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Comparison Of The Effectiveness Of Methanol And Ethanol As A Fixative In Cytological Smears

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ABSTRACT :
Background: *Cytology is the science of the interpretation of cells removed from the human body through clinical procedures or exfoliation. The quality of cytological diagnosis depends in equal measure on excellence of clinical procedures used to secure the sample and on laboratory procedures used to process the sample , these multiple steps being collection, proper fixation, staining and quality control.*
Fixatives play a very important role in cytopathology for its interpretation for an accurate and reliable diagnosis. Alcohols are most used fixative in cytopathology. Fixative of choice in cytology is 95% ethyl alcohol. But , it is expensive, inflammable and subject to pilferage due to its addictive properties. . Therefore, in search of an ideal fixative and to overcome limitations of ethanol, alternative alcohol fixatives like Propanol and methanol are being used. But propanol causes some cell shrinkage. If methanol is used in adequately ventilated laboratories equipped with exhaust fans, inhalational toxic effects are reduced. so methanol can be used as an alternate cheap fixative in cytopathology. The present study was done to compare the cytomorphological parameters between smears fixed in 95% ethanol and 100% Methanol on Papanicolaou stain and to document the efficacy of methanol as an alternate cheap fixative for cytological smears.
Aim : *The primary objective of the study was to asses and find out effectiveness of methanol as an alternate fixative comparing with ethanol in cytology smears .*
Methods: *This was a descriptive study. A total of 1188 cytological smears were received in our department during the period January 2022 to January 2023. Of these 409 cases were fine needle aspiration cytology,698 cases were fluid cytology and 81 cases of cervical cytology smears. Two smears were made, one fixed in 100% methanol and other fixed in 95% ethanol. After adequate fixation both smears were stained with Papanicolaou stain. Five different parameters (clarity of staining, uniformity of staining, preservation of morphology, cytoplasmic and nuclear features) were evaluated and scored and tabulated separately for ethanol and methanol fixed smears.*
Results*: Overall grading of smears in methanol and ethanol fixatives showed moderate similarity in both FNAC and fluid cytology. Clarity of staining and nuclear features were much better in methanol fixed smears. But uniformity of staining, preservation of morphology and cytoplasmic features were better with ethanol fixed smears. All parameters show a moderate to significant similarity between methanol and ethanol fixed smears of FNAC and fluid cytology. Cervical cytology smears did not show any significant similarity and association.*
Conclusion: *Methanol fixed smears showed moderate to substantial agreement in staining while comparing with 95% ethanol fixed smears in FNAC and fluid cytology. Clarity of staining and nuclear features were better with methanol fixed smears but uniformity of staining, preservation of morphology and cytoplasmic features were better in ethanol fixed smears, but showed moderate to substantial similarity between both fixatives. Hence methanol can be used as an alternate fixative in cytopathology being cheap and having comparable effectiveness with 95% ethanol.*
Keywords*: ethanol,methanol,cytology,fixative*

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I.INTRODUCTION

Cytology is the science of the interpretation of cells removed from the human body through clinical procedures or exfoliation. The quality of cytological diagnosis depends in equal measure on excellence of clinical procedures used to secure the sample and on laboratory procedures used to process the sample. These include multiple steps such as collection, proper fixation, staining and quality control.[1] Fixatives play a very important role in cytopathology apart from the quality of material collected and its interpretation for an accurate and reliable diagnosis. Immediate fixation of cytology smears is essential for better morphological details and correct interpretation. A discrepancy in any of these steps has an adverse effect on the final diagnosis.[2] Both air dried and wet fixed smears are prepared for cytological examination. Comparing with air dried smears wet fixed smears are

better for interpreting nuclear details. Alcohols are most used dehydrating agent in histopathological tissue processing. They are the mostly used fixative in cytopathology. Fixative of choice in cytology is 95% ethyl alcohol for a minimum of 15 to 20 minutes.[1]It is proven to be an efficient one. However, it is expensive, inflammable and subject to pilferage due to its addictive properties.[2] Also, ethanol is not freely available in some clinics and hospitals. So to overcome limitations of ethanol, alternative alcohol fixatives like Propanol and methanol are being used. Propanol is being used as an alternative, as it is a cheap and easily available. But it causes cellular shrinkage than ethanol and causes difficulty in interpretation.[3]

Most laboratories use methanol routinely for fixing blood and bone marrow smears. It can also used as a fixative in cytology. According to WHO methanol health and safety guidelines – 1997, toxic effects of methanol due to low dose inhalation or skin contamination have not been observed in our laboratory settings.[4]

If methanol is used in adequately ventilated laboratories equipped with exhaust fans, inhalational toxic effects are reduced. Methanol is well absorbed through intact skin. Skin contact is minimal in steps in the performance of the FNAC ,fixation and staining. It is safe for use in the laboratory unless it is deliberately ingested or inhaled.[4]Honey is a natural cytology fixative and most of the studies in literature are with honey which is being compared with the gold standard fixative -- 95% ethanol. Few studies are with neutral buffered formalin compared with 95% alcohol. There are only very few studies comparing cheap fixative methanol with expensive and standard 95%ethanol. The present study was done to compare the cytomorphological parameters between smears fixed in 95% ethanol and 100% Methanol on Papanicolaou (Pap) stain and to document the efficacy of methanol as an alternative cheap fixative for cytological smears.

II.Material and Methods

The descriptive study was carried out in cytological section of department of pathology in government medical college ,pariyaram , Kannur during 2022-2023. A total of 1188 cytological samples taken for the study.

- **Study setting** : department of pathology, Govt medical college , Kannur
- **Study duration** :january 2022 – january 2023
- Study design : Descriptive study
- Study population : all cytology smears in department of pathology ,Govt:medical college Kannur during period of 1 year.
- **INCLUSION CRITERIA**
- All cytology smears in department of pathology during the period of 1 year
- **EXCLUSION CRITERIA**
- 1. Air dried smears
- 2.Haemorrhagic smears
- **Sample size calculation** :
- $n = \frac{\left(z_{1-\frac{\alpha}{2}} + z_{1-\beta}\right)^2 pq}{d^2}$
- $Z_{1-\alpha/2}$=1.96 , Aproximate 5% level of significance ,$Z_{1-\beta}$= 0.84 ,80%POWER
- P= 97.5 , Q=2.5 ,D,difference of proportions = 1.[1]
- While putting all the values in Above mentioned formula Sample size calculated was 1911 during the study peried of 1year.
- By convenient sampling method,1188 cases were included in this study.

Procedure methodology :

This study was done in the Cytology section of the Department of Pathology. This was a descriptive study done over a period of one year, smears made from cytological samples comprising samples of various body fluids (peritoneal, pleural,

bronchoalveolar lavage,CSF and urine), cervical smears, and fine-needle aspiration samples.

Samples from the fluids were centrifuged at 2000 rpm for 10 minutes and smears were made from the sediment. Fine-needle aspiration was done on various lesions on the patients referred to the cytology laboratory with the help of a 23-gauge needle with a 10 ml syringe. Two smears were also made both from the body fluids as well as the fine-needle aspiration cytology (FNAC) material. The respective smears were labeled and were fixed in 95% ethanol and100% methanol for a minimum of 15 minutes.

The cervical smears prepared by the gynecologists in the Gynecological outpatient department were immediately wet-fixed in 95% ethanol and 100% Methanol.

All the smears, including the conventional as well as the additional smears prepared, were stained with Papanicolaou stain after the designated fixation period. The stained smears to be compared from both the fixatives.

According to preservation and evaluating different parameters are scored and tabulated separately for ethanol and methanol smears, as given below:[41]

Table 5.1:Modified evaluation criteria given by Singh et al. based on various features

FEATURES	SCORE	CRITERIA	REMARK
Clarity of staining	Score 1	Crisp and transparent staining	Present and adequate
	Score 0	Obliteration of nucleus and cytoplasmic staining	Absent and inadequate
Uniformity of staining	Score 1	Homogenous staining throughout the cells	Present and adequate
	Score 0	Different shades of color in individual cells	Absent and inadequate
Overall morphology	Score 1	Absence of folds, overlapping, or nuclear swelling	Preserved and adequate
	Score 0	Disintegrated cells with overlapping and folding	Unpreserved and inadequate
Nuclear features	Score 1	Round nuclei with smooth and clear nuclear membrane	Acceptable and adequate
	Score 0	Nuclear granularity and disintegration	Unacceptable and inadequate
Cytoplasmic features	Score 1	Intact cytoplasmic membrane with transparent cytoplasm	Acceptable and adequate
	Score 0	Disintegrated cytoplasmic membrane with out-of-focus granular cytoplasm	Unacceptable and inadequate
Total score	Score 5:	Excellent	
	Score 3-4:	Good	
	Score ≤ 2:	Poor	

The total score was obtained by adding each parameter and grading all the slides.

Steps in cytological examination :

- Collection of sample by fine needle aspiration, fluid cytology and gynecological smears
- Smear preparation: make two separate smears each for ethanol and methanol.
- Put in coplin jars, one with ethanol and other with methanol, at least for 15 minutes
- stained with Papanicolaou stain and labelled separately

The above procedures done in the laboratory of department of pathology in our institute.
All smears are examined under microscope and scored all parameters mentioned above separately and tabulated.

Statistical analysis

- Data will be entered in excel sheet and analysed using SPSS software.
- Descriptive statistics like frequency, percentage and standard deviation is preferred
- Chi square test and tests for agreement (kappa analysis) will be used for testing the significant difference between two groups.

Kappa	Interpretation
< 0	No agreement
0.0 - 0.20	Slight agreement
0.21 – 0.40	Fair agreement
0.41 – 0.60	Moderate agreement
0.61 – 0.80	Substantial agreement
0.81 – 1.00	Almost perfect agreement

P value < 0.05 considered as significant.

III. Result

A hospital based descriptive study was done to assess the effectiveness of methanol and ethanol as a fixative in cytological smears on 1188 cytological smears including fine needle aspiration cytology, fluid cytology and cervical smears during the period of ,January2022 – January,2023.
Scoring of cytomorphological features of each smear with methanol and ethanol fixatives were evaluated.

Observations and results are discussed under the following headings:
I. All smears with ethanol and methanol fixatives were compared for the following characteristics:
1. Clarity of staining.
2. Uniformity of staining
3. Overall morphology
4. Nuclear features
5. Cytoplasmic features
II.Comparison and effectiveness of fixatives were done for the different procedures
(FNAC,Fluid cytology & cervical smears)
All cytological samples which came to our department during the study period were taken for the study ---- Fine needle aspiration cytology, fluid cytology and cervical smears.

Table6.1: The details of the distribution of the total number of sample

CYTOLOGY	FREQUENCY	PERCENTAGE
FNAC	409	34.4
NGC	698	58.8
GC	81	6.8
TOTAL	1188	100

Table 6.2 shows **comparison of clarity of staining in all smears ,** The proportion of cases with adequate clarity are slightly higher in methanol fixed smears (98.3%) than in ethanol fixed smears (98%), and showed moderate agreement (kappa 0.6) and pvalue <0.001,hence shows a significant association.

Smears	**Ethanol (%)**	**Methanol (%)**
FNAC	98	98.3
NGC	97.6	98.1
GC	100	98.8
Sample type	Kappa	p value
FNAC	0.66	<0.001
NGC	0.591	<0.001
GC	0	NA

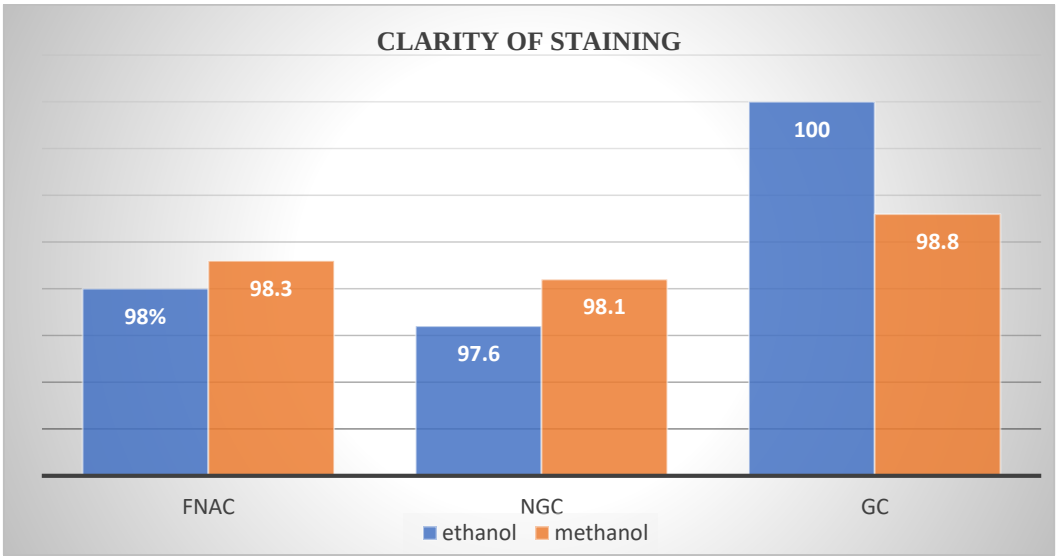

Table 6.3 shows comparison **of uniformity of staining in all smears** , Out of all smears examined adequate smears are slightly more with ethanol fixative than methanol in FNAC and fluid cytology smears.
But better uniformity in methanol fixed smears in cervical cytology.it does not showed any significance. (kappa -0)

SMEARS	ETHANOL	METHANOL
FNAC	96.1	95.6
NGC	95.1	94.8
GC	98.8	100

Sample type	Kappa	p value
FNAC	0.57	<0.001
NGFC	0.789	<0.001

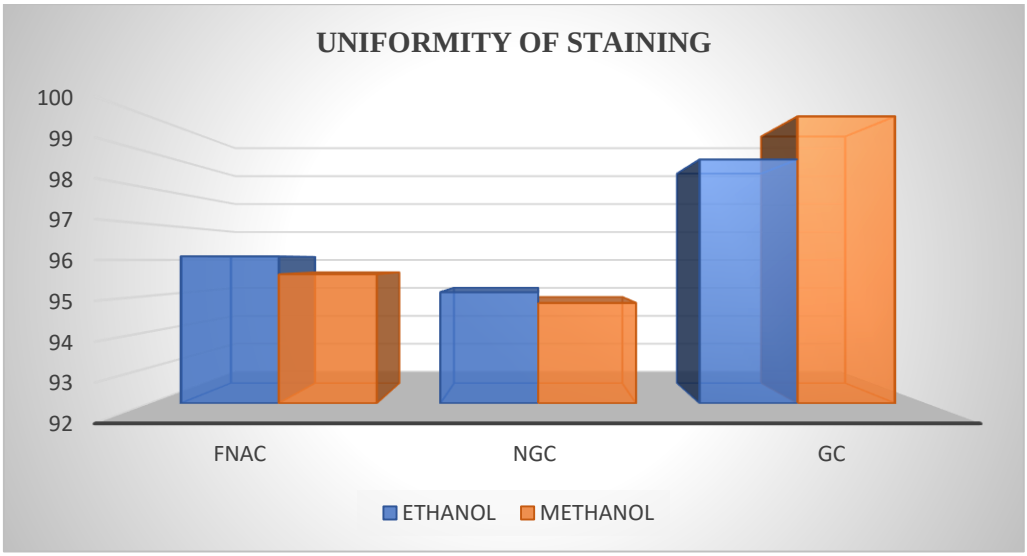

Table 6.4 showed **comparison of preservation of morphology in all smears ,** Ethanol fixed smears showed better morphology than methanol fixed smears and kappa value showed Moderate similarity in FNAC and Fluid cytology. Gynecological smears are better with methanol fixed smears, but similarity cannot prove while kappa was no agreement.

SMEARS	ETHANOL(%)	METHANOL(%)
FNAC	95.4	93.4
NGC	96	92.3
GC	97.5	100

Sample type	Kappa	p value
FNAC	0.586	<0.001
NGC	0.511	<0.001
GC	0	NA

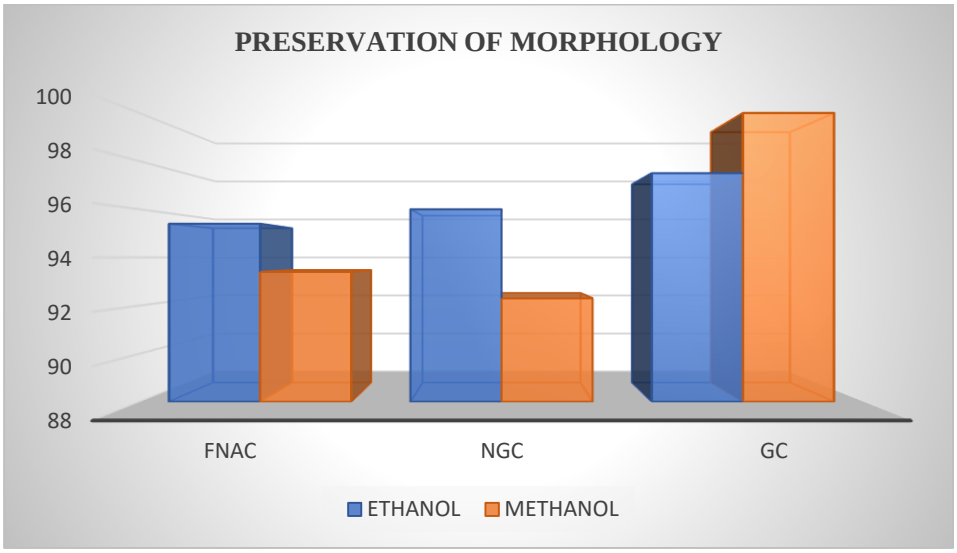

Table 6.5 showed **comparison of Nuclear features in all smears ,** Methanol fixed smears showed better nuclear features in all smears. Fluid cytology smears showed a moderate agreement while others with slight / no agreement and it is significant.

SMEARS	ETHANOL(%)	METHANOL(%)
FNAC	97.6	99.3
NGC	97.1	98.9
GC	98.8	100

Sample type	Kappa	p value
FNAC	0.144	<0.001
NGFC	0.419	<0.001
GPS	0	NA

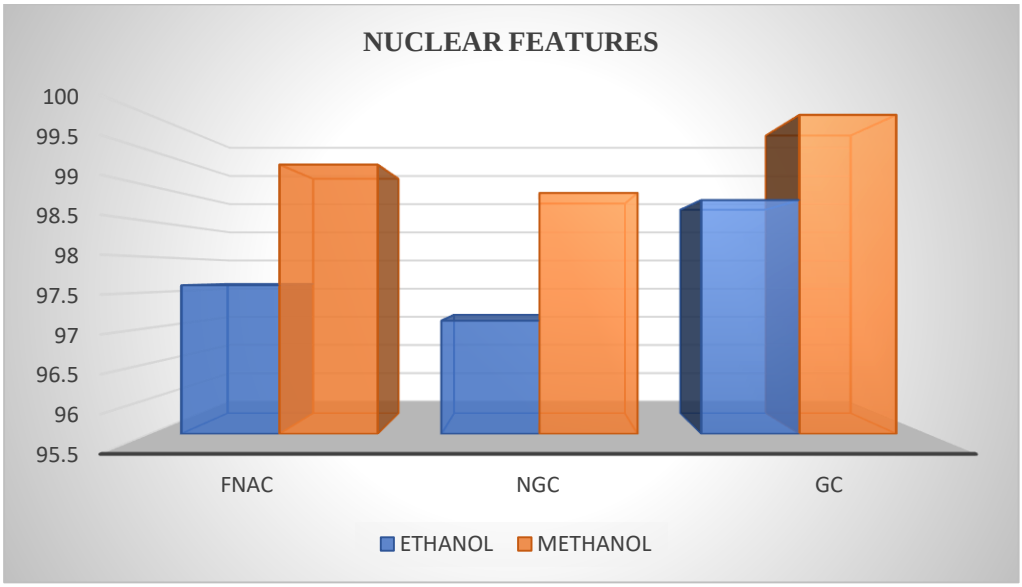

Table 6.6 showed **comparison of cytoplasmic features in all smears ,** Ethanol fixed smears showed better cytoplasmic features in all smears.

SMEARS	ETHANOL	METHANOL
FNAC	97.3	95.4
NGC	98	94.7
GC	100	98.8

Sample type	Kappa	p value
FNAC	0.448	<0.001
NGFC	0.495	<0.001
GPS	0	NA

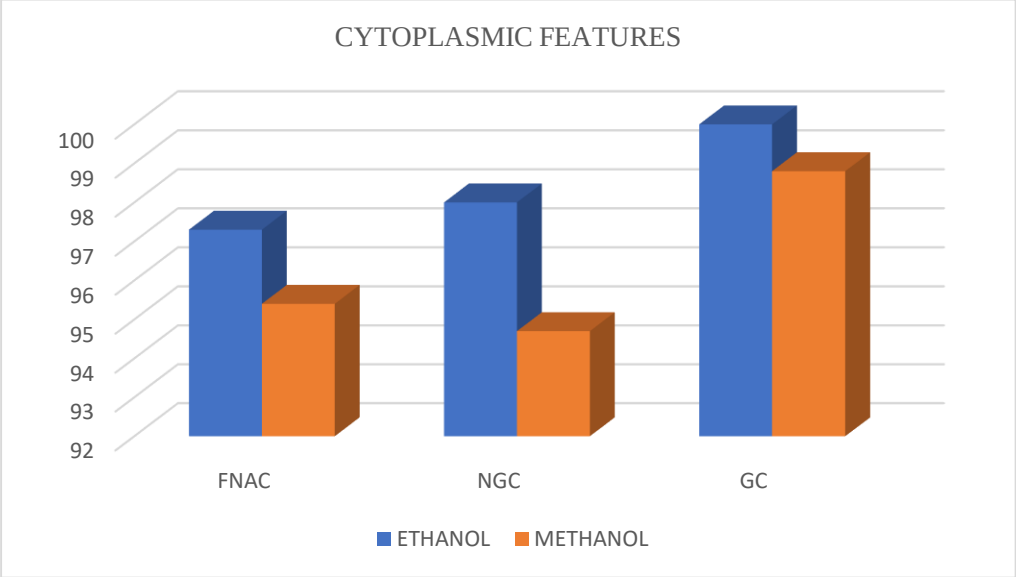

Figure 6.6: comparison of cytoplasmic features in all smears

Both FNAC and fluid cytological smears showed moderate agreement and significant association. But no significance in cervical smears.

Table 6.7: overall effectiveness in smears by different procedures

Sample type	Kappa	p value
FNAC	0.481	<0.001
NGC	0.484	<0.001
GC	-0.037	0.713

In FNAC and fluid cytology smears showed moderate agreement in methanol fixed smears when compared with 95%ethanol fixed smears and it is statistically significant. But cervical cytology smears did not showed similarity between both smears**.**

IV.Discussion

Cytology is the science of the interpretation of cells removed from the human body through clinical procedures or exfoliation. The quality of cytological diagnosis depends in equal measure on excellence of clinical procedures used to secure the sample and on laboratory procedures used to process the sample , these multiple steps being collection, proper fixation, staining and quality control.[1]

Fixatives play a very important role in cytopathology besides the quality of material collected and its interpretation for an accurate and reliable diagnosis. Immediate fixation of cytology smears is essential for better morphological details and correct interpretation.[2] Alcohols are most used dehydrant fixative in histopathological tissue processing. They are also the most used fixative in cytopathology. In all standard text books fixative of choice in cytology is 95% ethyl alcohol. Smears fixed for 15 to 20 minutes.

Ethanol and methanol are dehydrant coagulating fixatives that break the hydrogen bonds to precipitate proteins. Both ethanol and methanol have demonstrated their potential tissue fixation quality through routine use in cytology smears - [42]Fixative of choice being 95% Ethyl alcohol .Ethyl alcohol is expensive (20 times than methanol), inflammable and subject to pilferage due to its addictive properties.[2]100% methanol is an alternate fixative ,which is cheap and the toxic effects are low unless deliberately used. Although methanol is well absorbed

through intact skin, skin contact is minimal in steps in the performance of the FNAC / fixation and staining of slides with methanol. Its effectiveness as a fixative must be evaluated and compared with 95% ethanol.

Expenses for the public health system are the main concern in the establishment of methanol as a fixative of choice in cytology with comparable efficacy as with 95% ethanol.

All cytological smears by different procedures which came to our department during the study period were included in the study after applying inclusion and exclusion criteria mentioned earlier. These include fine needle aspiration cytology, fluid cytology and gynecological smears. Total of 1188 smears are included, out of which 409 cases were FNAC ,698 cases were fluid cytology and 81 cases of cervical smears. Scoring of all smears were done based on clarity of staining, uniformity of staining ,overall morphology , nuclear features and cytoplasmic features . All these scores were added together and smears were grouped into excellent, good and poor.

Given below are the details of comparison between various studies in search of an alternate efficacious fixative in cytology.

Table 7.1: Comparison of different studies based on cytology fixatives

.**Studies**	**Alternative fixative used**	**Total sample size**	**Types of smears**	**Statistical analysis: (Kappa value /p-value)**
Kumarasinghe MP et al (1997) .[1]	Methanol	108	FNA of thyroid	p > 0.05 (NS)
Ozkan et al (2012) .[43] formalin	10% honey NBF and alcoholic	7	Tissue samples each from the endometrium, breast, placenta, uterus,omentum, suprarenal,stomach, and lung	p > 0.05 (NS) 10% honey and alcoholic formalin), p <0.05 (S), 10% honey and NBF
Sabrinath et al (2014) .[44]	Formalin + honey	13 (formalin fixed tissue) & 17 (honey-fixed tissue	Maxillofacial tissue	p-value < 0.05 (S)
Singh A et al (2015).[41]	20% Honey	30	Buccal smears	p-value:0.47 (NS)
Avinash Priyadarshini et al (2022).[39]	20% unprocessed honey	300	Fluid + cervical smears + FNAC smears	Kappa value: overall grade F:0.800,CS:0.851, FN:0.880, p-value: NS
Lalwani et al (2015) .[45]	20% processed honey+ 20% unprocessed honey + formalin	36	Human tissue (oral epithelium, lymphoid, salivary gland, fat, muscle, and skin	p-value = 0.04 (NS)
Ishaq R et al (2016).[46]	20% honey	30	FNAC sample	p-value > 0.05 (NS)
Sona M et al (2017).[47]	20% Honey	194	Buccal smears of healthy individuals	Kappa value: 0.879, p-value: 0.842 (NS)
Pandiar D (2017) .[48]	20% honey and 30% aqueous jaggery solution	25	Oral smears of healthy individuals	p-value > 0.05 (NS)
Kuriachan et al (2017).[49]	Honey, jaggery, and sugar compared with formalin	40	Human gingival tissue	p-value: <0.05 (S); honey and jaggery gave superior results
Khan et al (2018).[50]	20% honey	200	Buccal smears	p-value: >0.05 (NS)
Nerune et al (2019).[2]	20% processed honey	50	Buccal mucosa	p-value: >0.05 (NS)
Sah et al (2022).[51]	20% processed honey and 20% jaggery	60	Buccal mucosa	Kruskal-Wallis test (X2): 4.93 p-value = 0.41 (NS)
Present study	**100% Methanol**	**1188**	**FNAC,fluid cytology,cervical smear**	**Kappa value: moderate agreement p value <0.001(s)**

The studies done by the above-mentioned authors were limited to one particular site or procedure with smaller sample size. In contrast to this, the present study was with material from various sites , various procedures and with a sufficient sample size (1188). Most of the studies mentioned above are statistically non- significant.

For a comparison based study of 95% ethanol and methanol as a fixative in cytological smears only two literatures has been published. One study by M P Kumarasinghe et al[1] in 76 thyroid fine needle aspiration cytology smears ,other study by Katia Ramos Moreira Leite et al[52] for validation of low cost methanol based fixatives in cervical screening program . The current study undertook all types of procedures including fine needle aspiration , fluid cytology and cervical cytology smears.

In our study both ethanol and methanol fixed smears in FNAC and fluid cytology smears were comparable in grading of smears (Excellent /good /poor) . Kappa value showed moderate agreement and p value was significant (<0.001).

Excellent grade in

FNAC -- ethanol fixed smears -- 90.2% and methanol fixed smears -- 89.7% .

Fluid cytology ---ethanol fixed smears --91.3% and methanol fixed smears ---87.9%

In the study by MP Kumarasinghe et al[1] where total score for *preservation* of cells was 98.9% for methanol and 98.2% for ethanol .In study by Katia Ramos Moreira Leite et al[41] (725 cases) 701 (96.7%) were *satisfactory* for cytological analysis by both fixatives.

Uniformity of staining , preservation of morphology and cytoplasmic features are more adequate with ethanol fixed smears than with methanol fixed smears among all category. It is similar to study by M P Kumarasinghe et al where cellular preservation and nuclear features are equal in both smears.

Clarity of staining is more with methanol fixed smears than in ethanol fixed smears both in FNAC and fluid cytology smears. In FNAC, ethanol fixed smears --- 98% and methanol fixed smears --- 98.3%. Fluid cytology smears showed 97.6% in ethanol and 98.1% in methanol. Kappa value showed substantial agreement and p value is significant, <0.001.

Nuclear features are better with methanol fixative in all smears. FNAC smears showed better results with methanol fixed smears (99.3%) than with ethanol (97.6%). Fluid cytology smears methanol fixed smears were better (98.9 %) than Ethanol fixed smears (97.1%). Kappa value showed moderate agreement and p value showed significant association ,(<0.001). But cervical smears did not show agreement (kappa value 0). In the Study by .M P Kumarasinghe et al nuclear features were equal in both fixatives.

All fine needle aspiration cytology and fluid cytology smears showed moderate agreement with kappa value and showed significant association with pvalue <0.001. But cervical smears doesn't reveal any significant association, p value 0.713 and kappa value 0.

Methanol is a cheap alcoholic fixative (even less than propanol) has moderate efficacy while comparing with 95%ethanol, so it can be used as an alternate fixative in cytological smears with a low expense and comparable effectiveness in fixation and helpful to common peoples.

V.Conclusions

- Methanol fixed smears showed moderate similarity in effective staining when compared with 95% Ethanol fixed smears.
- Clarity of staining and nuclear features were better with methanol fixed smears but uniformity of staining, preservation of morphology and cytoplasmic features were better in ethanol fixed smears.
- All these characters showed moderate to substantial agreement between both smears and showed significant association (p value <0.001) in FNAC and fluid cytology smears.

But cervical smears did not reveal any significant similarity or association.

Hence, we can use methanol as an alternate fixative in cytopathology being cheap and have comparable effectiveness with 95% ethanol.

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