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The stability of sample storage for complete blood count (CBC) toward the blood cell morphology

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ABSTRACT

Background: Peripheral blood smear examination had a pivotal role in determining diagnosis and as confirmation of the automatic hematology analyzer results. The storage process was highly influential on the morphological stability of the cell. This study aimed to assess the morphological changes in blood cells stored at certain period and temperature.

Method: Sample of 30 blood specimens of healthy people with *dipotassium ethylenediaminetetraacetic acid* (K_2EDTA) anticoagulants. The specimens were stored at room temperature (18-25ºC) and *refrigerator* temperature (2-8ºC) and were analyzed at the preliminary examination i.e., 8 hours, 16 hours, 24 hours, 48 hours, 72 hours, and 96 hours. Kappa test was used in validating the reading of PBS (Peripheral Blood Smear). The discrimination testing used were paired t-test and Kolmogorov Smirnov test with p value <0.005, which stated to be significant.

Result: The changes in erythrocytes morphology stored at room temperature (18-25ºC) was that the erythrocytes crenation started to happen at 8 hours storage with the grading scale of +2 that were found in 24 (80%) samples, whereas at refrigerator temperature (2-8ºC) the grading scale was +1 and found in 13 (43.3%) samples. Spherocytes on erythrocytes began to form at room temperature (18-25ºC) at 8 hours storage with grading scale of +1 and was found in 2 (6.7%) samples, whereas at refrigerator temperatures (2-8ºC) spherocytes on erythrocytes began to form at 24 hours storage with grading scale of + 2 and was found in 3 (10%) samples.

Conclusion: Peripheral blood smear (PBS) examination shall be done immediately to obtain significant results.

Keywords: Peripheral blood smear (PBS), K_2EDTA, complete blood count

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INTRODUCTION

The automatic hematology analyzer for complete blood count (CBC) analysis has rapidly developed and widely used by almost all clinical laboratory. Morphology of peripheral blood smear was still needed and useful since it could provide an estimation of the leukocytes and platelets counts, relative proportions of leukocytes type, and abnormal morphology of blood cells. Up till now, the automatic hematology analyzer still had not been able to conduct detail morphological examination of erythrocytes and leukocytes.[1,2,3]

The weakness of this automatic analyzer was its ability to identify cell morphology which resulted in the need of peripheral blood smear examination to be done. Peripheral blood smear examination was needed for confirming the *flagging* showed by the device or if influential factor was suspected to exist.[2,3] Peripheral blood examination should be done immediately since blood cell morphology would be affected by the storage process namely anticoagulant, temperature and period. The delays in peripheral blood smear examination might occur because the samples were referral from other health facilities, large number of examinations, and the period when *flagging* or critical value was detected. Some studies state that the best time to conduct peripheral blood examination is during the 2-8 hours storage for room temperature (18-22ºC) and 12-24 hours storage for refrigerator temperature (4-8ºC).[4] Based on the previous aforementioned above, this study aims to evaluate the changes in blood cells morphology based on blood storage period and temperature.

MATERIALS AND METHODS

The samples were derived from the results of laboratory examinations of 30 people that fit the normal range consecutively. The bloods of healthy adults (3 ml each) were taken using 2 tubes that contained *dipotassium ethylenediaminetetraacetic acid* (K_2EDTA) anticoagulant. The study was conducted in the laboratory of Dr. Soetomo Regional General Hospital, Surabaya from March to December 2017. Peripheral blood smear examination has been carried out from the beginning of the examination and the tubes that contained blood specimens with K_2EDTA anticoagulants were stored at room temperature (18-25ºC) and refrigerator temperature (2-8ºC). After that, peripheral blood smear examination was carried out at the storage period of 8, 16, 24, 48, 72, and 96 hours. Peripheral blood smear preparation was painted using

Wright paint, then examined under microscope with objective magnification of 10x and 100x.[4]

Semi-quantitative assessment of erythrocyte morphology was done based on Turgeon scale, while leukocytes and platelets morphology were assessed qualitatively. The calculation of the differential leukocytes and platelets count[5,6] and the reading of peripheral blood smears was carried out by 2 expert readers. From the result of the reading, *Cohen Kappa* was conducted and then the percentage of the results obtained was calculated. Discrimination test of leukocytes differential count between room temperature and refrigerator temperature storage was done using paired t-test and Kolmogorov Smirnov test with p <0.05 and showed a significant. This study has been approved by the ethics committee of Dr. Soetomo Regional General Hospital, Surabaya (164/Panke.KKE/III/2017) and the participants in this study had signed the *inform concert*.

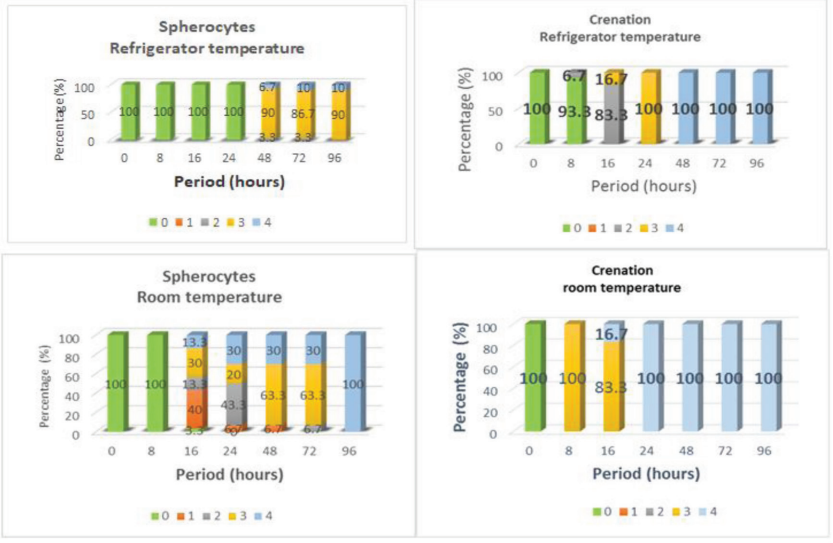

Figure 1 *Grading* scale of creantion and spherocytes found in peripheral blood smear

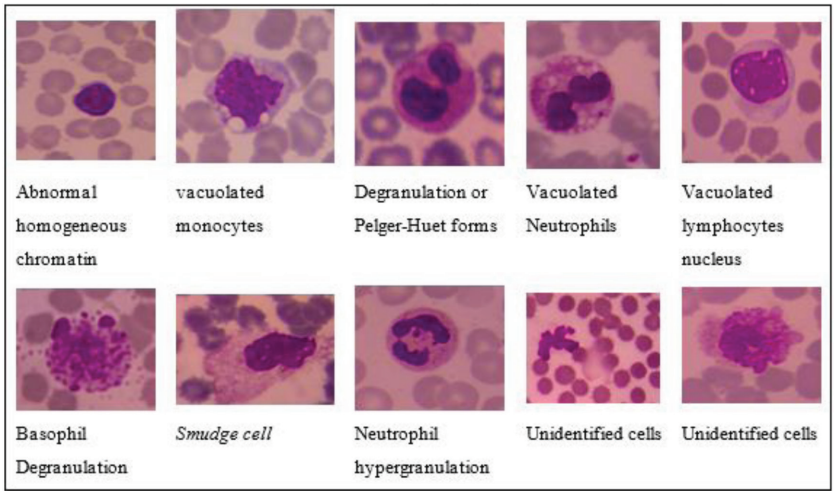

Figure 2 The morphological changes in leukocytes stored at room temperature

RESULTS

The morphological changes in erythrocytes were analyzed by peripheral blood smear as a result of storing complete blood count specimens toward the storage temperature and period. The assessment of morphological changes was done based on *Turgeon* grading system. The morphology of normochromic normocytic erythrocytes for blood specimens was examined after blood collection. The changes in erythrocytes morphology stored at room temperature for 8 hours was that the erythrocytes crenation with the grading scale of +3 was found in 30 (100%) samples; whereas for the specimen stored for 8 hours at refrigerator temperature, the erythrocytes crenation also began to be found in 2 (6.7%) samples with the grading scale of + 2. Spherocytes on erythrocytes was a morphological change found in specimens that had been stored for quite some time. In specimen stored at the room temperature storage, the spherocytes began to form at 16 hours storage period, i.e., with the grading scale of +1 in 12 (40%) samples, grading scale of +2 in 12 (40%) samples, grading scale of +3 in 9 (30%) samples, and grading scale of +4 in 4 (13.3%) samples. On the other hand, in the specimens stored at refrigerator temperatures the spherocytes began to form at 48 hours storage period, i.e., with the grading scale of +2 in 1 (3.3%) sample, grading scale of +3 in 27 (90%) samples, and grading scale of +4 in 3 (10%) samples. The morphological changes in erythrocytes was assessed based on the *Turgeon* scores which could be seen more clearly in bar charts i.e., 0 (green), +1 (orange), +2 (gray), +3 (yellow), and +4 (light blue) (Figure 1).

The morphological changes in leukocytes at storage temperature of 2-8°C began to happen at the storage period of 48 hours, while at storage temperature of 18-24°C the changes began to happen at the storage period of 8 hours. Some morphological changes in neutrophil were core lobes separation, damage to cytoplasmic boundaries, the granules disappeared and small vacuoles in the cytoplasm were visible. Morphological changes in monocytes were: there were small vacuoles in the cytoplasm and damaged irregular lobulated nuclei. The morphology of lymphocytes was slightly change, i.e., there were some vacuoles in the cytoplasm, homogeneous nucleus, and around 2-3 lobes started to form in some nuclei. The morphological changes in platelet were found in platelet enlargement and degeneration in the specimens stored at both temperatures (room and refrigerator temperatures) after 8 hours storage period (Figure 2)

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Table 1 The results of the leukocyte differential count at storage temperatures of 2-8°C and 18-24°C

Storage Period	Temperature	Lymphocytes		Monocytes		Stab neutrophil		Segmented neutrophil		Eosinophil		Basophil	
		Mean ± SD Median (Min - Max)	P	Mean ± SD Median (Min - Max)	P	Mean± SD Median (Min - Max)	P	Mean ± SD	P	Mean± SD Median (Min - Max)	P	Median (Min – Max)	P
0 hour	2-8°C	31.80 ± 6.810		5 (3 – 8)		6 (4 – 9)		48.50 ± 7.749		0.50 (0 – 2)		0.5 (0 – 1)	
	18-24°C	30.77 ± 5.309	0.152*	5 (3 – 7)	0.666*	6 (4 – 9)	1.000*	48.60 ± 8.561	0.943*	0.50 (0 – 2)	1.000*	1 (0 – 1)	0.480*
8 hours	2-8°C	31.50 (22 – 40)		6 (4 – 10)		14 (10 – 16)		56.43 ± 7.394		0.87 ± 0.681		1 (0 – 1)	
	18-24°C	31.50 (22 – 40)	1.000*	6 (4 – 10)	0.317*	14 (7 – 16)	0.887*	5.13 ± 6.404	0.756*	0.53 ± 0.571	0.057*	1 (0 – 1)	0.317*
16 hours	2-8°C	30.27 ± 6.772		6 (4 – 9)		14 (8 – 24)		55.33 ± 6.456		0.53 ± 0.571		1 (0 – 1)	
	18-24°C	31.00 ± 6.539	0.078*	6 (4 – 9)	0.635*	14 (4 – 16)	0.036*	56.43 ± 7.394	0.651*	0.87 ± 0.681	0.057*	1 (0 – 1)	0.166*
24 hours	2-8°C	28.93 ± 7.719		6 (4 – 10)		5 (3 – 8)		48.00 ± 7.566		1 (1 – 2)		1 (0 – 2)	
	18-24°C	30.60 ± 6.179	0.086*	7 (4 – 8)	0.714*	6 (4 – 8)	0.024	49.33 ± 8.281	0.335*	1 (0 – 2)	0.027	1 (0 – 2)	0.439*
48 hours	2-8°C	31.03 ± 7.337		6 (2 – 10)		12.03 ± 2.553		49.77 ± 9.435		1.03 ± 0.556		1 (0 – 1)	
	18-24°C	30.27 ± 6.772	0.155*	6 (4 – 9)	0.482*	6.67 ± 1.470	0.000	54.87 ± 6.947	0.002	0.53 ± 0.571	0.002	1 (0 – 2)	0.083*
72 hours	2-8°C	31 (17 – 43)		6.33 ± 1.626		12.60 ± 2.896		44.67 ± 9.271		0.53 ± 0.571		1 (0 – 2)	
	18-24°C	29 (16 – 43)	0.007	5.67 ± 1.373	0.001	7.43 ± 1.357	0.000	50.37 ± 8.841	0.010	1.03 ± 0.556	0.002	1 (0 – 2)	1.000*
96 hours	2-8°C	31 (17 – 45)	0.969*	6.53 ± 1.224		14 (8 – 16)		50.07 ± 7.965		1.03 ± 0.556		1 (0 – 2)	
	18-24°C	30 (20 – 44)		5.67 ± 1.493	0.000	13 (5 – 15)	0.000	54.67 ± 6.947	0.000	0.53 ± 0.571	0.002	1 (0 – 2)	1.000*

*p>0.05, not significantly different

The results of the leukocyte differential count between room and refrigerator temperature showed that the monocytes started to be significantly different after the storage period of 48 hours; stab and segmented neutrophil after 16 hours, segmented neutrophil after 24 hours, and eosinophil after 16 hours. Lymphocytes and basophils were stable until 96 hours storage period at both temperatures (Table 1)

DISCUSSIONS

Storage temperature and period affected the morphological stability of erythrocytes. The results of this study showed erythrocytes morphology began to change after 8 hours storage period and the crenation started to form at both *refrigerator* and room temperature storage. Crenation occurred at room *temperature* were clearer compared to crenation occurred at *refrigerator* temperature. The spherocytes was found at room temperature after 16 hours storage period, while at refrigerator temperature was found after 24 hours storage period. Morphological changes happened as the result of cells aging and death. Cell aging resulted in a progressive declined in the cell lifespan and functional capacity. The condition and natural balance of erythrocyte cells stored at refrigerator temperature could help the cells maintaining its lifespan longer. The morphological changes in erythrocyte cells were caused by membrane stiffness and the loss of erythrocyte cell membrane lipids. The morphological changes that occurred were started with an increase in the cell size so that it spherocytes and crenation were formed.[6-8]

Morphological changes in erythrocytes occurred in the storage of blood specimens with EDTA which were stored at room temperature for more than 6 hours. In a long storage periods, crenation and spherocytes would be formed. The morphological changes would still occur even though the specimens were stored at temperature 4ºC. Thus, peripheral blood smear examination shall be conducted immediately once the samples were collected. The recommended time was 4 hours for blood with anticoagulants.[9,10]

Rodak et al states that EDTA cause irreversible damage to structural, biochemical, and functional of blood and platelets and other cells. EDTA affected the erythrocytes in the form of crenation, spicules and *echinocytes* or *burr cells*. Red blood cell artifacts on peripheral blood smear examination from samples with EDTA occurred when the samples were stored at room temperature for more than five hours.[11]

Bafour et al confirm that storage of blood samples with EDTA as anti-coagulant at storage temperatures of 4-8ºC for 72 hours will cause some morphological changes in erythrocytes and cause extraordinary osmotic fragility. The results of peripheral blood smear examination on the first day showed normochromic normocytic erythrocytes. On the second day there was an enlargement of the red blood cells due to the degenerative changes that allow water to enter the cell.[2]

This results of this study found that the morphological changes in leukocytes occurred when the specimens were stored at refrigerator temperatures (2-8ºC) for 24 hours. When the specimens were stored at room temperature the morphological changes in leukocyte would begin after 8 hours and the changes were directly proportional with the length of storage.[6,7] It meant that the longer the storage period, the severe the morphological changes occurred. The result of the discrimination test in this study showed that leukocyte differential counts at both temperatures, namely segmented neutrophils, stab neutrophils, eosinophils, and monocytes showed different results during storage period of 8 and 24 hours. This difference occurs due to the morphological changes that began to occur, especially at room temperature so that the cell could not be identified.[2] Changes in lobulation, cytoplasmic irregularities, and vacuolization resulted in misidentification of cells. The changes in segmented neutrophil resulted in reading errors, in which it was read as stab neutrophil and monocytes instead of segmented neutrophil. On the other hand, monocytes that experienced morphological changes was identified as segmented neutrophil, stab neutrophil, and atypical lymphocytes. Lymphocyte degranulation showed cells that resemble neutrophils or monocytes.[6,7]

After blood specimens were collected, the blood experienced oxygen deprivation which led to decrease in oxidative phosphorylation and caused declined in *Adenosine triphosphate* (ATP). The decline in ATP would cause Na^+ pump to decrease which led to the increase in the entry of Ca^{2+}, H_2O, and Na^+ and also an increase in *Efflux of* K^+ as a result of cell swelling.[13,14] The decline in ATP also resulted in anaerobic glycolysis which caused decrease in glycogen, increase in lactic acid and decreases in pH which resulted in chromatin clustering.[13] This would affect the blood cells integrity which resulted in the changes of the shape of cell walls, chromatin, nucleus, and cell size. The changes in nucleus were one of three patterns which were caused by damage to deoxyribonucleic acid (DNA) and chromatin. First, the basophil color of the chromatin would fade (karyolysis), possibly occurred secondary to deoxyribonuclease (DNase) activity. The second was pyknosis which was degeneration of cell in

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which the nucleus shrinks in size and the basophils color darkened, *deoxyribonucleic acid* (DNA) turned into a solid mass and shrank. The third was karyorrhexis, i.e., fragmentation of the nucleus.[13,15]

Tsatsumi et al state that the morphological changes in neutrophils began to occur right after blood collection with EDTA. This change depended on the time when the peripheral blood smear was prepared and the EDTA concentration used for collecting blood specimens. There was not much difference between the morphology of leukocyte cells specimens stored at room temperature for 3 hours with the specimens that freshly (immediately) taken. The clear changes could be seen at 12-18 hours storage period. The morphology of neutrophil became unclear since some parts of the cell in the sample were destroyed after 3 hours storage period.[7,12]

CONCLUSION

Peripheral blood smear (PBS) examination shall be done immediately after the samples were taken. The ideal peripheral blood smear could be made after the samples being stored for 2-8 hours at room temperature and for 12-24 hours at refrigerator temperature (2-4^{o}C) after sample collection.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding manuscript.

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AUTHORSHIP CONTRIBUTIONS

AA, conceived and designed the study, analyzed the data, performed the statistical analysis and drafted the manuscript. AJ, MY, and AR analyzed morphology in blood smear evaluation. All Authors read and approved the final version of the manuscript.

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