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Effectiveness of Using Methylene Blue Dyes in Papanicolaou Staining in Cytology of Ascites Fluid

1st Acivrida Mega Charisma[1*]

2nd Melati Indah Permatasari[2]

3rd Yohanes Ardian Kapri Negara[3]

[1,2,3]Department of Medical Laboratory, Faculty of Health Sciences, Anwar Medika University

[*]email: *acie.vrida@uam.ac.id*

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Abstract

Papanicolaou staining is routinely used in staining ascitic fluid to identify malignant cells. However, pale nucleus arise as a problem associated with Harris hematoxylin, causing the sample to be hard to see under microscope. Methylene blue can be an alternative stain targeting nucleus in fluid sample. This study aims to evaluate the effectiveness of methylene blue in Papanicolaou staining for the cytology of ascitic fluid. The research uses an experimental approach by preparing four smears from each of 6 ascitic fluid samples obtained from the Laboratory of the Faculty of Medicine, at Airlangga University. The four smears were stained differently, consisting of Papanicolaou staining as control, and methylene blue replacing Harris hematoxylin with concentration of 5%, 10% and 15%. Slides were graded for its morphology with quality scores of 1,2,3 and 4, followed by statistical analysis. Based on the results of the Kruskal-Wallis test, the Sig value was 0.001, which was <0.005, showing difference between control and experimental group. Quality assessment revealed slides stained with 5% methylene blue solution to have clear shape of the cell nucleus, cytoplasm and the shape of the cell compared with slides stained with 10% and 15%. Hence, it can be concluded that 5% methylene blue solution can be used as an alternative solution.

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INTRODUCTION

Cytology examination involves the study of human body fluids that are processed through fixation and centrifugation (1), followed by slide smear preparation which is then microscopically observed. The cytology examination is useful for detecting the presence or absence of cancer cells in body fluids and can provide an overview of cell changes due to inflammation or infection. This examination is the gold standard for examining ascites fluid to detect malignant cells microscopically (2,3). Ascites is an accumulation of pathological fluid in the membrane lining the inner abdominal wall as a barrier from the organs in the abdomen. This fluid exists in patients with liver, heart, kidney, infection, and malignancy disease. Cytology examination of ascites fluid helps rule out misdiagnosis and establish accurate diagnoses (4,5). Papanicolaou staining is commonly used in cytology examination to clearly stain the cell nucleus, helping to detect it. The comparison dye used for staining has the advantage of being very contrasting to color the cytoplasm so that it can see the stacked cells.

Papanicolaou staining is carried out in five stages, namely fixation, nuclear staining, staining cytoplasm, clearing, and mounting (6). Papanicolaou staining has a drawback that there is a frequent problem in the results, particularly the pale nucleus, leading to difficulty in observing under microscope. It is revealed that this problem is associated with contamination of hematoxylin, leading to reduce its ability to penetrate the nucleus and the smear dries quickly before being fixed (7). One alternative proposed as an alternative of hematoxylin in staining ascites fluid is methylene blue. In Papanicolaou staining, the polychromatic staining method combines nucleus staining with hematoxylin and staining the cytoplasm with other types of stain. As a cation staining, methylene blue is used to blue stain the cell nucleus. The concentration of methylene blue used in this study includes solutions of 5%, 10%, and 15% methylene blue. The purpose of this study is to measure the effectiveness of methylene blue in Papanicolaou staining in the cytology of ascitic fluid.

METHODS

The types of research conducted was quantitative, utilizing an experimental design and had been ethically cleared with no. 0930/HRECC.FODM/VIII/2024. It was conducted from June to July 2024 at the Parasitology and Mycology Laboratory at Anwar Medical University campus.

This study involved the preparation of four smears from each of 6 ascitic fluid samples obtained from the Laboratory of the Faculty of Medicine, at Airlangga University. The four smears were stained differently, consisting of Papanicolaou staining as control, and methylene blue replacing Harris hematoxylin as experimental group with concentration of 5%, 10% and 15%. The materials used in this study included centrifuge tube, glass object, deck glass, microscope, vessel , dropper, staining jar, staining glass, timer, glass measuring, and sample pots. Reagents used in study included Harris hematoxylin, distilled water, 5% methylene blue, 10% methylene blue, 15% methylene blue, buffer pH 7.4, 96% alcohol, 70% alcohol, 50% alcohol, orange G6, EA, xylol, and entellan.

Slides prepared in control and experimental groups were graded for quality assessment with details in Table 1. The quality assessment was carried out double-blind by pathologist and lab assistant.

Table 1. Quality Assessment of Papanicolaou Staining Preparations (8)

No	Description	Quality	
		Ordinal scale	**Score**
1	Form cell No clear, intensity nucleus No clear, intensity color cytoplasm No clear, nucleus or chromatin No clear, still Lots seen erythrocytes.	Not good	1
2	Form cell not enough clear, intensity nucleus or the core is not clear, intensity color cytoplasm not enough clear, nucleus or chromatin not enough clear, still seen erythrocytes.	Not good	2
3	Form cell clear, intensity nucleus color cytoplasm clear, nucleus or chromatin clear, a little seen erythrocytes.	Good	3
4	Form cells are very clear, shape nucleus or core child more big, size cell normal nucleus, nucleus seen clear, cytoplasm clear.	Very good	4

The data obtained were then analyzed using a normality test and then continued with the Kruskal-Wallis test processed using the *Statistical program Products and Service Solution* (SPSS).

RESULTS AND DISCUSSION

This study utilized six ascites fluid samples from the Faculty of Medicine at Airlangga University in Surabaya, with each sample having a volume of 100 ml The focus of the research was to evaluate the effectiveness of using methylene blue staining in ascitic fluid cytology. The results included microscopic images of smear preparations, an assessment of the quality of these smears and a comparison of the results obtained using methylene solutions at concentrations of 5%, 10%, and 15%, as well as Harris hematoxylin as a control. These findings are illustrated in **Figure 1** as follows:

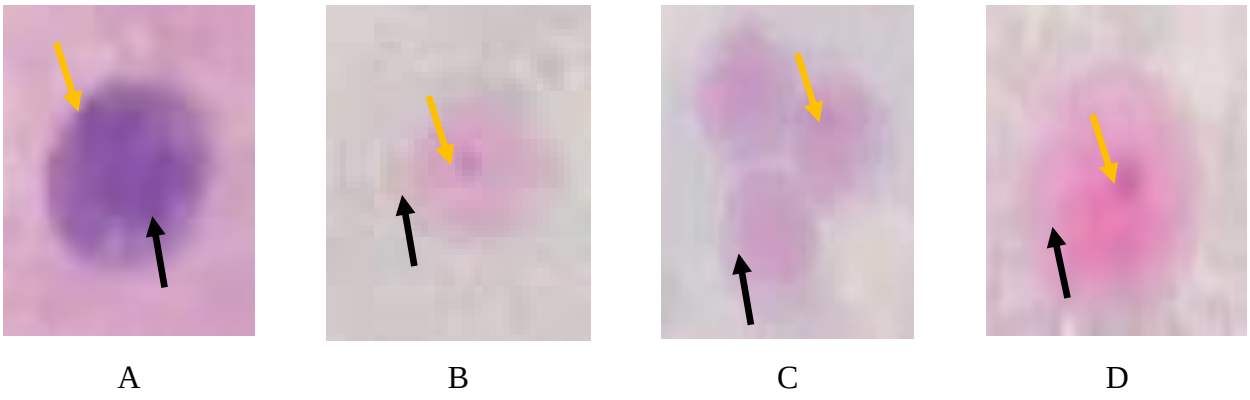

Figure 1 (a) Microscopic view of control, (b) Microscopic image smear results methylene blue 5%, (c) Microscopic image smear results methylene blue 10%, (d) Microscopic image smear results methylene blue 15%

Information: ▼ Cytoplasm

▼ Nucleus

The microscopic examination of the control revealed distinctly defined cell shapes, clear outlines of nucleus, and well defined cytoplasm colors, resulting in a score of 4 on a very good ordinal scale. In contrast, the microscopic results of the preparations with methylene blue at 5%, 10% and 15% showed unclear cell shapes, and ambiguous cytoplasmic colors. Despite these issues, the images were still diagnostic, earning them a score of 2 on a poor ordinal scale.

Smear preparations and comparison of the results using 5%, 10% and 15% Methylene blue solutions are presented in **Table 1** below:

Table 1. Results of Microscopic Assessment of Smear Preparations

Sample	Score Results			
	Harris control hematoxylin	**Methylene blue 5%**	**Methylene blue 10%**	**Methylene blue 15%**
A	4	2	2	2
B	3	2	1	2
C	3	2	2	1
D	3	2	1	1
E	3	2	2	1
F	3	1	1	1

The scores obtained were then processed using the SPSS program with the Kruskal-Wallis test. The result is presented in the following table:

Table 2 Kruskal-Wallis Test Analysis Result

	Ranks			Asymp . Sig
	Slide	N	Mean Rank	
Results	Control	6	21.50	0.001
	Methylene blue 5%	6	12.00	
	Methylene blue 10%	6	9.00	
	Methylene blue 15%	6	7.50	
	Total	24		

Normality of data was tested and known it was normal distributed. If the results of the study >0.005 are declared normal, then the test will use the ANOVA test , while the results of the study <0.005 are declared abnormal and will be continued with the Kruskal-Wallis test. The data normality test is the result of calculations that are detected by graphic analysis using *software spss*. The normality test can be seen from the graph and one sample Kolmogorov-Smirnov test. As for research that is free from normality tests, the data is normally distributed . If the normality test one sample Kolmogorov-Smirnov test shows that the a symp value. Sig >0.05, which is 0.110 > 0.05, means that the data is normally distributed. In the results of the Shapiro-Wilk normality test in the Harris control smear preparation hematoxylin obtained a sig of 0.000. In the results of the Shapiro-Wilk normality test on the smear preparation methylene blue 5% obtained sig 0.000. In the results of the Shapiro-Wilk normality test on the 10% smear preparation, sig 0.004 was obtained. In the results of the Shapiro-Wilk normality test on the 15% smear preparation, sig 0.001 was obtained, so the results of all smear preparations were declared abnormal because <0.005. This study uses a normality test to see whether the results of this study are normal or not. The sample used is small so that the results of the normality test can be seen Sig in the shapiro-wilk (9).

If the normality test results are not normal, the next test is the Kruskal-Wallis test is a test used for comparison to test more than two independent sample groups. The Kruskal-Wallis test is a non- parametric test. Kruskal-Wallis test if the Asymp.sig value >0.005 then there is no difference, whereas if the Asymp.sig value <0.005 then there is a difference. The results of the study obtained the results of the Kruskal-Wallis test, namely the Asymp.sig value of 0.001, states that there is a significant difference between the treatment and the assessment of the control stain and stain using 5%,10%, and 15% methylene blue solution in Papanicolaou staining for smear preparations on ascites fluids (10).

DISCUSSION

1. Smear Preparation Results

Microscopic examination of ascites fluid using Papanicolaou staining were excellent. Step Papanicolaou staining, cytology preparations as an assessment of whether or not the colors included in Papanicolaou staining are good. These are Papanicolaou staining steps, namely fixation, nuclear staining, cytoplasmic staining, clearing and mounting. Harris hematoxylin functions to color chromatin and nuclear membranes (blue-purple). Orange G functions to give a bright color to the cytoplasm (yellow - orange). Eosin has acidic properties, is orange-red to color the cytoplasm and will bind positively charged protein molecules in the cytoplasm and connective tissue. Eosin as a counterstain to color the cytoplasm, cells that have an affinity for eosin, namely the cytoplasm is acidophilic which means acidic to pink or yellow shadows and superficial cells are more acidophilic, while the cytoplasm is basophilic which means basic with a pale blue or greenish blue color, intermediate cells, parabasal (9). Methylene blue is an example of a cation dye or positive ion in tissue. This dye is basic and is generally used to color the cell nucleus, this is related to the concept of tissue staining, where the tissue is more acidic so that it is easily colored with basic dyes, namely bases (11).

2. Microscopic Image Scoring Assessment of Smear Preparations

Slide smeared by 5% methylene blue solution scored 3 since it has red cytoplasm and blue nucleus, which are clearly seen microscopically. Result, blue 5% got a clear red cytoplasm color result, the blue color in the cell nucleus was clear so that it got a score of 3 with a good ordinal scale. While the smear preparation stained with methylene solution blue 10% and 15% so that it gives a blue color to the cell nucleus less, the red color in the cytoplasm less gets a score of 2 with a less good ordinal scale, but in the image it can still be diagnosed. As in previous research conducted by Nurjanah (2020) that smear preparations using methylene solution blue 5%, 10% and 15% which produced the best was a concentration of 5% with good visible color intensity, the cell nucleus was clearly visible, the cytoplasm was pink with the cell structure still identifiable (12). Meanwhile, according to previous research Hormalia *et al.*, (2018) research results of supplies the smear has met the criteria for good preparation in terms of concentration methylene blue 5% and methylene blue 10%. However, at methylene concentration blue 20% almost all preparations can be said to be not good (do not meet the criteria for good preparations) (13).

According to Tri Rahmawati *et al. et al.* (2020), using a 1% methylene blue solution gives bad quality color for 60% of slides because the nucleus was not stained (14). While other experiments produced fairly good quality as much as 40% showing colored nucleus cells but the mitotic cell nucleus was not densely visible. Staining using hematoxylin - eosin produced good quality 93% colored and 7% fairly well colored. In addition, based on Kiuchi's research, (2016) maximum staining was obtained with methylene blue with the loffer formula showing the best results using potassium hydroxide to increase the pH to stain protein and nucleic acid components, so that all tissues were stained blue (15). But in this study methylene blue 10% and 15% get poor results, the color is not bright enough, the cell nucleus and cytoplasm

produced by the smear are not clear. can be caused by longer fixation time used while smearing, delayed staining will cause cell changes and also expired or expired dyes (7).

Slides stained by 5% methylene blue solution have the best result on coloring cells, giving rise to the best intensity of cytoplasm color. Slides stained by 10% and 15% of methylene blue solution have non-optimal staining compared to slides stained by 5% methylene blue solution. While in methylene blue 10% and 15% got poor results, because in the preparation the cell shape was less clear, the intensity of the cytoplasm was less clear, the color intensity in the nucleus or nucleus was less clear.

Poor or bad results in Papanicolaou staining can be caused by poor fixation or inadequate fixation can affect the color of the cytoplasm, due to poor fixation the cytoplasm becomes paler and fainter (16). Papanicolaou staining is a polychromatic staining combination of hematoxylin to color the cell nucleus and cytoplasm in other dye parts, the problem that often occurs in Papanicolou staining is that the nucleus is too pale, so slide difficult to examined microscopy, because it is contaminated with hematoxylin which reduces the ability to penetrate the nucleus and the paint dries before being fixed (7).

CONCLUSION

The concentration of methylene blue 5% is better than 10% and 15% concentrations with the results Kruskal-Wallis test obtained a significance value of 0.001 indicating a result of less than 0.005, meaning that there is a difference between the control slide and treatment slide. Based on quality assessment of the methylene blue, it can be concluded that the methylene solution blue 5% can be used as an alternative solution, because the shape of the cell nucleus is clearly visible, the cytoplasm is clearly visible and the shape of the cell is clearly visible.

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