characteristics (REAP stain,40x)

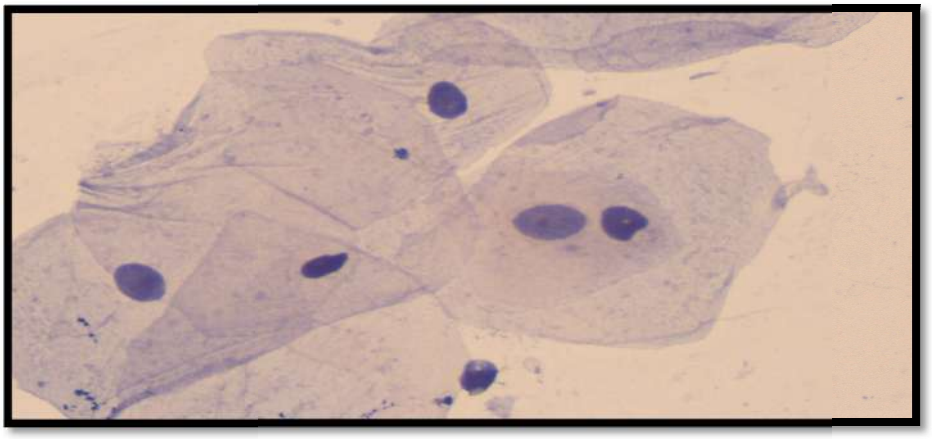


Photo 15: Crisp nuclear characteristics (RADPS stain, 40x)

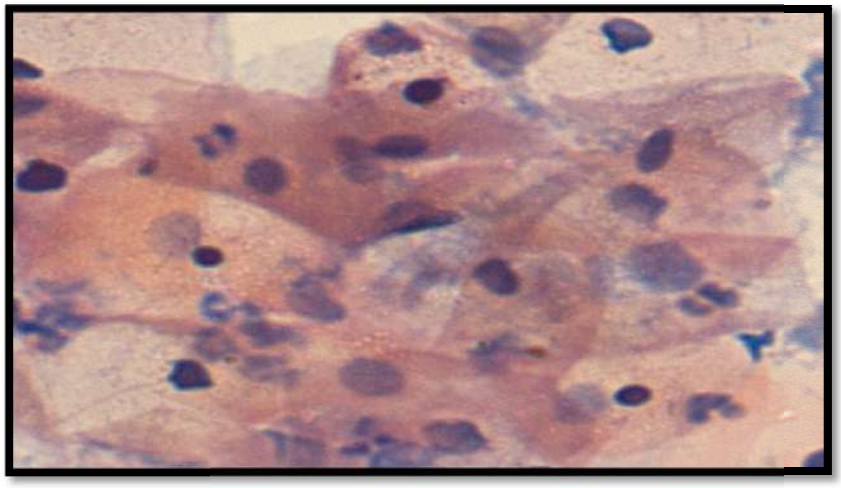


Photo 16: Indistinct Nuclear borders (CPAP stain, 40x)

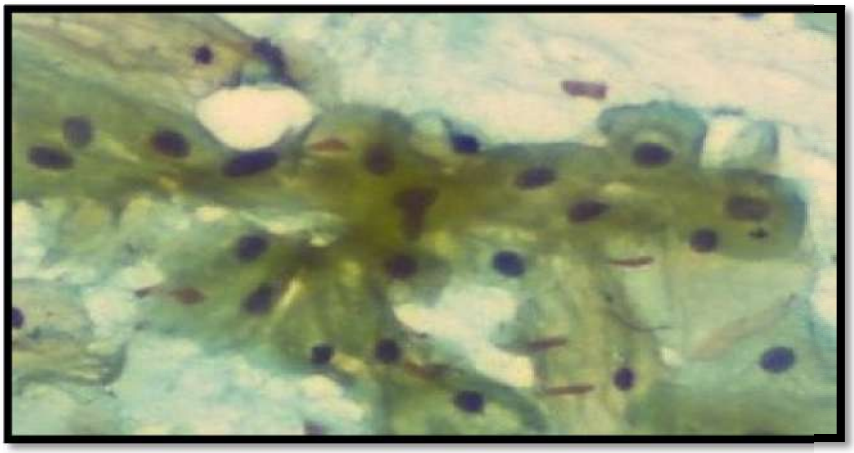


Photo 17: Indistinct Nuclear borders (REAP stain, 40x)

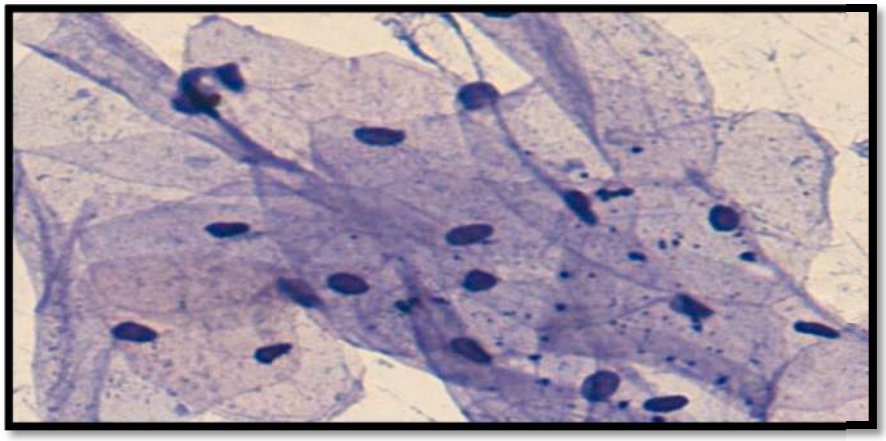


Photo 18: Distinct Nuclear borders (RADPS stain, 40x)

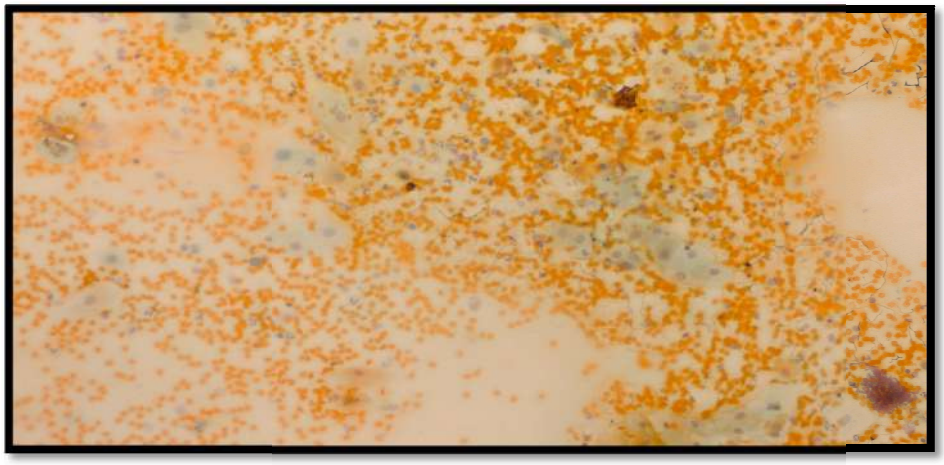


Photo 19: Hemorrhagic background (CPAP stain, 4x)

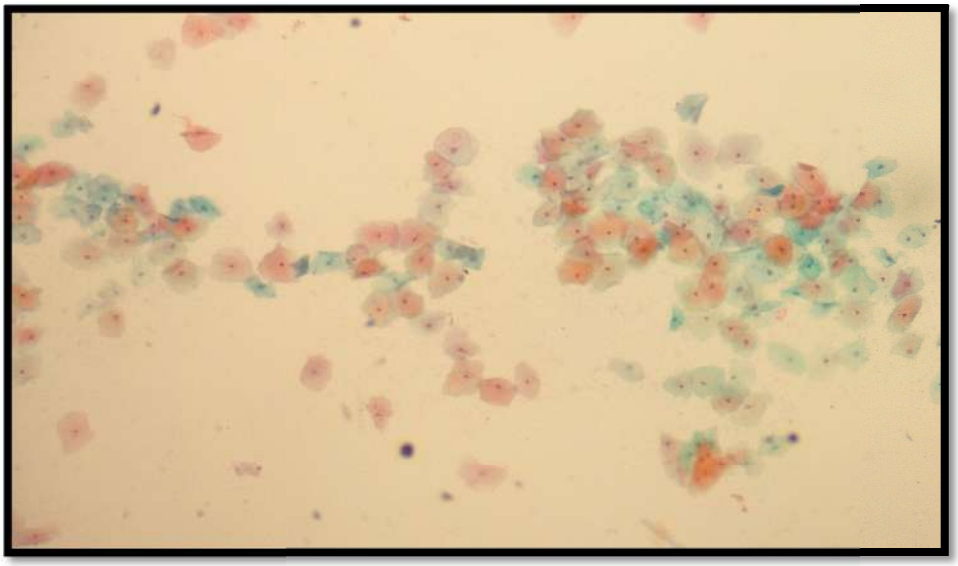


Photo 20: Clear background (REAP stain, 4x)

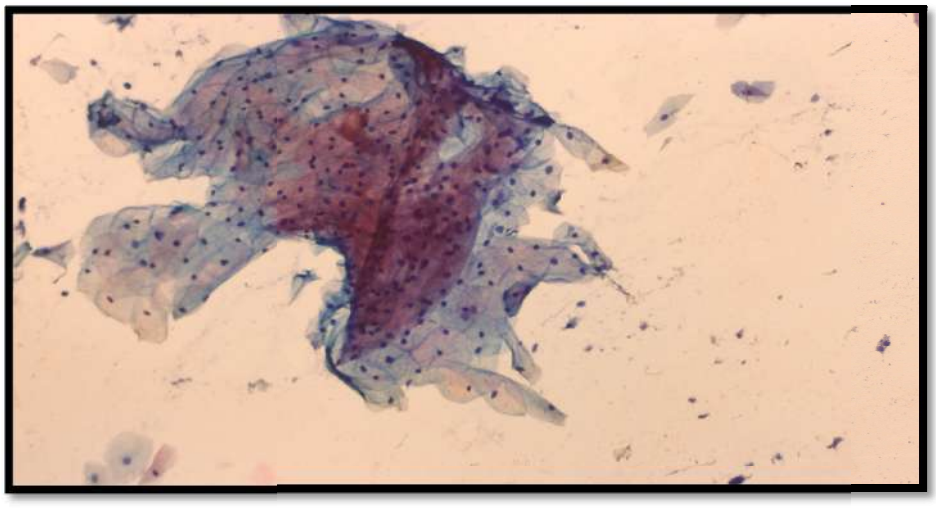


Photo 21: Clear background (RADPS stain, 4x)

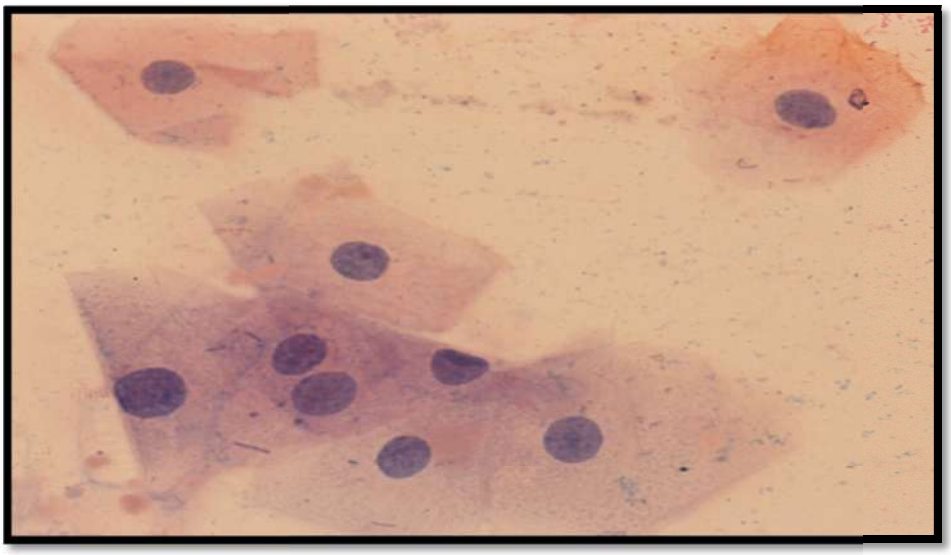


Photo 22: No air drying artifacts (CPAP stain, 20x)

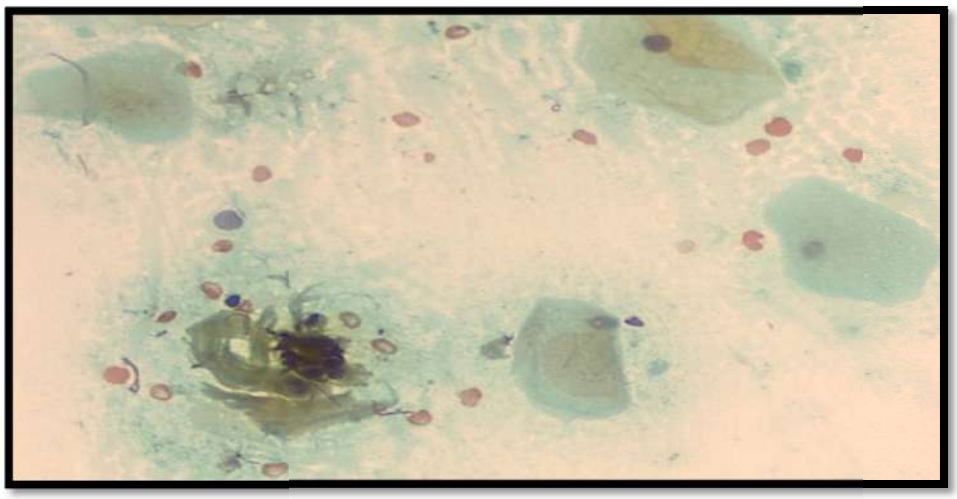


Figure 23: Few air drying artifacts (REAP stain, 20x)

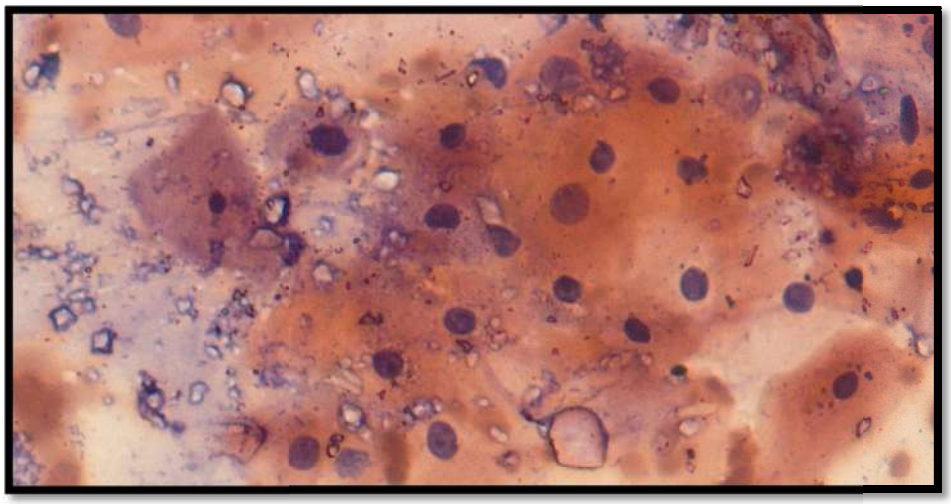


Photo 24: Air drying artifacts (RADPS stain, 20x)

DISCUSSION:

PAP smear is still considered a very simple as well as effective technique used for mass screening of cancer cervix. [66] Although the pap smear is widely used, in healthcare facilities like PHCs where resources are limited, pap smear collection, fixation & staining by the CPAP method may not be done properlyleadingto an incorrect diagnosis so, Proper training of medical & technical staff in smear collection, fixation & staining is essential.[67,68]Theconventional method of PAP staining has been inuse since 1954and since then, many modifications have been made according to the laboratory needs & requirements as CPAP requires alcohol in high quantities &longer duration for the stainingprocedure. [69,70] Some of the commonly studied modified staining techniques include REAP & RADPS.

REAP uses1%Acetic acid, a reagent that is less priced than alcohol & the procedure is time-saving, RADPSprovidesa clear background.[71, 72] Studies done by Kamble et al, Kapse et al, Kusuma et al, Garima et al, Jaiwong et al, Izhar et al, and Deshpande et al, found RADPS & REAP as better alternatives for CPAP.[73-79]

However, none of the above-mentioned studies, comparison of all the 3 staining techniques was done. In the present study, we analysed cyto-morphological features in the cytological pap smears stained by three different techniques namely, CPAP, RADPS& REAP.

Age-wise distribution in our study showed thatthe youngest patient was 30yrs&the oldest was 66 years old. (140) 56% were between 40-49yrs age group. This correlates with Jha et al study where the mean age was 45.5 years.[80]Out of 250 cases,191(76.40%) were diagnosed as NILM-Inflammatory smears. Total 249 cases were NILM& one case was diagnosed as HSIL.A similar observation was noted in the Jha et al study where 199 of 200 smears were cases of NILM& only 1 case (0.4%) was of HSIL.

As RADPSdo not require immediate fixation, the smears are collected leisurely with no hurry at the same time, air-drying causes RBC lysis, and also enhances adhesion of the smear to the slides, a high cellularity is expected when the smears are stained using this technique which was noted in Kapse et al study where RADPS smears were highly cellular in comparison to CPAP but in our study, Highly cellular smears were noted in 124 cases (49.6%) of CPAP withRADPSand REAP-stained smears showing moderate cellularity in 108(43.2%), and 158(63.2%) cases respectively which was statistically significant($p<0.0001$). However, studies conducted by several others like Gupta et al, Jha et al &Jaiwong et al

showed no suchstatistical significance. This observation noted in our study regarding cellularity may be attributed to split smear preparation that might have affected cellularity. [77, 80-84]

TheCyto-morphological features considered in our study were Over-all staining, Cell morphology, Cell borders, Cytoplasmic details, nuclear characteristics, nuclear borders,background&air-drying artifacts.

In ourstudy, overall staining with score-3 i.e. good staining was found in 79.60% of RADPS smears Whereas, 64.4% of CPAP & 60% of REAP cases showed moderate staining with a score of2. These values were comparable to a previous study by Gupta el al where statistically significant good overall staining was observed in RADPSsmears.[84] This good overall staining in RADPS smears can be attributed to enhanced &better adhesion of the cells to the slide.

Cell morphology was well preserved in 76.8% of RADPS smears followed by 47.2% of CPAP with REAP smears having the least number of well-preserved smears (19.2%). A similar observation was made by Izhar et al where REAP smears had the least number of cytologicallywell preserved smears. [78]This preservation of cell morphology observed in the RADPS smears may be due to the step of rehydration with Normal Saline used in this technique.

Distinct cell borders were seen in 94.4%of CPAP followed by 90% in RADPS & 80.4% in REAP in the present study which was not a statistically significant finding& wassimilartofindings observed in Zare et al study. [85]Kapse et al &Deshpande et al noted distinct cell borders in RADPS & REAP in comparison to CPAP which was statistically significantandcontradictory to the findings observed in our study &study done by Zare et al.[75, 79 85]Distinct cell borders are associated with a reduced number of air-dryingartifacts& cytolysis and in our study, CPAP showed fewerartifactsthat may have resultedina high number of cells showingdistinct cell borders.

The cytoplasmic details were superior in RADPS (61.2%) compared to CPAP (48.0%) in our study. Similar comparable results were found in studies by Sivaraman et al, Bahadur et al, and Zare et al.[85-87] However, Jaiwong et al & Gupta et al observed that both techniques had similar, satisfactory cytoplasmic staining& the findings in both studies were not statistically significant.[77, 84]The superior cytoplasmic staining seen in RADPS in our study

may be attributed to better penetration of stain &lysis of RBCs that removes obscuring factors 48% of REAP and33% of CPAP smears showed optimal cytoplasmic details in our study which contrasted the observations noted by Gachie et al.[71,77, 84] The decreased number of optimal cytoplasmic details noted in REAP smears may be due to factors like increased overlapping of cells & partial air drying that are associated with this technique.[88-90]

In RADPS smears, the cells appear flatter & nuclei appear shallower contributing to the characteristic crisp nuclear feature.[91,92] Thepresent studyshowed Crisp Nuclear Characteristics in 65.60% of RADPS followed by 41.20% REAP&39.20% CPAP smears which were similar to the results observed bythe Kamble et al study.[73]However, Jaiwong et al noted RADPS having a higher frequency of cells with hazy chromatin& Izhar et al and Deshpande et alnoted excellent nuclear features in REAP cases(50% & 98% respectively).[77-79]A study byZare et alshowed no such difference butthe Jha et al study showed crisp nuclear characteristics in both CPAP(100%)& RADPS (99%)smears.[85]

The present study showed distinct nuclear borders in 89.60% of RADPS smears similar to the Jha et al study which showed 93% & 96% in the Kapse et al study.[74, 80] 55.2% CPAP & 37.6% REAP smears showed distinct nuclear borders in our study which contrasted the results observed by Deshpande et al study (99% & 98% distinct nuclear borders in CPAP & REAP respectively).[78] Rehydration after air-drying causes the nucleus to appear distinct & larger leading toa greater number of RADPS smears showing distinct nuclear borders.

A clear background was seen in 92% of RADPS smears in our study which was similar to the observations made by Zare et al, Sivaraman et al, Gupta et al &Jaiwong et al.[77, 84, 85, 87]A haemorrhagicbackground was seen in 83.60% of CPAP smears in our study which was similar to the Jha et al study which noted 81% of smears with a haemorrhagic background.[80] Air-drying causes lysis of RBCs rendering a clean & clear background in RADPS smears.

CPAP smears showed < 50% (mild to absent) air-drying artifacts with scores2&3 in 87.60% of cases in our study with RADPS & REAP at 87.20% & 81.60% respectively whereas, >50% air-drying artifacts withscore seen in 18.4% REAP smears followed by 12.8% RADPS & 12.4% CPAPsmears whichwere not statistically significant. Similar findings were noted in Kusuma et al where mild air-drying artifacts were seen in both CPAP & REAP (100%) and same was also seen in the Jaiwong et al study.[75, 77]Inthe literature search, we noted that most of the studies analysedthe presence or absence of air-drying artifacts including

studies by Kamble et al &Kapse et al both of which concluded the increased presence of air-drying artifacts in CPAP smears in comparison to RADPS smears.[73, 74] In a study by Jha et al where air-drying artifacts were categorized as absent, mild &moderatea statistically significant difference was noted in air-drying artifacts between CPAP & RADPS which was contradictory to the findings in our study.[80]

In our study, the total time taken for REAP was the least i.e. 18-19 minutes where CPAP took a longer time of 30-35 minutes. The preparation cost per slidefor staining by CPAP & RADPS was Rs.48 whereas it costRs.8 for REAP which was 6 times less. Though the cost per liter of Ethyl alcohol was Rs.500and Acetic acid was Rs.600only 1% Acetic acid was used for staining in REAP technique. Thus, REAP was noted to be time & cost-effective.

Table 12: Time duration in three different techniques

	CPAP	RADPS	REAP
Fixation time	15mins	-	15mins
Staining time	15-20mins	15-20mins	3-4mins
Total time for the procedure	30-35mins	15-20mins	18-19mins

Table 13: Comparison of results in our study with other studies

	Present study	Other studies
High cellularity		
RADPS	43.2%	62% Kapse et al
CPAP	49.6%	48% Kapse et al
Good overall staining		
CPAP	85.2%	(90%) Kusuma et al
REAP	76%	(86%) Garima et al
Well preserved Cell Morphology		
CPAP	47.2%	91.6% Izhar et al
REAP	19.6%	33.5%Izhar et al
Distinct cell Borders		

RADPS	90%	97%Kapse et al
CPAP	94.4%	78% Kamble et al
REAP	80.4%	98.4% Deshpande et al
Optimal cytoplasmic details		
RADPS	61.2%	(65.8%) Zare et al
CPAP	33%	(81.7%) Gachie et al
REAP	48%	(86.7%) Gachie et al
Crisp Nuclear –characteristic		
RADPS	65.6%	(86.8%) Jha et al
CPAP	41.2%	(99.2%) Jha et al
REAP	39.2%	(50%) Izhar et al
Nuclear borders-Distinct		
RADPS	89.6%	(93%) Jha et al (96%) Kapse et al
CPAP	55.2%	(99%) Deshpande et al
REAP	37.6%	(98%) Deshpande et al
Hemorrhagic background		
RADPS	8%	(06%) Zare et al (07%) Kamble et al (37%) Jha et al (2%) Kapse et al

CPAP	83.6%	(81%) Jha et al (46%) Kamble et al (42%) Kapse et al
REAP	44%	(60%) Kusuma et al
Presence of Air DryingArtifacts		
RADPS	31% with <50%	(30%) Jha et al
CPAP	39.2%	(24%) Jha et al

LIMITATIONS:

1) Technical errors (delayed fixation, delayed rehydration) can influence the Cyto-morphological features and hence the results.
2) Inadequatematerial due to split sampling
3) Fading of stains if preserved for > 6 months
4) There can be inter-observer variability

CONCLUSION:

In our study, concerning cyto-morphological features, RADPS proved to be a better alternative to CPAP&REAP and was associated with good overall staining, well-preserved cell morphology, optimal cytoplasmic details, distinct nuclear borders&crisp nuclear characteristics with clear background. However, no statistical difference was noted about features like cell borders & air-drying artifacts.About timesaving&cost-effectiveprocedures, REAP proved to be better. Both the modified pap procedures were technician-friendly as they are easy to perform and can be used in developing countries like India where resources are limited & cancer prevalence is high.

SUMMARY:

A total number of 250 samples were collected and stained with 3 different techniques (CPAP, RADPS, REAP), and evaluated for the eight cyto-morphological features that included Overall staining, cell morphology, cytoplasmic details, cell borders, nuclear borders, nuclear characteristics, air-drying artifacts&background. Scoring for each cyto-morphological feature, total score &the Quality Index were statisticallyanalysed.

- RADPS smears showed a statistically significant difference with good overall staining, well-preserved Cell morphology, optimal cytoplasmic details, crisp nuclear characteristics, distinct nuclear borders, and clear background in comparison to CPAP & REAP.
- Distinct cell borders were found in more CPAP when compared to RADPS & REAP.
- Air drying artifacts were lesser in REAP smears when compared to CPAP & RADPS.
- Except for distinct cell borders &air-drying artifacts, all other 6 cyto-morphological features along with total score & Quality index were better in RADPS.
- REAP was proven better concerning time &cost-effectiveness.
- The present study showed better overall cyto-morphological results in RADPS-stained smears followed by CPAP & REAP and also REAP was both time &cost-effective. Therefore, both these modified techniques can be used as alternativestoCPAP in limited resource settings like PHCs.However, there isa need for more studies with a larger sample size comparing all 3 staining techniques to replace the currently used CPAP with these modified techniques.

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ANNEXURE I: Informed consent

Consent form format

KAHERs JNMC

BELAGAVI

INFORMED CONSENT FORM

"Comparison of cyto-morphological features in Conventional Papanicolaou Staining method with Rehydrated Air Dried Pap Smears (RADPS) and Rapid Economical acetic Acid Pap (REAP) Staining method in Cervical Smears: An observational study"

Name of Student/Principal Investigator:
Name of Guide/Co Investigators:

Objective: To compare the cyto-morphological features of cervical smears stained by Conventional Papanicolaou with that of modified Papanicolaou staining methods of RADPS, REAP

Introduction: Conventional method of staining Pap smear have been in use since 1943 to yields crisp nuclear and cytological features that can be easily interpreted by a trained pathologist[2].

This method has been modified since then according to the needs and resources of laboratories. Several Studies have reported the comparison of the use of single modified method like RADPS REAP with the conventional procedure. Here we are comparing both the modified methods with the conventional to know best one out of three that can be followed.

Explanation of procedure: Collection of pap smears is a simple OPD procedure. Ayres spatula and cytobrush for are used for collection of material from cervix. It is minimally invasive and less time consuming. After the smears are made, they are stained with pap in three different methods, observed under microscope and compared.

Withdrawal from participation in the study: Participation in this study is voluntary. You will be free to decide whether to participate in this study or continue participation once enrolled. In case you decide to withdraw your participation, you are free to do so. However, please convey the decision to the principal investigator.

Possible benefits from participating in the study: You will/will not have nor get any benefits by participating in this study. The data gathered will help the population at large.

Possible risks from participating in the study: There are no risks involved in participating in this study.

Privacy and confidentiality: The information collected from you will be coded, to prevent any person from identifying you. Your identity will never be revealed. The data collected from you will be kept confidential and only processed or aggregated data will be used for publication.

Financial incentives: You will not receive any payment for participating in this study.

Authorization for publication of aggregated data: Results obtained after processing of the aggregated data will be published for scientific purposes and or presented to scientific groups. However, your identity will never be revealed.

Questions: In case of any questions with regard to this study, you are free to contact: "Name of student/PI, mobile number, email ID" If you have any question or complaints with regard to your right as study participant you may contact Dr Harsha Hegde, Chairperson, Ethical committee of JNMC, 0831-2473777 Extension 4052.

Legal rights: By signing this consent form, we are not waving any of your legal rights.

CONSENT STATEMENT

I am making a voluntary decision to participate in the study "Comparison of cyto-morphological features in Conventional Papanicolaou Staining method with Rehydrated Air Dried Pap Smears (RADPS) and Rapid Economical acetic Acid Pap (REAP) Staining method in Cervical Smears: An observational study"

My signature below indicates that I have decided to participate and I have read the information provided above or the information provided above has been read to me in the language that I understand best. I was given the opportunity to ask questions and that they have been answered to my satisfaction.

Name of the participant:

Signature or left thumb impression of the participant:

Name of the witness:

Signature or left thumb impression of the witness:

Name of the investigator:

Name of the guide :

Signature of the investigator:

ANNEXURE II:

PROFORMA:

Patient details:

Name:

Age:

Test Results:

Cellularity- L/M/H

Overall staining- score: 1/2/3

Cell morphology- 1/2/3

Cytoplasmic details-1/2/3

Cell borders- 1/2

Nuclear characteristics-1/2/3

Nuclear borders-1/2

Background-1/2

Air-drying artifacts-1/2/3

Total score- 8-21

Quality Index- 0.3-0.8

ANNEXURE III:

KEY TO MASTER CHART:

S.NO: Serial number

L-Low

M-Moderate

H-High

CPAP- Conventional Papanicolou

RADPS- Rehydrated Air-dried Papanicolou Smears

REAP-Rapid, Economic, Acetic-acid Papanicolou

ADA- Air-drying Artifacts

QI- Quality Index

NILM- Negative for Intra-epithelial Lesion Malignancy

HSIL- High grade Squamous Intra-epithelial Lesion

ANNEXURE IV: Master chart