

The effect of excessive disodium ethylene diamine tetraacetic acid (Na₂EDTA) anticoagulant concentration on leukocytes profile in peripheral blood examination

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Abstrak

Akurasi pemeriksaan profil leukosit dipengaruhi beberapa faktor preanalitik diantaranya konsentrasi antiokoagulan. Antikoagulan yang paling sering dipakai pada pemeriksaan darah rutin adalah EDTA. Ketidaksesuaian perbandingan konsentrasi EDTA dengan bahan darah berefek terhadap hasil pemeriksaan darah tepi diantaranya parameter leukosit. Penelitian ini bertujuan untuk mengetahui apakah terdapat perbedaan antara hasil pemeriksaan profil leukosit pada bahan darah dengan berbagai konsentrasi antikoagulan Na₂EDTA yang berbeda. Penelitian ini merupakan penelitian potong lintang. Bahan penelitian berupa 33 sampel darah vena mahasiswa Fakultas Kedokteran UGM Yogyakarta. Dua mL darah dibagi ke dalam 4 tabung Na₂EDTA. Tabung pertama berisi Na₂EDTA konsentrasi standar, 2 mg/dl, tabung yang lain secara berurutan berisi Na₂EDTA dengan konsentrasi 4 mg/dl, 6 mg/dl, and 8 mg/dl. Sebelumnya dibuat sediaan hapus langsung dari setetes darah tanpa antikoagulan (sebagai kontrol). Darah dalam keempat tabung tersebut segera dilakukan pembuatan sediaan hapus dan diperiksa profil hematologi leukositnya menggunakan SYSMEX SE-9500 automatic analyzer. Terdapat perbedaan yang bermakna dari hitung leukosit, hitung jenis leukosit absolut dan prosentase monosit pada konsentrasi Na₂EDTA yang berlebihan. Prosentase netrofil relatif meningkat dan terdapat perbedaan yang bermakna. Prosentase limfosit, eosinofil dan basofil tidak berbeda secara bermakna. Pemeriksaan morfologi leukosit menunjukkan perubahan yang bermakna berupa tepi sitoplasma yang irreguler, vakuolisasi, dan lobus nukleus yang irreguler akibat pengaruh konsentrasi Na₂EDTA yang berlebihan. Disimpulkan bahwa penggunaan konsentrasi Na₂EDTA yang berlebihan pada preparasi spesimen darah menyebabkan perubahan profil leukosit sesuai peningkatan konsentrasinya. Konsentrasi standar tidak mempengaruhi hitung leukosit dan hitung jenis leukosit serta morfologinya, kecuali berpengaruh terhadap tepi sitoplasma yang irreguler dan lobus nukleus yang irreguler. (*Med J Indones 2007; 16:168-75*)

Abstract

Accuracy of leukocytes profile assessment is influenced by several pre analytical factors, among others, the anticoagulant concentration. EDTA is one of the most frequently used anticoagulant in peripheral blood examination. Several references stated that inappropriate concentration of EDTA anticoagulant in blood sample may affect the result of leukocytes profile in peripheral blood examination. The aim of this study was to evaluate whether there are differences among leukocytes profile in peripheral blood examination specimens, which were prepared with excessive Na₂EDTA anticoagulant in different concentration. This study was conducted in Faculty of Medicine, Gadjah Mada University. Blood samples from 30 subjects were taken using vein puncture. Two millimeters blood was divided into 4 Na₂EDTA-containing tubes. Before that, one drop of blood without Na₂EDTA anticoagulant was used to make blood film right after vein puncture, as control. Each tubes contained different concentration of anticoagulant. The first tube contained Na₂EDTA in standard concentration 2 mg/ml; the remaining tubes contained 4 mg/ml, 6 mg/ml, and 8 mg/ml respectively. These samples were immediately examined using SYSMEX SE-9500 automatic cell counter to measure the total and differential leukocytes count; and were stained with Wright staining for morphological examination under the microscope. These procedures were done before 20 minutes of vein puncture. There were significant decrement of total leukocytes count, absolute differential leukocytes count and monocyte percentage following excessive Na₂EDTA administration. Neutrophil percentage was found to be relatively increased and the difference was significant. Lymphocyte, eosinophil and basophil percentages were not significantly different. Morphological examination showed significant increment in irregular cytoplasm margin, vacuolation and irregular nuclei lobes following excessive Na₂EDTA administration. It is concluded that excessive concentration of Na₂EDTA used in blood specimen preparation, will lead to changes in leukocytes profile as the concentration increased. Standard Na₂EDTA anticoagulant concentration did not alter any leukocytes count and morphology, except for irregular cytoplasm margin and irregular nuclei lobes. (*Med J Indones 2007; 16:168-75*)

Keywords: Na₂EDTA, anticoagulant, leukocytes profile, leukocytes count, leukocytes morphology

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Peripheral blood examination is one of the important laboratory procedures in order to establish the diagnosis of various diseases, such as infection, tumor, degenerative diseases and others. This examination is usually used as first line procedure, because it is easy and relatively affordable for the patient.

One of the most important and routinely examined blood elements is the leukocyte. Leukocytes perform their role as host defender against various invaders. Abnormality in their quantitative and qualitative profile may lead to the understanding of body's response mechanism to foreign body. The leukocytes profile includes examination of cell morphology; and cell count that consists of total leukocytes count and differential leukocytes count.

Many pre analytical factors can influence the result of leukocytes profile examination, one of it is the concentration of anticoagulant being applied. National Committee for Clinical Laboratory Standards (NCCLS) has recommended EDTA as anticoagulant for full blood cell counts and white blood cell differential analysis, principally because of its cell preservation properties.¹

Most commonly used EDTA salt in hematology examination are disodium EDTA (Na_2EDTA) with concentration of 1.4 – 2.0 mg/ml blood, dipotassium EDTA (K_2EDTA) with concentration of 1.5 – 2.2 mg/ml blood, and tripotassium EDTA (K_3EDTA) with concentration of 1.5 – 2.2 mg/ml blood.²

The aim of this study is to know whether there are differences among leukocytes profile in peripheral blood examination specimens, which were prepared with excessive Na_2EDTA anticoagulant in different concentration.

METHODS

The design of this study was a qualitative non-experimental, performed by cross sectional method. It was conducted during September until November 2004 in the Faculty of Medicine, Gadjah Mada University.

There were 30 blood samples obtained from subjects with the following inclusion criteria; male, Malay race, age between 18 to 24 years old, healthy (proven by anamnesis and vital sign examinations), had been given informed consent prior to the study and agreed to join this research voluntarily as subjects, and the result of routine hematology examination using automatic

cell counter was within reference range. The exclusion criteria consist of history of allergy, recent disease, chronic disease and recent intake of medicine especially those that may influence hematology profile.

Variables being measured in this study include independent and dependent variables. Independent variables were Na_2EDTA anticoagulant in different concentrations, which were standard concentration (2 mg/ml), 2x (4 mg/ml), 3x (6 mg/ml), and 4x (8 mg/ml) of standard concentration. For morphology examination, there was non- Na_2EDTA specimen, as control in addition to the other 4 variables.

Dependent variables consist of leukocytes profile elements, that comprises total leukocytes count, differential leukocytes count and morphology variation of leukocytes. The differential leukocyte count was consists of absolute count and percentages of neutrophil, lymphocytes, monocytes, eosinophil and basophil. The evaluation of leukocytes morphology consists of irregular cytoplasm margin, vacuolation and irregular nuclei lobes.

Two ml of blood was drawn from the veins of antecubital fossa using aseptic technique of vein puncture for each subject. The blood samples were divided into 4 groups in 4 different tubes, 0.5 ml in each tube. These four tubes were prepared containing Na_2EDTA in standard concentration, 2x, 3x and 4x of standard concentration, respectively. Cell counting using SYSMEX SE-9500 automatic cell counter and blood smear specimen preparation were done as soon as possible before 20 minutes from blood sampling to minimize bias with the effect of preparation time. For the morphology examination, control was made from the first drop of blood without anticoagulant. Principally, SYSMEX SE-9500 occupies flow-through system that identifies cell on the basis of cell size and intracellular density by using Direct Current (DC) and Radio Frequency (RF) method.³

The slides were examined using binocular microscope, under 100x magnifications with immersion oil. The counting areas for leukocytes assessment were chosen, with respect to the well-preserved central pale area of single erythrocytes in the surroundings. The most efficient check for proper leukocytes counting areas was by low-power field inspection of the peripheral (side and feathered end) area for a disproportionate number of neutrophil.⁴ The morphologic variables were counted manually per 100 leukocytes. The results of examination of each slide were reported in the leukocytes morphology examination forms.

Data analysis was performed by using ANOVA (Analysis of Variant) for normally distributed data and nonparametric tests (Kruskal-Wallis and Mann-Whitney) for not normally distributed data.

RESULTS

Total Leukocytes Count

The graph 1 showed that the mean of total leukocytes count decreased significantly as the Na₂EDTA concentration increased beyond the standard concentration.

The percentage of total leukocytes count ($\times 10^3 \mu\text{L}$) in 3 groups of excessive Na₂EDTA anticoagulant concentration is lower than that in the control group (standard Na₂EDTA anticoagulant concentration) ($p < 0.0001$).

The result means that excessive concentration of Na₂EDTA will damage the leukocytes thus they cannot be detected any longer by the automatic cell counter, which result in the decrement of the total leukocytes count.

Differential Leukocytes Count

Absolute Differential Leukocytes Count

Figure 2 showed that the mean of absolute differential leukocytes count were decreasing significantly as the Na₂EDTA anticoagulant concentration increased beyond the standard concentration ($p < 0.05$). This result is linear with the result of the total leukocytes count analysis, because as we know, differential leukocytes count were the building blocks of the total leukocyte count. Therefore, decrement in the amount of differential leukocytes will consequently decreasing the amount of total leukocytes.

The result signified that excessive concentration of Na₂EDTA anticoagulant will damage the leukocytes in which Na₂EDTA distorted cellular morphology, thus the cells cannot be detected by the automatic cell counter, which result in the decrement of absolute differential leukocytes count.⁵ Some authors said that pseudoleukopenia may due to the gating out of large platelet-WBC masses.^{6,7}

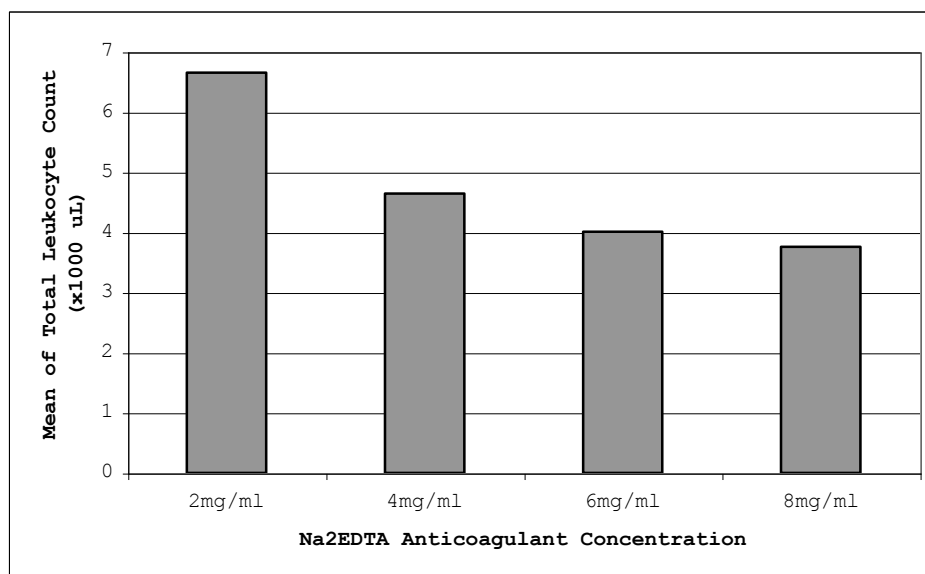


Figure 1. The relationship between mean of total leukocytes count ($\times 10^3 \mu\text{L}$) with an increasing Na₂EDTA anticoagulant concentration

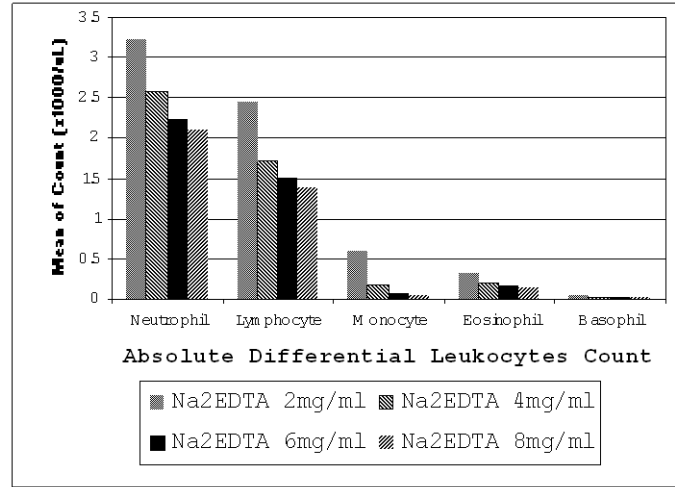


Figure 2. The relationship between mean of absolute differential leukocytes count ($\times 10^3 \mu\text{L}$) with an increasing Na₂EDTA anticoagulant concentration

Percentages of Differential Leukocytes Count

Figure 3 showed that the mean of differential leukocytes count percentages were not decreasing significantly as the Na₂EDTA anticoagulant concentration increased beyond the standard concentration ($p > 0,05$), except for monocyte percentage ($p < 0,05$). Monocyte percentage was the only variable that showed linear result with its absolute count.

The mean of neutrophil count percentage in the excessive group is higher than that in the control group ($p < 0,05$). Neutrophil percentage was increasing as the anticoagulant increased, which was not concomitant with its absolute count result. Actually, the

absolute neutrophil count did not increase. Neutrophil percentage was relatively increased because the proportions of other cells absolute counts in total leukocyte population were decreasing more sharply than absolute neutrophil count. We assume that this decrement was due to cellular susceptibility, so probably they were not recognized by automatic cell counter as lymphocyte, eosinophil and basophil.

Percentages of lymphocyte, eosinophil and basophil were found to be varied and not significantly different among groups with standard and excessive Na₂EDTA concentration. These results were also not linear with their absolute count results.

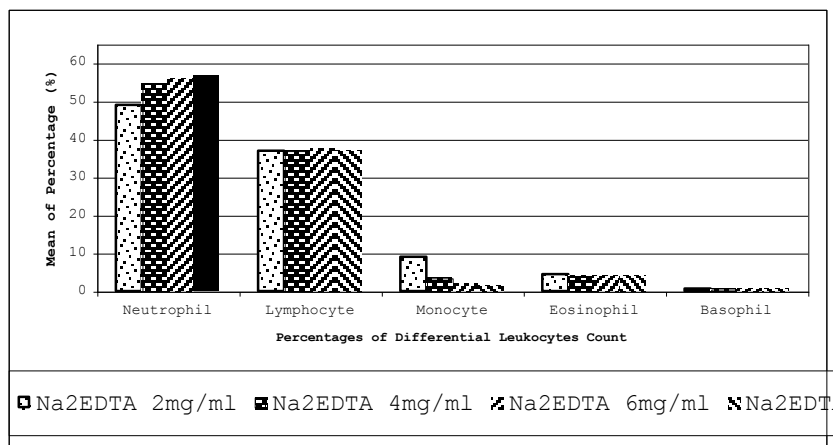


Figure 3. The relationship between mean of percentages of differential leukocytes count (%) with an increasing Na₂EDTA anticoagulant concentration

Morphology of Leukocytes

Figure 4 showed that the trend of mean of cells with irregular cytoplasm margin, vacuolation and irregular nuclei lobes per 100 leukocytes were increasing significantly ($p < 0,05$) following the administration of standard and excessive Na_2EDTA concentration, as compare to the control.

The descriptions of the leukocytes morphologic variables assessment were presented in Figure 5, 6 and 7. Since

we were not able to see the trend of morphologic changes following different excessive Na_2EDTA administration, we preferred to choose some of these figures, only to represent the view of the evaluated variables.

Figure 5 showed a lymphocyte with irregular cytoplasm margin. Unlike the smooth and regular cytoplasm margin found in normal lymphocyte, this irregular cytoplasm margin of lymphocytes, protrudes, perhaps due to hypertonic environment.

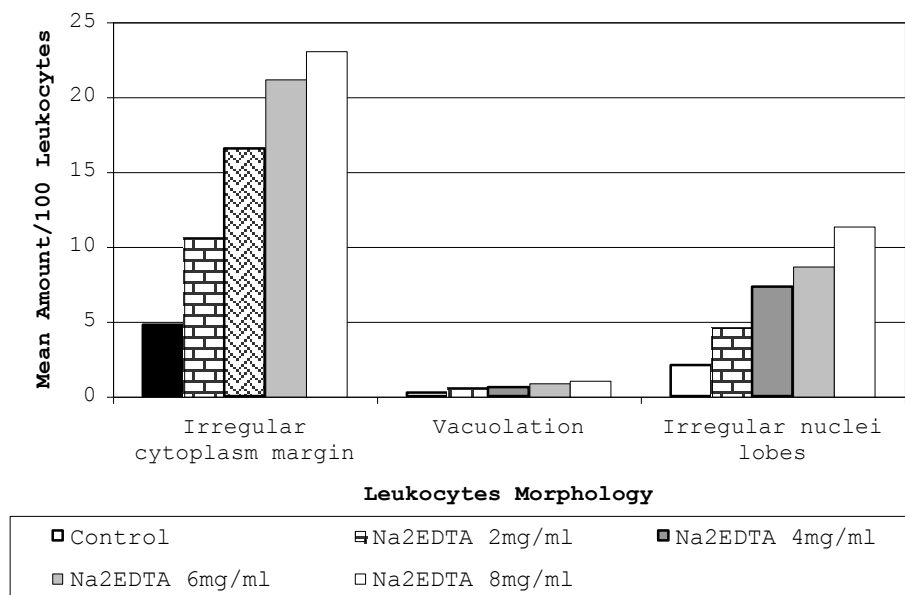


Figure 4. The relationship between mean of leukocytes morphologic variables per 100 leukocytes with various Na_2EDTA anticoagulant concentration

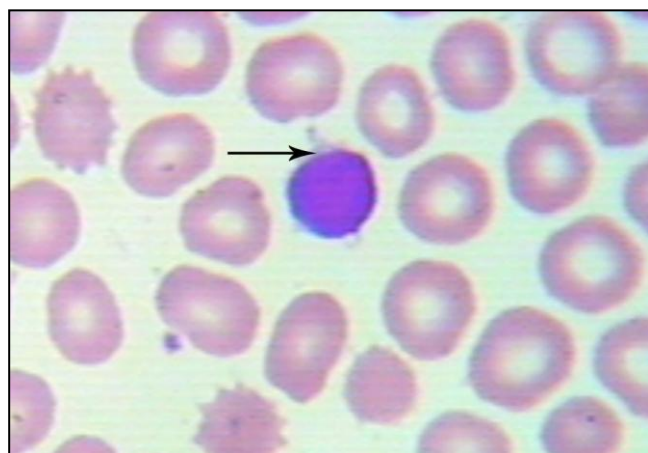


Figure 5. Photograph of Microscopic view at 100x magnifications. Irregular cytoplasm margin found in lymphocyte. Specimen Preparation with Na_2EDTA 6mg/ml

Figure 6 and 7 represents anticoagulant-induced vacuolation and irregular lobulation found in neutrophil. In the microscopic examination, we found that lymphocyte experienced irregularity of cytoplasm margin more than other leukocyte elements. On the other hand, neutrophil was the most cells that underwent irregular nuclei lobes, while vacuolation mostly appeared in neutrophil and monocyte.

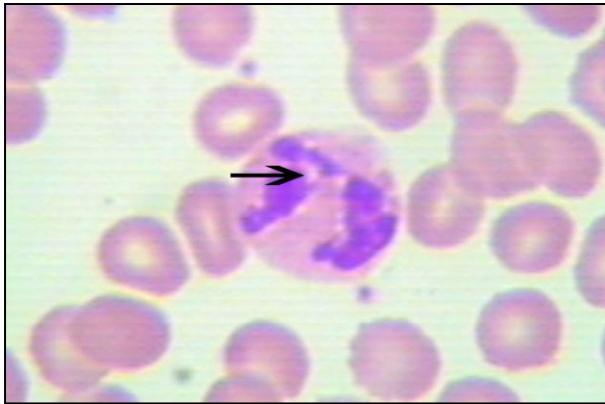


Figure 6. Photograph of Microscopic view at 100x magnifications. Vacuolation found in neutrophil. Specimen Preparation with Na_2EDTA 4mg/ml. Specimen Preparation with Na_2EDTA 6mg/ml

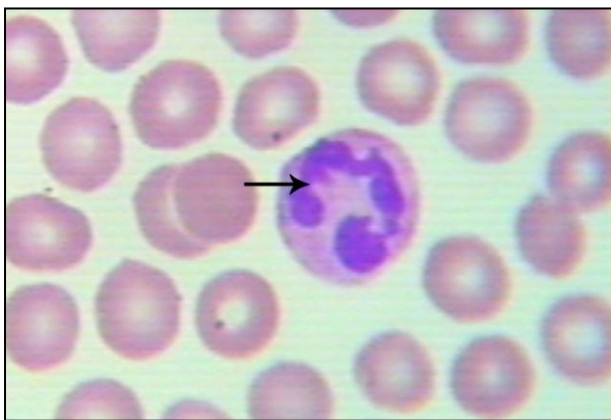


Figure 7. Photograph of Microscopic view at 100x magnifications. Irregular nuclei lobes found in neutrophil. Specimen Preparation with Na_2EDTA 8mg/ml

DISCUSSION

Though unclear, cellular properties of each cell such as cytoskeleton and granularities, may have specific roles in determining the results of absolute counts and percentages following excessive Na_2EDTA administration. The basic principle of SYSMEX SE-9500 automatic

cell counter in the detection of cells can also influence the result.

In clinical setting, the decrement of total leukocytes count is known as leukopenia. This quantitative alteration had been reported in some pathologic conditions such as decreased production and increased destruction of leukocyte following infection, post chemotherapy, glucocorticoid or ACTH administration and hyperthyroidism.⁴

Erroneous percentages of differential leukocytes count may also result in misdiagnosis. In this study, we found that there were excessive anticoagulant-induced neutrophilia and monocytopenia. Pathologic neutrophilia may be due to generalized or localized infections, inflammatory response to tissue destruction, neoplastic growth, metabolic disorders, acute haemorrhage and corticosteroid or lithium administration, while monocytopenia can be found in overwhelming infections and following glucocorticoids administration.^{4,8}

These findings indicate that excessive Na_2EDTA administration may cause misdiagnosis, because of the misinterpretation of leukocytes profile results. Patient with leukocytosis such as in acute bacterial infection and inflammation, might be misinterpreted as having leukopenia because of excessive Na_2EDTA administration to the blood specimen.

In patient with monocytosis such as in tuberculosis, dengue hemorrhagic fever in 6th and 7th days, syphilis and inflammatory responses,⁴ the result of peripheral blood examination may changed into monocytopenia if the specimen was prepared with excessive Na_2EDTA , thus will lead to misinterpretation of the patient's condition.

Like other human cells, leukocytes are eucaryotes cells that possessed cellular components, including plasma membrane, cytoplasm, cytoskeleton, organelles and nucleus. One of the cell components that is important to be focused on, in relation to the irregularity of cytoplasm margin and nuclei lobes, is the structure of phospholipid bilayer plasma membrane in the outer part of the cell and the nucleus membrane.⁹

Calcium is thought to be involved in the regulation and stabilization of membrane phospholipid structure. In an electron microscopic study concerning the effect of EDTA, cations, and various buffers on the morphology of erythrocyte membranes done by Pinteric et al, it was known that some ions, especially calcium, reversed the effect of EDTA that cause stromalytic forms of

erythrocytes membrane. In other words, EDTA which is a chelating agent that binds to calcium in preventing blood coagulation, is able to influence the stability of erythrocytes membrane, and perhaps also leukocytes membrane.¹⁰

Our knowledge about leukocytes membrane, its properties and the possible relationship with Na₂EDTA was not so extensive compare with those of erythrocytes. By considering the situation, we were only able to made assumption on how Na₂EDTA concentration can influence the leukocytes.

This assumption may describe why the increment of leukocytes cytoplasm margin and leukocytes nuclei lobes irregularity occurred linearly with the increment of Na₂EDTA anticoagulant concentration. However, the exact mechanism is not yet known. These irregularities also may describe why the leukocyte count decreased. As mentioned earlier, the cells would have been abnormal in shape and configuration, thus automatic cell counter was not able to detect them anymore.

Elevated pH in plasma leads to echinocytes formation in erythrocyte morphology. This finding also supported by other studies that proved that alteration in external PH cause changes in erythrocytes morphology, low PH produced stomatocytes whereas high PH produced echinocytes as observed in intact cell experiment.¹¹ This Na₂EDTA anticoagulant was assumed to produce increase pH, because sodium ion is the component of alkali. Echinocytes formation related with EDTA anticoagulant concentration involved mechanism that changed membrane structure of erythrocyte, mainly cytoskeleton and Ca²⁺ components.^{10,12}

In our observation, the qualitative alterations of leukocytes in the presence of excessive Na₂EDTA, when carefully examined, were not similar with those of pathologic condition. Stiene-Martin stated that vacuolation due to delay tends to be autophagocytic, which are small, evenly distributed vacuoles and the vacuoles reported to stain yellow rather than red with neutral red.⁴ We found that the vacuolation occurred due to excessive Na₂EDTA administration were similar to this description. We assume that excessive Na₂EDTA may induce vacuolation that resembled autophagocytosis, which may be found in prolonged storage of cells and exposure to certain toxins.

In clinical application, the presence of neutrophil vacuolation could not signify pathologic condition if it is less than 10% of the neutrophil population.⁴

Consequently, based on this study, vacuolation induced by excessive Na₂EDTA anticoagulant, was not qualitatively significant until it reach 4x concentration (8 mg/ml) to cause a misdiagnosis. However, inexperienced observer or laboratory workers may misclassify the findings as a sign of pathology thus result in overestimation of anticoagulant-induced vacuolation. This will lead to false positive cases emergence, and will be harmful for the patient.

Based on the result of this study, we must aware to maintain proper blood to anticoagulant ratio. It is suggested that blood collection procedure is directly performed into the commercially prepared negative-pressure vacum tubes (vacutainer tubes). In reading the result of leukocytes profile analysis from automatic cell counter, it is recommended to confirm the percentages of differential leukocytes count with absolute differential leukocytes count.

CONCLUSION

Excessive use of Na₂EDTA in blood specimen preparation, will lead to changes in leukocytes profile as the concentration increased. Standard Na₂EDTA concentration did not alter any leukocytes profile, except for irregular cytoplasm margin and irregular nuclei lobes.

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