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Research Article

Variations in Erythrocyte Count and Hemoglobin Levels in Blood Samples Collected Using Conventional Anticoagulants and Vacutainers with Different Secondary Homogenization Techniques

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ABSTRACT

The choice of anticoagulants and the methods of homogenization are critical pre-analytical steps that can significantly influence the accuracy of laboratory results. This study aims to assess the differences in erythrocyte count and hemoglobin levels among blood samples processed with conventional anticoagulants and Vacutainer tubes, taking into account variations in secondary homogenization techniques. A laboratory experimental design was employed, utilizing 32 blood samples acquired from eight participants. The analysis was conducted using an automated hematology analyzer. Data were subjected to a Paired T-Test, indicating no significant differences in erythrocyte counts (p=0.617) and hemoglobin levels (p=0.510) between conventional EDTA samples that were homogenized four times and those homogenized eight times. However, a significant difference was noted in erythrocyte counts (p=0.018), while hemoglobin levels remained unaffected (p=0.393) in EDTA vacutainer samples subjected to four versus eight rounds of secondary homogenization. Additionally, a significant difference was found in both erythrocyte counts (p = 0.008) and hemoglobin levels (p < 0.0001) between conventional EDTA samples and those subjected to four rounds of secondary homogenization using vacutainers. Similarly, there was a significant difference in erythrocyte counts (p = 0.006) and hemoglobin levels (p = 0.014) between conventional EDTA samples and Vacutainer samples that underwent eight rounds of homogenization.

Keywords*: Blood Specimen Collection; EDTA; Erythrocyte; Hemoglobin; Secondary Homogenization*

INTRODUCTION

Hematology examinations are among the most commonly conducted tests in healthcare institutions, serving as a foundation for clinicians in managing patient care. Therefore, the examination procedures must be conducted properly to ensure that the results obtained (Kuman, 2019). There are two categories of laboratory examinations within the field of hematology: routine

hematology tests and comprehensive hematology tests. Routine examinations encompass the counting of erythrocytes, platelets, and leukocytes, along with measurements of hemoglobin, hematocrit, and erythrocyte indices. In contrast, complete hematology examinations include routine blood tests that are enhanced by leukocyte differential counts, peripheral blood smears, blood morphology assessments, and peripheral blood profiles (Wahyuni & Aliviameita, 2021).

The accuracy of examination results is influenced by several factors, spanning the pre-analytical, analytical, and post-analytical stages (Sebayang et al., 2021). The addition of anticoagulants to blood samples represents a crucial pre-analytical step that significantly affects the precision of test outcomes (Ayuningsih et al., 2023). Another vital pre-analytical factor impacting examination accuracy is sample homogenization. This process is classified into two types: primary homogenization, which occurs directly after mixing the sample with the anticoagulant, and secondary homogenization, which involves re-homogenization prior to analysis by the instrument (Sebayang et al., 2021).

There are several types of anticoagulants commonly used in hematology examinations, including Ethylenediaminetetraacetic Acid (EDTA), Sodium Citrate, Sodium Oxalate, and Heparin. Among these, EDTA is the most widely utilized anticoagulant for hematological assessments, particularly in its Sodium EDTA (Na2EDTA) and Potassium EDTA (K2EDTA/K3EDTA) forms. EDTA is typically available in powder form, which can be conveniently dissolved into a 10% solution (conventional EDTA) or utilized directly in vacutainer tubes (Kuman, 2019). For adequate anticoagulation, 1 mg of EDTA is suitable for preventing coagulation in 1 ml of blood. In contrast, in a 10% solution, only 0.01 ml is needed per 1 ml of blood (Mentari et al., 2020). Notably, EDTA does not adversely affect blood cells, making it highly appropriate for hematology examinations (Hariyanto et al., 2024).

According to the Indonesian Minister of Health Regulation No. 43 of 2013, blood samples collected in tubes containing anticoagulants should be homogenized through inversion (flipping) 10-12 times. The Clinical and Laboratory Standards Institute (CLSI) guidelines from 2017 and BD Vacutainer recommend homogenizing these samples by inversion 8 to 10 times. Additionally, research by Lieseke and Zeibig recommends a similar inversion process of 5 to 10 times (Nuraeni & Septie, 2023).

Research conducted by Lilis Septiana Dewi, Tantri Analisawati Sudarsono, Retno Sulistiyowati, and Dita Pratiwi Kusuma Wardani in 2022 found no significant difference ($p = 0.822$) in erythrocyte count results when comparing EDTA vacutainers with conventional methods (Dewi et al., 2022). In a separate study by Rindy Arista Mustika Dewi in 2017, a significant difference ($p = 0.001$) in hematocrit levels was observed between EDTA vacutainers and conventional methods (Dewi, 2017). A 2023 study by Maria Nuraeni and Lidwina Septi found a significant difference ($p = 0.000$) in erythrocyte counts when comparing secondary homogenization techniques performed four times versus eight times (Nuraeni & Septie, 2023). Additionally, research by Rosnita Sebayang, Hotman Sinaga, and Mustika Sari Hutabarat in 2021 revealed no significant difference ($p = 0.938$) in hemoglobin levels across secondary homogenization performed three, five, seven, or eight times (Sebayang et al., 2021).

The findings from these studies indicate that specimen homogenization methods can contribute to variations in test results. The inversion technique for homogenization is commonly employed, with varying numbers of secondary inversions on specimens in vacutainers—typically ranging from 8 to 10 times, 10 to 12 times, or 5 to 10 times. Other studies have utilized different inversion frequencies, such as four and eight times, five and eight times, or three, five, seven, and

eight times. These discrepancies in the number of inversions have been shown to lead to variations in test outcomes. However, there is still a scarcity of studies that simultaneously evaluate the effects of specimen handling and secondary homogenization techniques during the pre-analytical stage. Consequently, further research is warranted to assess the impact of anticoagulant use and variations in secondary homogenization on complete blood count test results in whole blood specimens.

MATERIAL AND METHODS

This research has successfully passed the feasibility assessment conducted by the Research Ethics Committee for Research and Health at the Faculty of Dental Medicine, Airlangga University, Surabaya, bearing the certificate number 0349/HRECC.FODM/IV/2024. The study design employs a quantitative analysis utilizing laboratory experimental methods. It was carried out at the Clinical Pathology Laboratory of the D-IV Medical Laboratory Technology program at UMSIDA in May 2024.

For this study, a purposive random sampling technique was applied, with inclusion criteria stipulating that participants be male, aged 17 to 25 years, willing to participate, and able to complete and sign the informed consent form. Exclusion criteria included being female or being unwilling to participate in the research.

Blood samples were collected from 8 respondents, with a total volume of 8 mL, which were divided into four portions: 2 mL in two K3EDTA Vacutainer tubes and 2 mL in two vials containing 20 µL of 10% Na2EDTA anticoagulant. This study resulted in a total of 32 samples for examination. After secondary homogenization of the samples, the examination of erythrocyte count and hemoglobin levels was performed using an automatic method with a hematology analyzer (Medonic M-32). Statistical data analysis was conducted using SPSS version 23, with normality testing executed via the Shapiro-Wilk test, followed by a paired T-test at a significance level of 95%.

RESULTS

Data Analysis

Table 1. Mean Erythrocyte Count ± Standard Deviation

Variable	Mean Erythrocyte Count (cellsl/μl) ± Standard Deviation
Vacutainer Secondary Homogenization 4 times	5.462.500 ± 287.836,75
Vacutainer Secondary Homogenization 8 times	5.505.000 ± 275.369,88
Conventional Secondary Homogenization 4 times	5.330.000 ± 306.873,63
Conventional Secondary Homogenization 8 times	5.345.000 ± 283.700,04

According to Table 1, the average erythrocyte count in EDTA vacutainer blood samples subjected to secondary homogenization four times is 5,462,500 cells/µl. For samples with secondary homogenization performed eight times, the average count increases to 5,505,000 cells/µl. In conventional EDTA blood samples, the average count following secondary homogenization four times is 5,330,000 cells/µl, while for those homogenized eight times, the average count is 5,345,000 cells/µl.

Table 2. Mean Hemoglobin Levels ± Standard Deviation

Variable	Mean Hemoglobin Levels (g/dL)±Standard Deviation
Vacutainer Secondary Homogenization 4 times	16,16 ± 1,11
Vacutainer Secondary Homogenization 8 times	16,28 ± 1,24
Conventional Secondary Homogenization 4 times	15,65 ± 1,21
Conventional Secondary Homogenization 8 times	15,73 ± 1,14

According to Table 2, the average hemoglobin levels in EDTA vacutainer blood samples subjected to secondary homogenization four times are 16.16 g/dL. In contrast, for EDTA vacutainer blood samples that underwent secondary homogenization eight times, the average level rises to 16.28 g/dL. For conventional EDTA blood samples, the average hemoglobin level after four times of secondary homogenization is recorded at 15.65 g/dL, while for those subjected to eight times of homogenization, the average levelis 15.73 g/dL.

Table 3. Normality Test Result for Erythrocyte Count

Variable	Normality Test Result (*Shapiro-Wilk*)
Vacutainer Secondary Homogenization 4 times	0,878
Vacutainer Secondary Homogenization 8 times	0,946
Conventional Secondary Homogenization 4 times	0,821
Conventional Secondary Homogenization 8 times	0,467

Based on the results presented in Table 3, the normality test for erythrocyte count data indicates that the values for EDTA Vacutainer blood samples, subjected to secondary homogenization performed four times, are 0.878. For those samples with homogenization carried out eight times, the value is 0.946. In the case of conventional EDTA blood samples with secondary homogenization performed four times, the value is 0.821, whereas for those with eight homogenization iterations, it is 0.467. Since all results exceed 0.05, we can conclude that the data are typically distributed.

Table 4. Normality Test Result for Hemoglobin Levels

Variable	Normality Test Result (*Shapiro-Wilk*)
Vacutainer Secondary Homogenization 4 times	0,720
Vacutainer Secondary Homogenization 8 times	0,149
Conventional Secondary Homogenization 4 times	0,453
Conventional Secondary Homogenization 8 times	0,155

In Table 4, the results of the normality test for hemoglobin level data show that the value for EDTA vacutainer blood samples with secondary homogenization performed four times is 0.720. For samples with homogenization conducted eight times, the value drops to 0.149. In conventional EDTA blood samples, the values for four rounds of secondary homogenization and eight rounds are 0.453 and 0.155, respectively. Given that all these results are greater than 0.05, we can also conclude that this data is usually distributed.

Table 5. Statistical Test Results for Erythrocyte Count

SPSS Paired T-Test Result	
Compared Variables	*p* value
Vacutainer with Conventional Homogenization 4 times	0,008
Vacutainer with Conventional Homogenization 8 times	0,006

Vacutainer Homogenization 4 times with 8 times	0,018
Konvensional Homogenization 4 times with 8 times	0,617

ccording to the findings presented in Table 5, the results of the paired t-test reveal a significant difference when comparing EDTA Vacutainer blood samples to conventional samples subjected to secondary homogenization performed four times (p = 0.008). Likewise, a significant difference is observed for samples with homogenization conducted eight times (p = 0.006). Furthermore, a significant difference exists between the EDTA vacutainer samples that underwent secondary homogenization four times and those that were homogenized eight times (p = 0.018). However, no significant difference is noted between the conventional EDTA samples subjected to secondary homogenization four times and those homogenized eight times (p = 0,617).

Table 6. Statistical Test Results for Hemoglobin Levels

SPSS Paired T Test Result	
Compared Variables	*p* value
Vacutainer with Conventional Homogenization 4 times	0,000
Vacutainer with Conventional Homogenization 8 times	0,014
Vacutainer Homogenization 4 times with 8 times	0,393
Konvensional Homogenization 4 times with 8 times	0,510

Turning to Table 6, the results of the paired t-test indicate that for EDTA vacutainer blood samples compared to conventional blood samples with secondary homogenization performed four times, a significant difference is present (p = 0.000). Similarly, a significant difference is also found for samples with secondary homogenization performed eight times (p = 0.014). However, there is no significant difference between the EDTA vacutainer samples subjected to secondary homogenization four times and those homogenized eight times (p = 0.393). Additionally, no significant difference is observed between the conventional EDTA samples with secondary homogenization performed four times and those with secondary homogenization performed eight times (p = 0.510).

DISCUSSION

Based on the conducted research, several findings indicate the presence of differences, while others show no significant variations. In practice, the use of vacutainer tubes is generally recommended over conventional anticoagulant, as various factors can influence results when using the latter. It is crucial to consider the position of the pipette during anticoagulant pipetting; an angled pipette can reduce the volume of the solution being pipetted from the intended amount, thereby affectingthe examination results. Although vacutainer tubes offer distinct advantages in terms of practicality, human error can still impact the accuracy of examination results, whether conventional anticoagulants or vacutainer tubes are used. Therefore, precision in the procedure is essential (Faradilla, 2018).

In the realm of hematology laboratory examinations, EDTA is the most commonly employed anticoagulant. Several forms of EDTA are available, including Na2EDTA, K2EDTA, and K3EDTA. All EDTA salts possess hyperosmolar properties that can cause erythrocytes to undergo crenation. Notably, Na2EDTA and K2EDTA are more acidic than K3EDTA, which

demonstrates better stability due to their pH being closer to that of blood (Dewi, 2017). A study by Wina Syahrial Winarzat in 2021 revealed no significant differences in erythrocyte profile examination results, including erythrocyte count (p = 0.856), hemoglobin level (p = 0.997), hematocrit (p = 0.987), MCV (p = 0.934), MCH (p = 0.997), and MCHC (p = 0.733) when comparing Na2EDTA, K2EDTA, and K3EDTA, all of which were analyzed automatically using a hematology analyzer (Winazart, 2021).

The findings of this study reveal notable differences in erythrocyte count and hemoglobin levels between blood samples collected using EDTA vacutainers and those gathered with conventional EDTA methods. This finding contrasts with the research conducted by Lilis Septiana Dewi, Tantri Analisawati Sudarsono, Retno Sulistiyowati, and Dita Pratiwi Kusuma Wardani in 2022, which concluded that there were no differences (p = 0,822) in erythrocyte count results between the EDTA vacutainer and conventional methods. In their study, erythrocyte counts from EDTA vacutainer samples were found to be lower than those from conventional EDTA samples. This discrepancy may be attributed to the unsuitable blood volume in the vacutainer's EDTA, as well as the potential influence of technician fatigue, given that the testing method employed was manual (Dewi et al, 2022).

Conversely, this study aligns with the research by Rindy Arista Mustika Dewi in 2017, which identified significant differences (p = 0,001) in hematocrit levels when using EDTA vacutainers versus conventional methods. These variations could stem from inaccuracies in measuring the conventional EDTA anticoagulant, potentially causing erythrocytes to undergo crenation and resulting in lower hematocrit values. Thus, the precision of the conventional EDTA measurement plays a critical role in influencing the results (Dewi, 2017).

If the volume of the anticoagulant used is excessive, it can lead to hypertonicity in the blood sample. Significant hypertonicity causes fluid to flow out of cells to maintain osmotic pressure, resulting in cellular shrinkage or crenation, which may ultimately lead to hemodilution and a decrease in erythrocyte and hemoglobin counts (Esaputri et al., 2022).

The findings of this study show that there are no significant differences in erythrocyte counts in blood samples containing conventional EDTA anticoagulant after undergoing secondary homogenization for 4 and 8 cycles. Furthermore, no differences were observed in hemoglobin levels between blood samples collected with Vacutainer EDTA and those collected with conventional EDTA, regardless of whether they were subjected to secondary homogenization 4 or 8 times. This finding aligns with research conducted by Rosnita Sebayang, Hotman Sinaga, and Mustika Sari Hutabarat in 2021, which reported no differences (p = 0.938) in hemoglobin level assessments with secondary homogenization performed 3, 5, 7, and 8 times. The homogenization was conducted using the inversion technique, which is the preferred method for this purpose. Since there is no established gold standard for the number of secondary homogenizations, the researchers referenced the primary homogenization standard as a basis for comparison. Based on this, the process was deemed adequate to achieve thorough homogenization without impacting the examination results (Sebayang et al., 2021).

The findings of this study reveal variations in erythrocyte counts in blood samples utilizing EDTA Vacutainer anticoagulant following secondary homogenization performed 4 and 8 times. This result supports the research conducted by Maria Nuraeni and Lidwina Septi in 2023, which demonstrated significant differences (p = 0,000) in erythrocyte count results when employing secondary homogenization techniques for both 4 and 8 times. Factors contributing to these discrepancies may include the sedimentation process and incomplete sample homogenization.

When blood is not examined promptly, it undergoes sedimentation in three stages: rouleaux, sedimentation, and compaction. Consequently, the denser erythrocytes settle at the bottom of the tube. Conducting secondary homogenization four times may not achieve adequate homogenization, which could impact examination results (Nuraeni & Septie, 2023). The rouleaux stage lasts for 10 minutes, the sedimentation stage for 40 minutes, and the compaction stage takes an additional 10 minutes (Viani et al., 2022).

It is crucial that blood samples treated with anticoagulants, in accordance with the required volume and nature of the examination, be analyzed immediately, as delays can compromise hematology test results (Nuraeni & Septie, 2023). If a sample is left to stand for an extended period, sedimentation of blood cells occurs, resulting in the separation of blood cells from the plasma. Therefore, a secondary homogenization process becomes essential. Inadequate homogenization of anticoagulated blood can cause clotting, resulting in falsely low results, while excessive homogenization may lead to hemolysis (Sebayang et al., 2021).

Blood coagulation can be inhibited by introducing anticoagulants into the bloodstream. Homogenization is essential to ensure that coagulation factors are uniformly mixed, thereby preventing the formation of clots that could compromise the accuracy of examination results. This process is critical for maintaining the quality of analytical outcomes; inadequate homogenization may lead to sample clotting that might not be immediately apparent. Consequently, homogenization must be carried out according to established standards to preserve sample integrity and avoid complications such as hemolysis (Nuraeni & Septie., 2023). Hemolyzed samples can result in a reduction of erythrocyte counts, although hemoglobin levels might not be consistently affected (Lippi et al., 2015).

Variations in homogenization speed and stability across samples can also contribute to discrepancies in examination results. If the homogenization process between the blood sample and anticoagulant is insufficient, clot formation may occur, which can prevent the detection of cells by the hematology analyzer (Septie et al., 2022). Numerous factors can influence erythrocyte counts and hemoglobin levels, including the precise weighing of anticoagulants when preparing standard EDTA, the techniques employed in sample homogenization, and the methods of pipetting. The color density of the blood can impact hemoglobin measurements, and inaccuracies in EDTA preparation can cause erythrocytes to swell, which in turn affects both erythrocyte counts and hemoglobin levels (Hariyanto et al., 2024).

This study has limitations, including a restricted number of samples and test types. For future research, it is advisable to increase the sample size and explore a broader range of treatments and test parameters to yield more representative and valid results.

CONCLUSION

Based on the conducted research, the following conclusions can be drawn: There is no significant difference ($p = 0.617$) in erythrocyte counts between conventional EDTA blood samples subjected to four rounds of homogenization and those subjected to eight rounds of secondary homogenization. Similarly, there is no significant difference ($p = 0.510$) in hemoglobin levels between these two groups of conventional EDTA blood samples.

However, a significant difference ($p = 0.018$) in erythrocyte counts was observed between vacutainer EDTA blood samples that underwent four rounds of secondary homogenization and those that underwent eight rounds. Notably, there is no significant difference ($p = 0.393$) in

hemoglobin levels between vacutainer EDTA blood samples with secondary homogenization four times and those with it eight times.

Furthermore, significant differences were found in erythrocyte counts (p = 0.008) and hemoglobin levels (p = 0.000) when comparing conventional EDTA blood samples to Vacutainer EDTA blood samples with secondary homogenization performed four times. Additionally, significant differences were identified in erythrocyte counts (p = 0.006) and hemoglobin levels (p = 0.014) between conventional EDTA blood samples and Vacutainer EDTA blood samples subjected to eight rounds of secondary homogenization.

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