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Original Research

Analysis of the Leukocyte Profile for Peripheral Blood Smear Stained with Diff-Count Based on Fixation Time Variation

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ABSTRACT

Background: Peripheral blood smear examination (HDT) is a test to observe the morphology of blood cells microscopically. A peripheral blood smear is a simple method, and its examination is widely available in laboratories. The advantage of peripheral blood smear examination is its ability to assess various components of peripheral blood cells, such as cell morphology (erythrocytes, leukocytes, and platelets). One of the commonly used methods for peripheral blood smear examination is Giemsa staining. The process of Giemsa staining fixation aims to preserve the cell structure and its components before the staining is performed. The precise fixation time is key to obtaining optimal staining results, which allows for accurate observation of cell morphology. The aim of this study is to analyze the morphology of leukocyte profiles in peripheral blood smears stained with Giemsa based on variations in fixation time to determine the optimal time. **Methods:** This research was conducted in August-September 2024. The sample in this study was whole blood taken from the academic community of the Department of Medical Laboratory Technology and examined with a peripheral blood smear using Giemsa staining, and analyzed using descriptive statistical tests with the SPSS for Windows 22 software. **Results:** The research results showed that at a fixation time of 3 minutes, 60% were good and 40% were less good; at 5 minutes, 100% were good; at 10 minutes, 100% were good; at 15 minutes, 40% were good and 60% were less good; and at 20 minutes, 20% were good and 80% were less good. **Conclusion:** The best time variation in Giemsa staining using phosphate buffer is fixation for 5 minutes and 10 minutes, where all preparations (5 preparations) are 100% in the good category.

Keywords: Morphology; leukocytes; variation; time; fixation

1. INTRODUCTION

White blood cells, or leukocytes, are one of the components of human blood that play a primary role as the body's immune system. Based on the shape of their nuclei, leukocytes are divided into two groups: granulocytes and agranulocytes. Granulocytes consist of neutrophils, eosinophils, and basophils. Agranulocytes consist of lymphocytes and monocytes.[1] Peripheral Blood Smear Examination (HDT) is an examination to observe the morphology of blood cells microscopically.[2] Peripheral blood smear is a simple examination and is widely available in laboratories.[3] The peripheral blood smear examination (HDT) is very important for diagnosing various types of diseases such as cancer, hemolytic anemia, thrombotic microangiopathy, and leukemia, or it can be independently performed by laboratory staff as a confirmation test when there is a discrepancy in the results of an automated complete blood count (CBC), such as

a very low platelet count (thrombocytopenia) or a very high leukocyte count (leukocytosis).[4] Another advantage of peripheral blood smear examination is its ability to assess various components of peripheral blood cells, such as cell morphology (erythrocytes, leukocytes, and platelets), determining the number and type of leukocytes (neutrophils, basophils, eosinophils, monocytes, and lymphocytes), estimating the number of platelets, and identifying the presence of parasites.[5]

One of the commonly used methods for examining peripheral blood smears is Giemsa staining.[6] Giemsa staining is a staining method that has a composition of azure methylene, methylene blue, and eosin, which is useful for staining blood through fixation with absolute alcohol. Giemsa staining involves several important stages, including fixation, staining, and washing.[7] The fixation process aims to preserve the cell structure and its components before staining is performed. The correct fixation time is key to obtaining optimal staining results, which allows for accurate observation of cell morphology. According to Nagata (2014), blood smears are fixed and immersed in methanol for 2–3 minutes.[8] Prihantoko, K. D., et al. (2020) stated that fixation using absolute methanol lasts for 10–20 minutes, while another approach involves fixation for 15 minutes before the staining process.[9] However, in general, the fixation process with absolute methanol is typically carried out for five minutes. This aims to open the cell wall so that Wright's stain can enter the cell, thereby staining the cell nucleus, cytoplasm, and cell granules.[10] Variations in fixation time can affect the quality of staining results and the interpretation of cell morphology.

Methanol that is good for the fixation process has a water content of less than 3%. Methanol that is left open for too long will evaporate and experience a decrease in concentration, leaving water in the fixation process and affecting cell morphology. By finding the optimal fixation time, it is hoped that the quality of leukocyte cell morphology examination can be improved, thereby making a significant contribution to clinical diagnostics. This is important considering that the accuracy of diagnosis in microscopic examination highly depends on the quality of the prepared specimens.[11] The poor quality of preparations due to non-optimal fixation time can lead to misinterpretation and diagnostic errors. Therefore, understanding and determining the ideal fixation time will provide significant benefits for diagnostic laboratories and clinicians to achieve accurate and efficient results in hematology diagnostics, particularly in peripheral blood smear examinations.[12] Based on the above background, this study aims to analyze the morphology profile of leukocyte cells in peripheral blood smear examination with Giemsa staining based on fixation time variations to find the optimal time.

2. METHODS

2.1 Study Design

This research is designed as an experimental laboratory study with a cross-sectional approach. The aim of this study is to observe the morphology of leukocyte cells in peripheral blood smears stained with Giemsa, using five different fixation times: 3 minutes, 5 minutes, 10 minutes, 15 minutes, and 20 minutes.

2.2 Research Settings

This research was conducted from August 2024 to October 2024 at the Hematology Laboratory, Medical Laboratory Technology Department, Health Polytechnic, Ministry of Health, Surabaya. The study population consisted of the academic community majoring in Medical Laboratory Technology, while the samples used were EDTA whole blood samples that did not undergo hemolysis. A total of 25 samples were used in this study.

2.3 Data Collection and Analysis

Data was obtained from the results of peripheral blood smear examinations using Giemsa staining based on five different fixation times and the type of white blood cells. The data was then analyzed by applying the criteria outlined in Table 1, followed by descriptive analysis using Microsoft Excel and IBM SPSS 27.

2.4 Ethical Clearance

This research has received ethical approval from the Research Ethics Commission of Poltekkes Kemenkes Surabaya under approval number EA/3012/KEPK-Poltekkes_Sby/V/2024.

3. RESULTS

The results of the examination of blood cell morphology when removing the edge of blood using Giemsa staining, based on variations in fixation time, are presented in Table 2. At a fixation time of 3 minutes, the blood cell staining results were 60% good, 40% not good,

and 0% bad. At a fixation time of 5 minutes, the blood cell staining results were 100% good. At a fixation time of 10 minutes, the blood cell staining results were also 100% good. At a fixation time of 15 minutes, the blood cell staining results were 40% good and 60% not good. At a fixation time of 20 minutes, the blood cell staining results were 20% good and 80% not good.

Table 1. Criteria for peripheral blood smear evaluation

No.	Criteria		Assessment description
	Cell type	Result	
1	Erythrocytes	Brownish red	a. Good: meets > 6 points
2	Thrombocytes	Light purple and pink	b. Not Good: meets 3 –5 points
3	Leukocytes	The nucleus is purple	c. Bad: meets 1-2 points
4	Basophils	dark blue of granules	
5	Eosinophils	Granules are brownish red	
6	Neutrophils	Granules are brownish red	
7	Lymphocytes	Cytoplasm is pale blue	
8	Monocytes	Cytoplasm is blue	
9	Background	Clean preparation, pale blue	

Table 2. Results of blood cell morphology on Giemsa staining based on variations in fixation time

No.	Treatment	Assessment category	Amount of stock	Presentation
1	3 minutes fixation	Good	3	60%
		Not good	2	40%
		Bad	0	0%
		Total	5	100%
2	5 minutes fixation	Good	5	100%
		Not good	0	0%
		Bad	0	0%
		Total	5	100%
3	10 minutes fixation	Good	5	100%
		Not good	0	0%
		Bad	0	0%
		Total	5	100%
4	15 minutes fixation	Good	2	40%
		Not good	3	60%
		Bad	0	0%
		Total	5	100%
5	20 minutes fixation	Good	1	20%
		Not good	4	80%
		Bad	0	0%
		Total	5	100%

Table 3 presents the results of the morphological examination of leukocyte profiles. Leukocytes have purple nuclei, basophils have dark blue granules, eosinophils have brownish-red granules, neutrophils have brownish-red granules, lymphocytes have pale blue cytoplasm, and monocytes have blue cytoplasm when stained with Giemsa, based on variations in fixation time.

The results of the leukocyte profile examination in peripheral blood with a fixation time of 3 minutes are shown in Figure 1. Part (a) displays lymphocyte cells with pale blue cytoplasm, while part (b) shows neutrophil cells with brownish-red granules.

For the leukocyte profile with a fixation time of 5 minutes, Figure 2 shows that part (a) contains monocyte cells with pale blue cytoplasm, while part (b) shows lymphocyte cells with pale blue cytoplasm.

Table 3. Morphological results of leukocyte cell profiles on Giemsa staining based on variations in fixation time

Standard criteria for leukocyte assessment	Staining results based on variations in fixation time				
	3 minutes	5 minutes	10 minutes	15 minutes	20 minutes
Leukocytes: Purple nuclei	5 slides (100%)	5 slides (100%)	5 slides (100%)	5 slides (100%)	5 slide (100%)
Basophils: dark blue granules	0 Slide (0%)	0 Slide (0%)	0 Slide (0%)	0 Slide (0%)	0 Slide (0%)
Eosinophils: Brownish red granules	4 slides (80%)	5 slides (100%)	5 slides (100%)	5 slides (100%)	5 slide (100%)
Neutrophils: Brownish red granules	0 slide (0%)	5 slides (100%)	4 slides (80%)	2 slides (40%)	1 slide (20%)
Lymphocytes: pale blue cytoplasm	5 slides (100%)	5 slides (100%)	5 slides (100%)	5 slides (100%)	5 slides (100%)
Monocytes: blue cytoplasm	3 slides (60%)	5 slides (100%)	5 slides (100%)	5 slides (100%)	5 slides (100%)

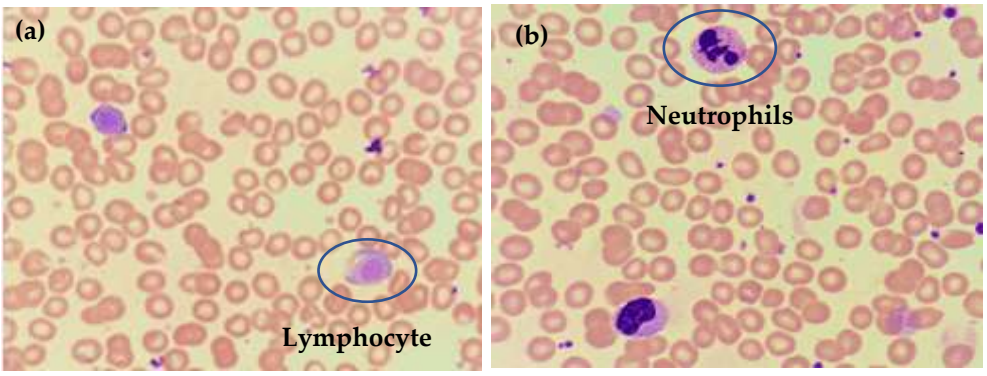

Figure 1. Leukocyte morphology with a fixation time of 3 minutes: (a) Lymphocyte cells and (b) Neutrophil cells

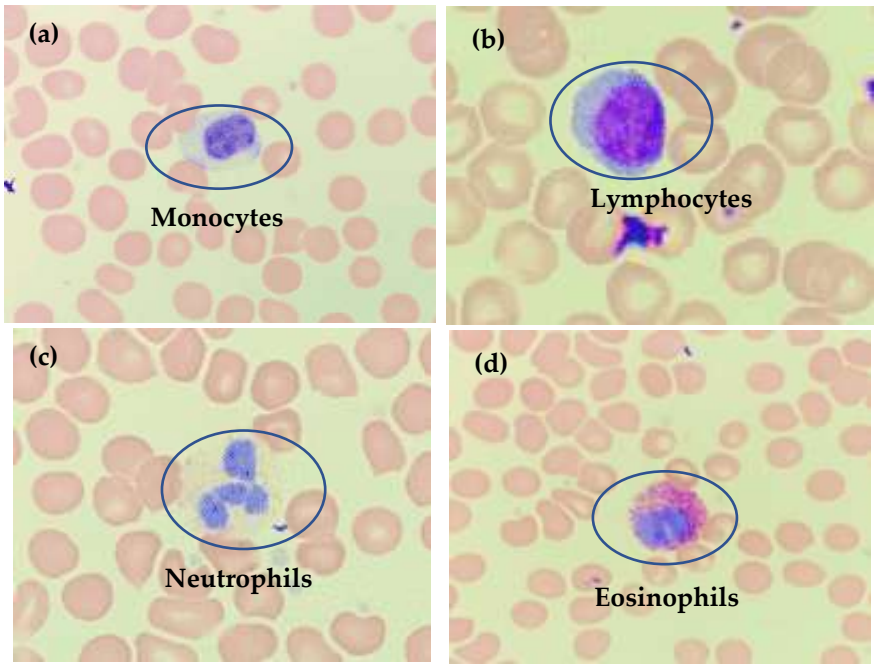

Figure 2. Leukocyte morphology with a fixation time of 5 minutes: (a) Monocyte cells, (b) Lymphocyte cells, (c) Neutrophil cells, and (d) Eosinophil cells

The results of examining the leukocyte profile in peripheral blood with a fixation time of 10 minutes are shown in Figure 3. Part (a) displays neutrophil cells with brownish-red granules, while part (b) shows lymphocyte cells with blue cytoplasm.

The results of examining the leukocyte profile in peripheral blood with a fixation time of 15 minutes are shown in Figure 4. Part (a) displays neutrophil cells with brownish-red granules, while part (b) shows monocyte cells with pale blue cytoplasm.

The results of examining the leukocyte profile in peripheral blood with a fixation time of 20 minutes are shown in Figure 5. Part (a) displays monocyte cells with pale blue cytoplasm, while part (b) shows neutrophil cells with brownish-red granules.

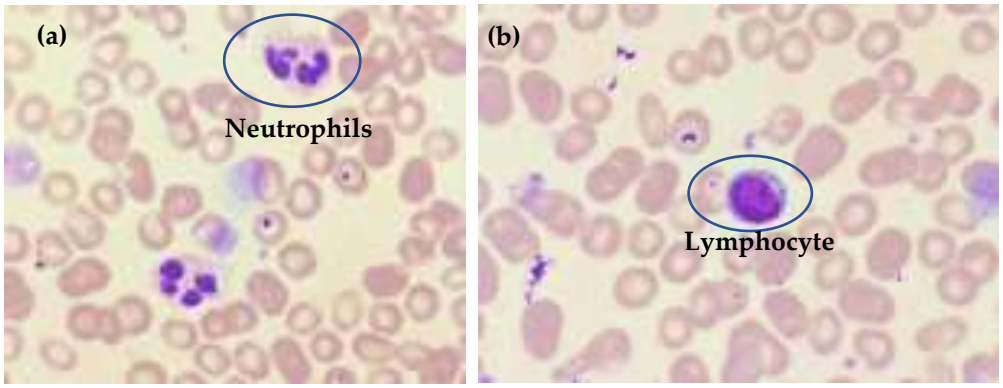

Figure 3. Leukocyte morphology with a fixation time of 10 minutes: (a) Neutrophil cells and (b) Lymphocyte cells

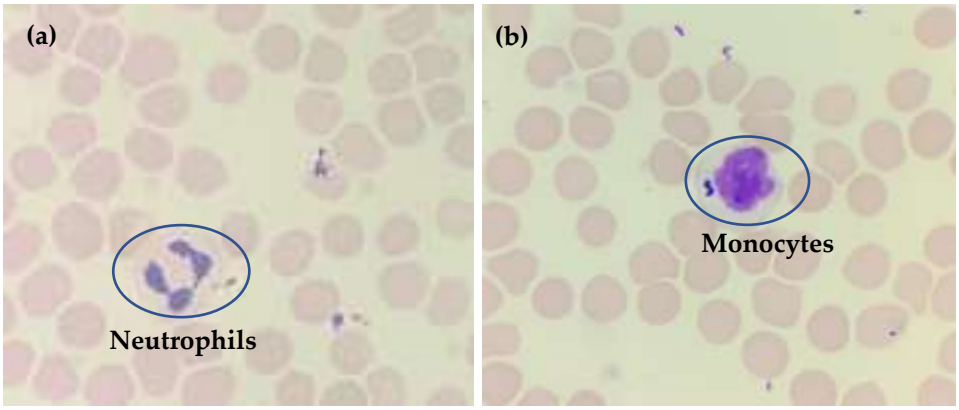

Figure 4. Leukocyte morphology with a fixation time of 15 minutes: (a) Neutrophil cells and (b) Monocyte cells

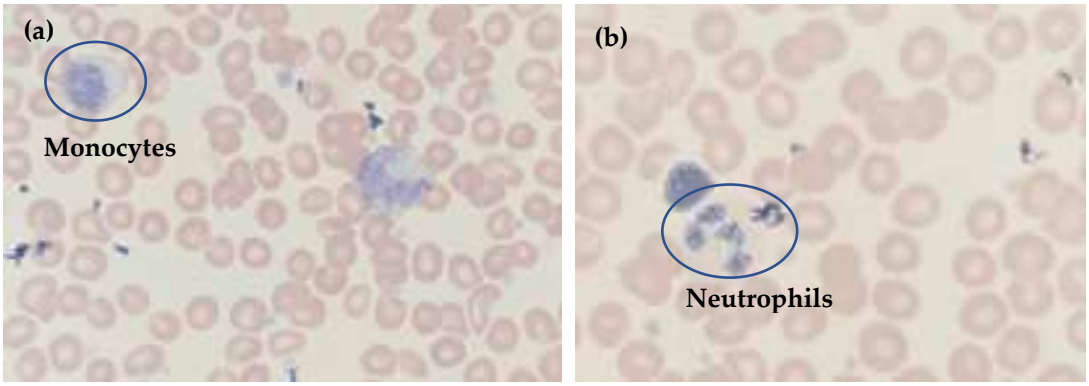

Figure 5. Leukocyte morphology with a fixation time of 20 minutes: (a) Monocyte cells and (b) Neutrophil cells

4. DISCUSSION

The fixation time was 3 minutes, with 60% of the smear preparations categorized as good, while 40% were classified as less good, and there were no preparations classified as poor. Fixation aims to quickly stop the metabolic process, prevent tissue damage, and maintain the actual state of the cells so that the internal cell structure remains normal and can absorb the dye well. Poor fixation can cause changes in cell morphology and the color of the preparation.[13-15] The correct fixation time is crucial to obtaining optimal staining results. Fixation using alcohol, especially ethanol, serves to precipitate proteins and preserve cell structures. However, fixation durations that are too short or too long can negatively impact staining quality. Fixation that is too brief may not be sufficient to effectively precipitate proteins, causing the cells to lose their structural integrity. This can cause morphological distortion and unclear staining results, making it difficult to identify cell characteristics.[16-18] The quality assessment of Blood Smear Preparations (HDT) is based on the previously established 9-point criteria.[19]

At a fixation time of 5 minutes, all smear preparations (100%) were deemed good, indicating that this time is optimal for achieving maximum quality in observation. The same applies to a fixation time of 10 minutes, where all observations remained in the good category. These findings support the hypothesis that the appropriate fixation time contributes to the accuracy and quality of the analysis.[20] Within this time frame, alcohol is quite effective in precipitating proteins without damaging the cell structure. Muflihah *et al*. research shows that fixation at the right time can produce better and clearer staining, as well as allow for more accurate visualization of cellular details.[21]

The decline in quality begins at a 15-minute fixation, where only 40% are deemed good and 60% less than good. Although there were no preparations classified as poor, this significant decline can be interpreted as the impact of fatigue or loss of concentration after a certain period of time. This is further reinforced by the results at the 20-minute fixation, where only 20% of the preparations were deemed good and 80% were less than satisfactory. This drastic decrease indicates that prolonged fixation time can reduce the quality of HDT, thereby affecting microscopic observations. Based on the results of this study, it can be concluded that the optimal fixation time range is 5–10 minutes, which is in line with Faradisa *et al*. who stated that there is an optimal fixation time range that supports good HDT results, namely between 3 and 10 minutes.[22]

Leukocyte Profile Description for Each Treatment. In the treatment with 3-minute fixation, leukocyte cells with purple-colored nuclei were found in 5 preparations (100%). This indicates that the fixation performed in 3 minutes is quite effective in preserving the structure of the leukocyte cell nucleus. This purple color is produced by the interaction between Giemsa stain and DNA within the cell nucleus.[23] In all preparations, basophil leukocytes were not found, so the researchers could not observe whether there were well-stained basophils marked by dark blue granules. The absence of basophils may be due to the normal value of this type of leukocyte being low, ranging from 0-1 cells.[14] Eosinophils were detected in 4 preparations (80%). Eosinophils appear to have granules that are reddish-brown in color. This indicates that fixation within 3 minutes is still possible to preserve the color of the granules, but the percentage of eosinophils not reaching 100% in all five preparations suggests that there is variation in cell morphology that may be caused by inconsistent fixation.

Neutrophil cells with reddish-brown granules were not found in any of the preparations (0%). This result indicates that a 3-minute fixation may not be sufficient to preserve the structure of neutrophil granules. This is consistent with findings that neutrophils require a longer fixation time for optimal results.[24] Lymphocyte cells with pale blue cytoplasm were found in all preparations (100%). This result indicates that although a short fixation time was used, the lymphocyte cytoplasm was well-stained. This suggests that the lymphocyte cytoplasm is not significantly affected by the short fixation time.[24] Monocyte cells with blue cytoplasm were found in 3 out of 5 preparations (60%). These results indicate that 3 minutes of fixation can be used to display the morphology of monocytes, although with a suboptimal percentage. The suboptimal percentage may be due to insufficient time to fully preserve the cellular structure.[1]

In the treatment with 5-minute fixation, leukocyte cells with purple nuclei were found in 5 preparations (100%). Good staining ensures that the cell nucleus can be easily identified, which is an important factor in the diagnosis of various pathological conditions. Basophils were not found in 5 preparations (0%). The absence or undetection of basophils may be due to factors such as

the naturally low number of basophils in the blood or suboptimal staining techniques for this type of cell. Eosinophil cells with reddish-brown granules were found in all 5 preparations (100%). This indicates that the staining process with a 5-minute fixation time successfully displayed the morphology of eosinophils well. Reddish-brown granules of neutrophils were also observed in all 5 preparations (100%). Lymphocyte cells with pale blue cytoplasm were found in all 5 preparations (100%). This result indicates that staining with 5-minute fixation is effective in marking lymphocytes. Monocyte cells with blue cytoplasm were found in 4 out of 5 preparations (80%). This result may be caused by improper smear preparation (non-gradient), which can affect the quality of the staining results. The percentage of leukocyte profile quality in fixation with a time of 5 minutes shows the best results. This is in line with Chen *et al*., who stated that the fixation process with absolute methanol for 5 minutes serves to open the blood cell walls so that Giemsa stain can enter and color the cells. Methanol left too long in open air will evaporate (resulting in a decrease in concentration) and absorb water, which will affect the morphology of the blood cells.[25]

In the treatment with 10 minutes of fixation, leukocyte cells with purple cell nuclei were found in 5 preparations (100%). This result indicates that the fixation time used is optimal enough to preserve the structure of the cell nucleus, making it easier to identify and analyze morphology. Basophils were not found in 5 preparations (0%). The loss or undetection of basophils may be due to the naturally low presence of basophils in the blood circulation or staining technique factors that can affect the visibility of basophil granules. Eosinophils with reddish-brown granules were found in 5 preparations (100%). This result indicates the effectiveness of the Giemsa staining technique with a 5-minute fixation time in identifying eosinophils, which often increase in response to allergies or parasitic infections. Brownish-red neutrophils were detected in 4 out of 5 preparations (80%). Although the presentation of neutrophils is good, this figure indicates that one preparation may have issues with fixation or staining that result in reduced granule visibility. Lymphocyte cells with pale blue cytoplasm were found in 5 preparations (100%). This result indicates that a fixation time of 5 minutes can maintain the morphological quality of lymphocyte cells in Giemsa staining. The successful staining of lymphocytes is very important because lymphocytes play a crucial role in the adaptive immune response. Monocyte cells with blue-stained cytoplasm were found in 4 out of 5 preparations (80%). This result indicates that monocytes were well-identified, although one preparation showed variation that might be related to the fixation method.[26-28]

In the treatment with 15 minutes of fixation, leukocyte cells with purple nuclei were found in 5 preparations (100%). This result indicates that all cells were detected well. Basophils were not found in 5 preparations (0%). This could be due to insufficient staining to detect basophils, or perhaps the basophil population in this sample is indeed low. Eosinophils with reddish-brown granules were found in 5 preparations (100%). Neutrophils with reddish-brown granules were found in 2 out of 5 preparations (40%). Lymphocytes with pale blue cytoplasm were found in 5 preparations (100%). These results indicate that lymphocytes were well-detected. Monocytes with blue cytoplasm were found in 4 preparations (80%). In this 15-minute fixation, there was a significant decrease in the quality of the leukocyte profile in the HDT preparations, where only 40% of the results were good and 60% were deemed less satisfactory, although no smear preparations were classified as poor. This decline indicates that an excessively long fixation process can affect the staining results.

In the treatment with 20 minutes of fixation, leukocyte cells with purple cell nuclei were found in 5 preparations (100%). Basophil cells were not found in 5 preparations (0%). This may be due to the low population of basophils in the blood or errors in the staining process that could affect the visibility of granules. Eosinophil cells with reddish-brown granules were found in 5 preparations (100%). Neutrophil cells with reddish-brown granules were found in 1 out of 5 preparations (20%). Lymphocyte cells with pale blue cytoplasm were found in 5 preparations (100%). Monocyte cells with blue cytoplasm were found in 5 preparations (100%). At 20 minutes of fixation, the quality of the leukocyte profile in the HDT preparation showed a more pronounced decline, with only 20% good results and 80% poor results. This indicates that the longer the fixation time, the worse the quality of the preparation. This phenomenon can be explained by the loss of clarity and detail necessary for accurate analysis.[29,30]

The limitation in this study is that the sample used is limited to healthy people so that basophil cells are not

found, where basophil cells in normal people are only about 0-2% so researchers cannot assess the morphology of basophil cells.

5. CONCLUSION

The best time variation for Giemsa staining using phosphate buffer is at fixation time 5, where all preparations (5 preparations) are 100% in the good category. And the leukocyte profile in minutes after staining showed results (nuclei colored purple) in 5 (100%), Basophils with dark blue granules were not found, Eosinophils (granules colored reddish-brown) in 5 preparations (100%), Neutrophils with reddish-brown granules in 5 preparations (100%), Lymphocytes (cytoplasm colored pale blue) in 5 preparations (100%), Monocytes (cytoplasm colored blue) in 5 preparations (100%). For further researchers, it may be possible to use sample characteristics, namely samples in normal people and samples in people who are experiencing abnormalities.

Ethical Approval

This research has received ethical approval from the Research Ethics Commission of Poltekkes Kemenkes Surabaya under approval number EA/3012/KEPK-Poltekkes_Sby/V/2024.

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Competing Interests

All the authors declare that there are no conflicts of interest.

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Underlying Data

Derived data supporting the findings of this study are available from the corresponding author on request.

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