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Cytomorphological Comparison between Well-fixed Smear and Air-dried Smear

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ABSTRACT

INTRODUCTION: Cervical cancer is the leading cause of cancer related death, especially in developing countries. The aim of this study was to compare well-fixed smear and air-dried smear. **MATERIALS AND METHODS:** A prospective study was conducted in Kasturba Medical College, Manipal, India, from paired cervical smears made from 200 women, who attended OPD of Obstetrics and Gynecology during a period of 3 months from November 2010 to January 2011. Routine smears were fixed immediately in 95% of ethanol and stained by Pap stain. Air-dried smears were rehydrated by immersing the slides in normal saline for 30 seconds, fixed in 95% of ethanol and stained by Pap stain. All slides were examined and assessed for various cytological parameters by the pathologist. Bethesda system was followed for reporting the cytology smears. **RESULTS:** Among the 200 paired smears 199 were negative for epithelial lesion or malignancy. One case (0.5%) had epithelial abnormality in both wet-fixed (WF) and air-dried (AD) smears. However the AD smears showed more number of abnormal cells and cells were of higher grade than the WF smear. Detection rate of Candida was similar (12.5%) in both AD and WF smears. Identification of Trichomonas was also similar (1.5%) in both types of smears. Candida could be more easily identified in AD smears as compared to WF probably because AD had a clearer background. **CONCLUSIONS:** In air-dried smears, individual cells are clearly seen. The cellular and nuclear size is comparable to tissue sections.

Keywords: Bethesda system, cancer of cervix, pap smear

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INTRODUCTION

Cervical cancer is the leading cause of cancer related death, especially in developing countries. Cytological screening is effective in preventing cervical cancer as majority of cancers are preceded by a long standing precancerous lesion, which shed abnormal cells that can be detected on cytological examination. Cervical smear or papanicolau (PAP) smear is widely acknowledged as one of the most effective methods of cancer screening. PAP is used as a routine investigation for the early detection of precancerous and cancerous lesions of uterine cervix. Smears are collected by gynaecologists or paramedical staffs in clinics, hospitals or health canters and camps. In the preparation of PAP smears, slides are immediately fixed (wet fixation) in 95% ethanol and sent to the laboratory for staining and evaluation by pathologist. However the fixation process has to be immediate as air drying artefacts result in unsatisfactory specimen for interpretation. Unfortunately, improper fixation and drying artefacts are common due to inadequate training of workers or heavy workload. This causes unsatisfactory staining and diagnostic difficulties, thereby requiring repeating of smears. This in turn increases the workload of laboratory and clinical personnel, jeopardizing patient compliance. Rehydration of air-dried smears has been reported as an excellent alternative to the wet-fixed method with excellent clinical applications in various types of cytological specimens. There are several reports describing the utility of rehydration of air-dried smears obtained from fine needle aspiration (FNA), effusion cytology and exfoliated cells [1,2,3]. Though rehydration of vaginal pool smears was attempted using tap water [4], very few reports regarding it as a substitute technique for cervical smears are available. As immediate fixation is not required, AD technique offers a simple and convenient method of smear preparation at the outpatient clinic. Furthermore, air-dried smears are easier to transport from distant areas. Due to these advantages AD method can be an alternate method that may replace wet fixation in cancer screening [5,6,7]. The objective of the current study was to compare the cytomorphologic quality parameters of air-dried smears (AD) with wet-fixed smears (WF) in paired cervical smears taken from the same patient.

MATERIALS AND METHODS

Study design and setting

A prospective study was conducted at Kasturba Medical College, Manipal, India from paired cervical smears made from 200 women, who attended OPD of Obstetrics and

Gynecology, who required a pap smear over a period of 3 months, from November 2010 to January 2011.

Participants, sample size and sampling technique

Two cervical smears were obtained by gynaecologists through direct vision with Ayres's spatula from each woman. One set of slides were labelled as A-Routine, which was wet fixed immediately and other as B-Air dried, which was rehydrated and then fixed. Routine smears were fixed immediately in 95% of ethanol and stained by Pap stain. Air-dried smears were rehydrated by immersing the slides in normal saline for 30 seconds, fixed in 95% of ethanol and stained by Pap. All slides were examined and assessed for various cytological parameters by the pathologist.

Data collection procedure and study variables

Bethesda system was followed for reporting the cytology smears. The smear was evaluated for cellularity (Low/Medium/High), presence of cytolysis, air drying artefact and RBC in the background. The preservation of cytoplasm was assessed by the staining quality of cells (unsatisfactory/satisfactory/excellent) and distinctness of cell border (distinct/indistinct). The preservation of the nuclei was assessed by the distinctness of nuclear chromatin (crisp/hazy). The cytomorphology of squamous epithelial cells and endocervical cells, when present were separately evaluated. The presence of any degree of indistinctness of cytoplasm or nuclear border or haziness of chromatin was recorded.

Statistical analysis and data management

Data was entered in Microsoft Excel Sheet and analysed in Statistical Package of Social Sciences (SPSS) version 16. Frequency and percentage were presented.

Ethical consideration

Ethical approval was obtained from Institutional Review Committee, of Kasturba Medical College, Manipal, India.

RESULTS

Out of the 200 paired smears, collected from women attending OPD of department of obstetrics and gynecology, the mean age of women between the age group 21-70 years was 45.5 years. 34 women were post - menopausal. Among the 200 paired smears 199 were negative for epithelial lesion or malignancy. One case (0.5%) had epithelial abnormality in both wet-fixed (WF) and air-dried (AD) smears. However, the AD smears showed more number of abnormal cells and cells were of higher grade than the WF smear. Detection rate of Candida was similar (12.5%) in both AD and WF smears. Identification of Trichomonas was also similar (1.5%) in both types of smears. Candida could be more easily identified in AD smears as compared to WF probably because AD had a clearer background. One of the problems in identifying Trichomonas in AD smear was, that air dried inflammatory cells were difficult to differentiate from Trichomonas vaginalis organisms.

Cellularity

Presence or absence of cytolysis was observed in both AD and WF smears. Cytolysis was present in 13.5% of AD smears while 24.5% of WF showed cytolysis. In 86.5% of AD smears cytolysis was absent while 75.5% of WF smears did not have cytolysis. This difference between the two groups was statistically significant ($p= 0.007$).

Presence of Air drying artifact was recorded as mild and moderate. If no air drying artifact was present it was recorded as absent. 72.5% of AD smears and 48% of WF did not show any air drying artifact. Mild air drying artifact was seen in 24% of AD and 30% of WF smears. Moderate air drying artifact was seen in 3.5% of AD smears and 22% of WF smears. This difference was statistically significant ($p<0.001$). Since mild air drying artifact does not hinder the morphology significantly, these cases can be considered as adequate smears. Therefore, when combined percentage of absent air drying and mild air drying artifacts are taken, we have observed that only 3.5% of AD smears showed air drying artifact of considerable significance as against 22% of WF smears. The possible reason why the air drying artifacts are less in AD smear may be due to the fact that the smears are made at leisure, thus allowing preparation of thin and uniform smears.

Hemorrhage in the smears may mask diagnostic cells, leading to difficulty in interpretation. All the smears were evaluated for presence or absence of RBC in the background. As RBC in the background mask the morphology of the diagnostic epithelial cells, a technique where a clean background with lyses of the RBC would be of great advantage. In the present study RBC background was absent in 63% of AD smears while 19% of WF smears RBC was absent in the background. In 81% of WF smears RBC was present in the background while in 37% of AD smears RBC was present. This difference between the two groups was statistically significant ($p<0.001$) indicating that the AD smears had a clear background devoid of RBC. Even in the 37% of cases where RBC was present in the background, it was very minimal (Table 1).

Differential cytoplasmic staining is one of the important properties of Pap stain. Too much blood or air drying usually alters the cytoplasmic staining and all the cells appear eosinophilic or orangophilic, giving rise to interpretive errors like overcalling of the air dried cells as dyskaryotic cells. In the present study cytoplasmic staining was unsatisfactory in 9% of AD smears and 7.5%

Table 1| Comparison of cellularity, cytolysis, air drying artifact and red blood cell background in the wet fixed and air dried smears

Cellularity	**Wet fixed**		**Air dried**		p-value
Low (61)	36	18%	25	12.5%	-
Intermediate (249)	114	57%	135	67.5%	
High (90)	50	25%	40	20.0%	
Cytolysis					0.007
Present (76)	49	24.5%	27	13.5%	
Absent (324)	151	75.5%	173	86.5%	
Air drying artifact					<0.001
Absent (241)	96	48.0%	145	72.5%	
Mild present (108)	60	30%	48	24%	
Moderate present (51)	44	22%	7	3.5%	
Red blood cell background					<0.001
Present (236)	162	81%	74	37.0%	
Absent (164)	38	19%	126	63.0%	

Table 2| Comparison of cytoplasmic staining, nuclear border squamous cell, endocervical cells, nuclear chromatin squamous cell and endocervical cells in the wet fixed and air dried smears

	Wet fixed		**Air dried**		p-value
Cytoplasmic staining					0.835
Unsatisfactory (33)	15	7.5%	18	9.0%	
Satisfactory (342)	173	86.5%	169	84.5%	
Excellent (25)	12	6.0%	13	6.5%	
Nuclear border squamous cell					0.840
Distinct (374)	188	94.0%	186	93.0%	
Indistinct (26)	12	6.0%	14	7.0%	
Endocervical cells					0.919
Absent	33	16.5%	30	15%	
Distinct	166	83.0%	169	84.5%	
Indistinct	1	0.5%	1	0.5%	
Nuclear chromatin squamous cell					0.499
Crisp (398)	200	100%	198	99.0%	
Hazy (2)	0	0.00%	2	1.0%	
Endocervical cells					0.999
Absent (61)	31	15.5%	30	15%	
Crisp (337)	168	84.0%	169	84.5%	
Hazy (2)	1	0.5%	1	0.5%	

of WF smears.84% of AD smears were satisfactory and86.5% of WD smears were satisfactory.

Excellent staining was observed in 6.5% of AD smears and 6.0% of WF smears. The difference between AD smears and WF smears was not statistically significant with respect to cytoplasmic staining (p=0.835). One of the important criteria for the diagnosis of abnormal cells is nuclear details. Well preserved nuclear structure and nuclear details are very important for the diagnosis of abnormal cells in Pap smear. Therefore, any method of fixation should take into account how well the nuclei are preserved. In the present study distinctness of nuclear borders and crispness of nuclear chromatin of squamous and endocervical cells have been evaluated. 93% of AD group and 94% of WF group had distinct nuclear borders and the difference was not statistically significant (p= 0.84) inferring that AD did not alter the nuclear characteristics. Endocervical cells were present in 170 of AD smears and 167 of WF smears. 84.5% of AD smears and 83.0% of WF smears showed distinct nuclear borders. This difference was not statistically significant (p= 0.919) inferring that type of fixation did not influence the nuclear morphology. In the present study nuclear chromatin of squamous cells was crisp in 99% of AD smears and 100% of WF smears inferring that there was no difference between AD and WF smears with respect to nuclear chromatin. Of the 200

paired smears endocervical cells were present in 170 cases of AD smears and 169 of WF smears. Crisp staining, which is an important criteria for diagnosis of atypical cells was present in 84.5% of AD smears and 84% of WF smears (p=0.499). Therefore there was no difference between AD and WF smears with respect to endocervical nuclear morphology (0.999) (Table 2).

DISCUSSION

Papanicolaou (Pap) stain is a multichromatic staining technique first described by Dr George-N papanicolau, the father of cytopathology. Pap staining is a very reliable technique used for screening of cervical cancer. One of the most important steps in Pap staining is immediate fixation of the wet smear in 95% of ethanol. The study of a well stained smear is a pre-requisite for accurate diagnosis and fixation is a primary event for any smear to be processed and examined microscopically [8, 9,19,11,12]. A well fixed tissue should be close to its living, should not change shape or volume of the cells, and should facilitate staining procedures. Our observations are similar to Gupta et al. [6] and Joiwong et al. [13]. Gupta et al. studied 950 paired smears and found 5.9% of AD and 4.9% of WF were highly cellular while 15.9% of AD and 14.9% showed moderate cellularity. Highly cellular smears were seen in78.2% of AD and 80.2% of WF smears [6]. There were no statistically significant differences between the two groups (p= 0.53). Joiwong et al. [13] studied 172 paired smears and reported low cellularity in 8.13% of AD smears, 11.04% in WF smears. Moderate cellularity was seen in 84.30% of AD smears and 81.39% of WF smears. Highly cellular smears were seen in 7.55% of AD smears and 7.55 of WF smears. In their study also there was no statistically significant difference between the two groups. If the intermediate and high cellularity is combined the AD group had more number of cases falling in this group as compared to WF smears (87.5% vs 82%). Though this difference was not statistically significant, WF smears were less cellular than AD smears probably due to loss of material during fixation in WF smears. Our results on air drying artifact were similar to the studies done by Chan et al. [1], Ng et al. [2], Dahlstrom et al. [3] Shidham et al [16],, Sivaraman et al. [5] and Gupta et al. [6], where rehydrated smears had less number of air drying artifacts. However in contrast to our study and other studies as mentioned above Joiwong et al. [13] reported increased frequency of cases with at least some degree of air drying artifacts in AD group as compared to WF group. The authors have explained this variation may be due to environmental factors like humidity and temperature which probably affected the speed of smear drying and process of cellular fixation in the dried state. Joiwong et al[13]n their study reported presence of cytolysis in 47.67% of AD and 34.88% of WF smears while cytolysis was absent in 52.32% of AD smears and 65.11% of WF. They also observed that the difference between AD and WF group was not statistically significant with respect to cytolysis (p value 0.016). In the study done by Gupta et al [6] 17.8% of the AD smears and 25.7% while 82.2% of AD smears and 82.2% of WF smears did not have cytolytic changes in the smears. 98.8% of AD smears had satisfactory staining in Joiwong et al's serie[13]while 95.3%of WF smears were satisfactory .Excellent staining was seen in 1% of AD smears and 5% of WF smears. The difference was not statistically significant. They did not report any unsatisfactory smears. In Gupta et al. [6] study showed a significantly higher percentage of excellent cytoplasmic staining in AD smears 26.5% vs. 15.4% in WF smears which was statistically significant. 61.3% were satisfactory in AD group vs. 63.6% WF smears. However unsatisfactory smears were more in WF smears 21% vs. 12% in AD. They concluded that cytoplasmic staining was better in AD as compared to WF smears. They postulated that the improved staining is due to thin and uniform nature of the smears as they are made in leisure, diminished cell loss and RBC lyses. Sivaraman et al. [5], also observed more number of cases with excellent cytoplasmic staining in AD smears as compared to WF smears (14% vs 6.2%) while satisfactory staining was seen in equal frequency . 60% of AD smears vs. 59% of the WF smears. Dahlstrom et al. [3], also reported significantly better cytoplasmic staining in AR slides as compared to WF smears which was statistically significant($p < 0.05$). Studies on large number of smears [14,15,16,17,18,19] and by Randall and Amerongen [20], carried out a retrospective matched study of 6,788 air-dried \rehydrated smears and 1,691 traditionally WF fixed smears. They found no significant difference in percentage of abnormalities between the two techniques. Cytology-biopsy correlation remained in 98-99%. They concluded that AD\rehydrated technique is a viable alternative. Bales and Durfee [21], opined that AD smears were difficult to interpret and should not be used as routine, but should be restricted to those smears that were inadequately fixed. Bonime's technique was used by them, where slides were immersed in 50% glycerine in water for 3 minutes followed by 2 minutes in 95% ethanol and then stained by Pap. With this method morphology of squamous cells was satisfactory while that of secretary cells was poor. However, Gill [17], felt that the quality of staining with AD rehydrated smears differs from WF smears. He stated that, when a cell is air dried, the surface tension forces denature and disrupt proteins, thereby altering the chromatin display relative to alcohol fixation. The air dried cells flatten more than wet fixed cells, but

shrink more if suspended in mucus, albumin or a tissue fragment than WF smears. Further they opined that AD smears appear more eosinophilic throughout as compared to WF Pap smears. Finally he stated that morphologically AD smears are more interpretable if rehydrated, and that diagnostic outcomes may be comparable to WF cohorts, but they are not identical to WF smears. A study to evaluate the possibility of routine use of AD smear instead of WF smear was done by Shidham et al. [16], intra-operative scrape smears and FNAC smears were studied. ADS were rehydrated in saline, post fixed in 95% ethanol with 5% acetic acid and stained with H & E/Pap, they were compared to corresponding stained WF Smear. Additional AD smear were stored up to 72 hours at room temperature prior to rehydration and staining to evaluate the effect of postponing rehydration and staining. They found, following characteristics in air-dried smears as compared to WF smears. Preparation without air drying artefacts was easier, cellular and nuclear sizes were larger, nuclear details were better, micro fragments showed better morphological details, non- cellular stroma was better seen, and RBC interference was less. Sivaraman et al. [5], Chan et al. [1], Ng et al. [2], Gupta et al. [6] and Joiwong et al. [13] and Zare-Mirzaaie et al. [12] made similar observations and opined that the frequency of RBC background was less in AD smears as compared to WF smears. In the study done by Sivaraman et al. [5] only 2% ADF smears had a hemorrhagic background while 7.8% of WF smears had a hemorrhagic background. Joiwong et al. [13] reported 3.49% of the AD smears and 13.94% of WF had a hemorrhagic background. AD technique promotes red cell lyses [2,9,10] , The cleaner background facilitated the interpretation of smears. Application of AD technique for markedly blood stained smears should be considered [2,10,11]. Lyses of RBC not only provide cleaner background in AD smears but leads to more vivid morphological interpretation, especially in malignancy where most often the smears are hemorrhagic.

Similar to our observationson cell borders, Joiwong et al. [13] reported distinct cell borders in 75.58% in AD smears and 80.23% in WF smears and the difference was not statistically significant. Gupta et al. [6] also reported distinct cell borders in 81.8% of AD smears and 83.1% of WF smears and difference was not statistically significant (p= 0.51). Dahlstrom et al. [3], in their study of 55 paired smears of gastric brush cytology also demonstrated distinct cell borders in 66.3% of WF smears and 69.1% of AD smears and the difference was not statistically significant. Therefore distinct cell borders which is an important parameter for cell morphology was almost similar in both the groups in the present study and is supported by various other studies.

Sivaraman et al. [5], had exactly same number of cases in both groups showing distinct nuclear borders (262/397) while Gupta et al. [6] reported 76.1% of AD smears and 78.6% of WF smears had squamous epithelial cells with distinct nuclear borders, the difference being not statistically significant (p= 0.21) A. Zare –Mirzaie et al. [12] in their study of 117 paired smears found same frequency of distinct nuclear borders in both groups. However Joiwong et al. [13] found distinct nuclear borders in the WF group 97.09% as compared to AD group which was 88.95%. This difference was statistically significant with a p value of 0.003 inferring that WF group showed better nuclear preservation.

Dahlstrom et al. [3], A. Mirzaie et al. [12], Sivaraman et al. [5] and Gupta et al. [6] did not find any statistically significant differences between AR and WF slides. Joiwong et al. [13] described increased frequency of distinct nuclear chromatin among the WF group 96.51% as compared to 87.79% of AR group, with a p value of 0.003 which was statistically significant.

Other workers have observed varying results with fluid specimens and aspiration smears [3,10]. In their study on gastric brush cytology opined that rehydration smears showed decrease in chromaticity of both nuclear and cytoplasmic staining. In their study nuclear membrane, chromatin net and nucleoli were more lightly stained and the cytoplasmic vacuoles were less distinct. In the study done by Joiwong et al[9]distinct nuclear borders were seen in 79.8% of the AD smears and 86.5% of WF smears. Though they got higher frequency among WF smears as compared to our study, this difference was not statistically significant. AD smears stored up to 72 hours showed cytomorphology comparable to well-fixed smears. Excellent morphological features including nuclear grooves and pseudo inclusions were demonstrated in AD smear from papillary carcinoma of thyroid. Special stain for fungi showed good morphology. In their study rehydration time of 15-20 seconds was considered optimal and post fixation in 5% acetic acid in 95% ethanol for a minimum of 1 minute was critical. Safneck et al. [18], did not get good results with fine needle aspiration biopsy (FNAB). They rehydrated the slides with normal saline and post fixed in formal- alcohol/water. As this had to be done quickly the smear quality was not of optimum quality. Further rehydration with 50% glycerin solution gave poor result staining.

CONCLUSIONS

In developing countries like India and Nepal hospital are far from lots of place, hence the time we can be used for preparation of air-dried smears for diagnosis.

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Data Availability: Data will be available upon request to corresponding authors after valid reason.

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