

Research article**Comparison of the Quality of Pap smear Slides Taken from the Cervix Using Two Fixation Methods****Keshavarz Najmeh*¹, Abdali Khadijeh², Akbarzadeh Mojgan³,
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*Corresponding Author Email: keshavarz_najmeh@yahoo.com, Tel :+989179281638**ABSTRACT**

Introduction: Pap smear or Pap test is a cytological method based on sampling from the cervix (or neck of the womb) in women and preparation of smear and direct observation under microscope. Pap smear is now used as a common test for screening in women. Since the Pap smear method has been used as a screening test for early detection of cervical cancer, the incidence of aggressive cervical cancer and mortality caused by it has reduced by 70%. So, conducting of experiments to improve the quality of prepared slides and reduction of red blood cells by proper fixators for better diagnosis of the disease are the purposes of this research. This study is aimed to comprise the adequacy of Pap smear taken from the cervix using conventional fixative spray and Carnoy's fixative methods. **Methods:** this study was performed on 202 blood Pap smear slides taken from 101 cervixes of women with abnormal uterine bleeding. These patients referred to Zeinabieh and Shahid Faghihi Hospitals related to Shiraz University of Medical Sciences in 2012 and 2013. After observation of two bleedings, two Pap smear slides were prepared from each sample; both of prepared slides from cervical cells of each sample were fixed at first by conventional fixative spray and one of them was then placed in Carnoy's solution for 22 minutes and air-dried. The slides were then stained with Papanicolaou stain and examined by two different cytologists in a double-blind fashion. The obtained data were analyzed by SPSS. **Results:** the results showed that the presence of squamous cells ($p=0.004$), glandular cells ($p=0.001$), transformation zone cells ($p=0.001$), cellular inadequacy ($p=0.002$), distribution of inflammatory cells ($p=0.001$) and metaplastic cells ($p=0.001$) were more on the spray-fixed slides. The mean percentage of blood on the slides ($p=0.091$) and distribution of microbial agents ($p=1$) on the spray-fixed and Carnoy's-fixed slides did not show statistically a significant difference. **Discussion and Conclusion:** based on the results of this study, Carnoy's solution do not help to diagnosis of endometrial cells and their malignancies in women with abnormal uterine bleeding.

Keywords:Quality of Slides, Pap smear, Cervix, Fixation**INTRODUCTION**

Fixation is one of the most aspects of histology and is an essential step in preparation of histological specimens and diagnosis of them. Fixation has a crucial role in identification of cells that should be considered. Proper and correct fixation helps cytopathologists to observe quickly and easily and to track cellular changes. Proper fixation seems to be necessary to obtain this goal

[1]. The purposes of fixation are preservation of autolysis steps, bacterial attack and shape and volume of cells during the fixation process, so that different sections stain easily and the cells remain unchanged [2]. Based on studies, Carnoy's solution is recommended in case that examination of nucleic acid is desired. Carnoy's fixative does not interfere with activity of nucleases but may

wrinkle the tissue. Carnoy's fixative has high rate and is used during paraffin steps of emergency biopsy for 5 hours. The sections that are fixed for an hour in Carnoy's solution can be directly transferred into absolute alcohol or into alcohol-chloroform solution in 1:1 ratio. In addition, since Carnoy's solution contains acetic acid in its compositions so this solution has a property to lyse proteins due to the presence of the acid. This property can be used to clean blood-stained samples [2]. On the other hand, Papanicolaou test is one of the most effective tools available for early diagnosis of cancer [3]. This test has been reduced the incidence of cancer by approximately 79% and reduced the mortality from it by approximately 70%, since 1995 [4]. False-negative results take place due to errors in sampling, interpretation, preparation of glass slides, improper fixation, drying the samples in air and covering the surface of glass slides by mucus, secretions and blood [5, 6]. Presence of blood on the prepared slides from cervical cells has negative impact on quality and results of smear [7]. As stated in previous studies, 85% of glass slides have small to medium (3 to 7%) smearing to blood and cellular abnormalities of bloody slides are further, which indicates that a decline in smearing to blood can be effective in better results of the test [8]. Bleeding occurs usually after sampling process in 22% of patients with abnormal Pap smear test, so blood is present in most cases and reduction of it will increase the quality of the prepared slides [9]. According to earlier studies, blood-stained slides of specimens prepared from Pap smear reduce the ability of diagnosis in tests. So finding a solution for removal of blood from the samples will reduce the related costs [10]. Different studies, including the study conducted by Nguyen GK et al. in 2005 on thyroid nodule specimens prepared by fine-needle aspiration (FNA) method showed that putting blood specimens in Carnoy's solution for 3-5 minutes prior to staining lyses the red blood cells. So, the use of Carnoy's solution may effectively improve diagnosis of uterus diseases and can

reduce the costs. This study is aimed to compare two methods including fixation on bloody Pap smear slides using Carnoy's solution and conventional fixative spray on specimens taken from women with abnormal uterine bleeding.

METHODS

This research is an experimental study performed in 2012 and 2013 in gynecological clinics of Zeinabieh and Shahid Faghihi Hospitals related to Shiraz University of Medical Sciences.

This study was conducted on 202 blood slides taken from the cervix cells in 101 women with abnormal uterine bleeding. They had experienced bleeding during the sampling period. According to resolution of the slides in spray-fixed and Carnoy's -fixed methods, the sample size was determined with a difference of 18% between the two methods and test power and significance level were 80% and 95%, respectively. The studied samples were included all the slides taken from the cells covering the neck of the womb, which during cervical sampling were smeared with blood due to the abnormal uterine bleeding. The obtained cells were uniformly coated on the glass slides and covered a thin layer. The specimens were fixed immediately using conventional fixative spray and Carnoy's solution. Women with the following characteristics were included in the study and otherwise were excluded: the women aged 40 to 60 years, they were married at least for one time and their disease was abnormal and irregular uterine bleeding, they did not have sexual relationship 24 hours prior to the sampling time, they did not use vaginal douching and/or any type of lubricant 48 hours prior to the sampling, they did not also use vaginal cream for a week.

Written informed consent was obtained from every patient to participate in this clinical trial. A questionnaire containing demographic information, technical information related to gynecological diseases, a report related to observations of sampler and report related to cytology expert was then completed for each patient. Each patient was lying in lithotomy

position and speculum was slowly inserted into vagina. No tool was used for insertion of speculum and warm water used if lubrication was needed. The cervix and vagina status was examined carefully. After observation of uterine bleeding, two specimens of Pap smear were prepared in the conventional method. Both slides prepared from cervical cells of each patient were fixed by conventional fixative spray at first. Then one of the two slides was also placed in Carnoy's solution and dried in air. To avoid bias, the slides were fixed by conventional fixative spray and Carnoy's solution in a crisscross manner. Determination of the percentage of blood on the slides was performed by a pathologist and based on the observation of the number of red blood cells in per microscopic field of vision [10]. According to some criteria including the percentage of blood on the slides and staining quality of the nucleus and cytoplasm, the resolution of the slides in this study was categorized as follows: the percentage of blood on the slides was in the range of fine resolution 0-40%, moderate resolution 40-70% and bad resolution 70-100%. The nucleus and cytoplasm staining was in the range of fine resolution (proper staining), moderate resolution (weak/proper staining) and bad resolution (weak staining of both the nucleus and cytoplasm) [8, 10]. The prepared specimens were then stained by Papanicolaou method and examined by two different cytologists in a double-blind fashion and the results were recorded. The obtained data were

analyzed by statistical package for social sciences (SPSS). The results of the study on spray-fixed and Carnoy-fixed slides were compared through two methods. The statistical tests used in this research include, paired t-test, McNemar's test and Wilcoxon's signed rank test.

RESULTS

McNemar's test was used to evaluate cellular adequacy in the conventional fixative spray group and spray + Carnoy's solution group. Chi-square test (or Fisher's exact test) was used for comparison of Carnoy's solution in the studied groups. The results are listed in Table 1.

According to the recorded data in Table 1 and on the bases of McNemar's test, there is a significant difference in comparing distribution of squamous cells on the spray-fixed and the Carnoy's-fixed slides. The presence of squamous cells is further on the spray-fixed slide (p=0.004). Difference in comparing distribution of glandular cells on the spray-fixed and the Carnoy's-fixed slides is statistically significant and the presence of glandular cells is further on the spray-fixed slide (p=0.001). Difference in comparing distribution of transformation zone cells on the spray-fixed and the Carnoy's-fixed slides is statistically significant and the presence of transformation zone cells is further on the spray-fixed slide (p=0.001). The difference in distribution of endometrial cells on the spray-fixed and the Carnoy's-fixed slides is not statistically significant (p=1).

Table 1- The results related to cellular adequacy in the two fixation methods used in this study

Groups The examined factors		Spray		Spray + Carnoy's solution		Total		Carnoy's p-value
		Number	Percentage (%)	Number	Percentage (%)	Number	Percentage (%)	
Squamous cells	Presence	63	62.4	48	47.5	111	55	0.024
	Absence	38	37.6	53	52.5	91	45	
	Total	101	100	101	100	202	100	
	p-value	0.004						
Glandular cells	Presence	64	63.4	47	46.5	111	55	0.775
	Absence	37	36.6	54	53.5	91	45	
	Total	101	100	101	100	202	100	
	p-value	0.001						
Transformation zone cells	Presence	41	40.6	25	24.8	66	32.7	0.024
	Absence	60	59.4	76	75.2	136	67.3	
	Total	101	100	101	100	202	100	
	p-value	0.001						

Endometrial cells	Presence	1	1	1	1	2	1	0.674
	Absence	100	99	100	99	200	99	
	Total	101	100	101	100	202	100	
	p-value	1						

Difference in comparing distribution of cellular inadequacy on the spray-fixed and the Carnoy's-fixed slides is statistically significant and cellular inadequacy is more on the Carnoy's-fixed slide (p=0.002) (Table 2). There is statistically no significant difference in comparing the mean percentage of blood on the spray-fixed and the Carnoy's-fixed slides (p=0.091) (Table 3). According to the results recorded in the above table and using the Wilcoxon signed-rank test, the difference of resolution in comparing the mean and quartile range on the spray-fixed and the Carnoy's-fixed slides is not statistically significant (p=0.051) (Table 4). The difference in comparing distribution of inflammatory cells on the spray-fixed and the Carnoy's-fixed slides is statistically

significant and distribution of these cells is further on the spray-fixed slides (p=0.001). There is statistically a significant difference in comparing the distribution of metaplastic cells on the spray-fixed and the Carnoy's-fixed slides and the distribution of these cells is further on the spray-fixed slides (p=0.001). There is statistically no significant difference in comparing the distribution of keratinized cells on the spray-fixed and the Carnoy's-fixed slides (p=1). There is statistically no significant difference in comparing the distribution of microbial agents on the spray-fixed and the Carnoy's-fixed slides (p=1). No cases of abnormalities in the epithelial and the cylindrical cells are found in this study (Table 5).

Table 2- The results related to comparing the distribution of cellular inadequate on the spray-fixed and the Carnoy's-fixed slides

		Spray		Spray + Carnoy's solution		Total		Carnoy's p-value
		Number	Percentage (%)	Number	Percentage (%)	Number	Percentage (%)	
Cellular inadequacy	Yes	38	37.6	55	54.5	93	46	0.003
	No	63	62.4	46	45.5	109	54	
	Total	101	100	101	100	202	100	
	p-value	0.002						

Table 3- The mean of blood percentage on the slides in the two fixation methods

	Spray		Spray + Carnoy's solution		Carnoy's p-value
	Mean	Standard deviation	Mean	Standard deviation	
Blood percentage on the slides	62.87	27.21	67.02	32.83	0.001
p-value	0.091				

Table 4- The results related to the resolution of the slides in the two fixation methods

		Middle	Quartile range	Carnoy's p-value
Resolution of the slides	Spray	2	(2-3)	---
	Carnoy's solution	3	(2-3)	0.002
	p-value	0.051		---

* 1: good resolution; 2: medium resolution; 3: bad resolution

Table 5- The results related to the distribution of inflammatory cells, metaplastic cells and keratinized cells as well as microbial agents in the studied groups

Methods Criteria			Total				p-value
			Number	Percentage (%)	Number	Percentage (%)	
Inflammatory cells	Spray	Presence	43	42.6	83	40.9	---
		Absence	58	57.4	120	59.1	
	Carnoy's	Presence	27	26.7	65	32	

	solution	Absence	74	72.3	138	68	
	p-value		0.001		----		----
Metaplastic cells	Spray	Presence	42	41.6	82	40.4	----
		Absence	59	58.4	121	59.6	
	Carnoy's solution	Presence	25	24.8	63	31	
		Absence	76	75.2	140	69	
p-value		0.001		---		---	
Keratinized cells	Spray	Presence	2	2	3	1.5	----
		Absence	99	98	200	98.5	
	Carnoy's solution	Presence	1	1	2	1	1
		Absence	100	99	201	99	
p-value		1		----		----	
Microbial agents	Spray	Presence	3	3	6	3	----
		Absence	98	97	197	97	
	Carnoy's solution	Presence	3	3	5	2.5	0.495
		Absence	98	97	198	97.5	
p-value		1		----		----	

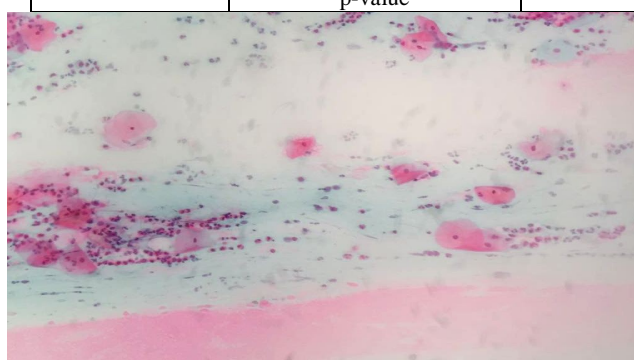


Figure 1: Spray+carnoy's-fixed smears

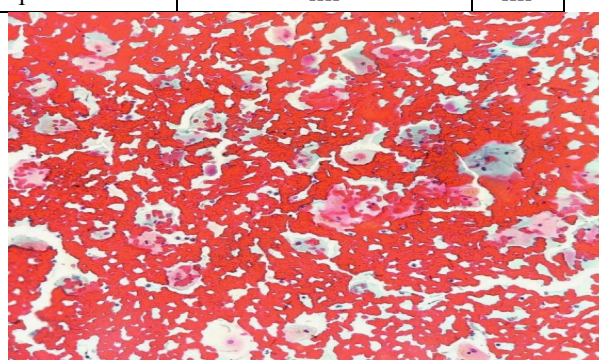


Figure 2: Spray-fixed smears



Figure3: Spray+carnoy's-fixed smears

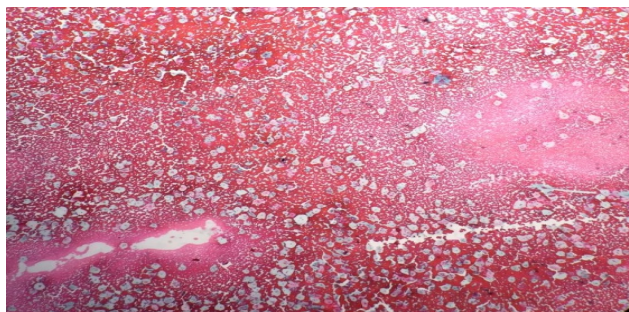


Figure 4: Spray-fixed smears

DISCUSSION

The results of this study formed based on findings of comparing the fixation of Pap smear blood samples taken from cervical cells of women with abnormal uterine bleeding referred to the gynecological clinics related to Shiraz University of Medical Sciences. The specimens were studied using two conventional fixative spray and spray + Carnoy's methods. The presence of squamous cells, glandular cells and transformation zone cells

were considered in this study as the criteria of cellular adequacy.

In comparing the distribution of squamous cells on the spray-fixed and the Carnoy's-fixed slides, the presence of these cells were further on the spray-fixed slides ($p=0.004$). The above results revealed that the distribution of squamous cells on the Carnoy's-fixed slides was more favorable. Underlying blood may remove from the slides by Carnoy's solution, and create the above condition.

The distribution of glandular cells on the surface of spray-fixed slides was more than on the surface of Carnoy's-fixed slides ($p=0.001$). A statistically significant difference was also observed in the distribution of transformation zone cells on the surface of spray-fixed slides and spray + Carnoy's-fixed slides; so that the presence of these cells was further on the surface of spray-fixed slides. Based on the evaluation performed on cellular inadequacy, it became clear that a significant difference existed between cellular inadequacies. There was also no significant difference between the mean percentage of blood on the surface of spray-fixed and the Carnoy's-fixed slides. This can be due to the used method in this study; so that the slides were fixed using fixative spray and then placed in the Carnoy's solution. Fixation of cells with spray prevented the effectiveness of Carnoy's solution and reduced the cleansing effect of the blood. Studies have suggested that putting blood samples in Carnoy's solution for 3-5 minutes and prior to staining lyses the red blood cells [11] and thereby improves quality of the cells and the prepared slides. As mentioned earlier, a fixative spray was used in this study prior to the use of Carnoy's solution. This decreased the quality of the slides.

The results showed that there is statistically no significant difference in terms of resolution between the spray-fixed and the Carnoy's-fixed slides. In some studies in which the specimens were directly placed in Carnoy's-fixed solution, an increase in resolution of the slides was observed due to the presence of chloroform in the composition of the solution [12, 13]. This agrees with the results of the current study. This probability can be due to the indirect contact of the cells with Carnoy's solution in this research.

Other studies have reported that the resolution of Carnoy's-fixed slides was further than the spray-fixed slides, while in this study no significant difference was statistically observed between the resolution of the spray-fixed and the Carnoy's-fixed slides [13]. This may be due to the presence of abundant blood on the slides. Note that the

slides were prepared from women with abnormal uterine bleeding. The fixation process was performed one-time with spray and then the process continued again with Carnoy's solution. The solution could not well erase the abundant volume of blood on the slides and consequently the slides resolution did not change.

The distribution of inflammatory cells on the spray-fixed and the Carnoy's-fixed slides had statistically significant difference and the distribution of the cells was further on the spray-fixed slides ($p=0.001$). No significant difference was statistically found in comparing the distribution of microbial agents on the spray-fixed and the Carnoy's-fixed slides.

This result was different from results of the study conducted by Shamsi. The distribution of microbial agents and inflammatory cells on the Carnoy's-fixed slides was more than the spray-fixed slides in the later study. Reduced underlying blood and increased resolution of the slides were two reasons that expressed for the event and they led to better identification of these factors. Probably due to the presence of thick layer of blood smeared on the slides (due to heavy bleeding in patients with abnormal uterine bleeding to patients with contact bleeding), the possibility of diagnosis of microbial agents and inflammatory cells did not achieve even with the use of Carnoy's solution. Kanbe and Kurosawa [15] also claimed that Carnoy's fixative solution reduced the number of chymase-positive mast cells derived from umbilical cord blood and nasal mucosa cultures and Carnoy's fixative solution had a negative effect on the number of chymase-positive mast cells. There is statistically no significant difference between comparing the distribution of endometrial cells on the spray-fixed and the Carnoy's-fixed slides. The result is quite similar to the result obtained by Shamsi. It can be concluded from this comparison that Carnoy's solution cannot well diagnosis the endometrial cells and their malignancies.

CONCLUSION: This research showed that a desirable result could be achieved with indirect

use of Carnoy's solution, but in some cases including cellular adequacy (the presence of squamous cells, glandular cells and transformation zone cells) the results were completely reverse; and fixation of the blood slides with conventional fixative spray showed better results than Carnoy's fixative solution. The identification of microbial agents and inflammatory cells was not possible in this research even with the use of Carnoy's fixative solution. This may be due to the presence of a thick layer of blood smeared on the slides. According to the results, it should be stated that Carnoy's solution did not help in diagnosis of endometrial cells and their malignancies in women with abnormal uterine bleeding. So, conducting experiments and tests about direct use of Carnoy's solution is recommended to enhance the quality of Pap smear slides.

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