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CONTENTS

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INDONESIAN JOURNAL OF
CLINICAL PATHOLOGY AND MEDICAL LABORATORY
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RESEARCH

LEUKOCYTE INTERFERENCE ON HEMOGLOBIN EXAMINATION IN HEMATOLOGY MALIGNANCY

(Pengaruh Jumlah Leukosit terhadap Kadar Hemoglobin pada Keganasan Hematologi)

Trinil Sulamit, Fery H. Soedewo, Arifoel Hajat

ABSTRAK

Pasien keganasan hematologi sering menunjukkan gejala anemia dengan hasil laboratorium leukositosis. Tolok ukur penentu anemia salah satunya hemoglobin, namun pemeriksaannya terganggu oleh jumlah leukosit tinggi yang menyebabkan hemoglobin tinggi palsu. Penelitian ini bertujuan mencari berapa jumlah leukosit minimal penyebab hemoglobin tinggi palsu pada keganasan hematologi. Sampel darah EDTA pasien keganasan hematologi yang memeriksakan darah rutin di Laboratorium Patologi Klinik RSUD Dr. Soetomo bulan April–Mei 2016. Penelitian analisis observasional dengan membandingkan hemoglobin tanpa pemusingan dengan hemoglobin setelah pemusingan serta dibuang buffy coat-nya. Terdapat 93 sampel dengan leukemia 43%, limfoma 16,12% dan terduga leukemia 38%. Rerata selisih hemoglobin yaitu 0,06±0,089; 0,025±0,05; 0,1±0,081; 0,15±0,07; 0,81±0,63; 0,94±1,13; 1,13±0,718; 1,6±0,818 g/dL pada peningkatan kelompok jumlah leukosit secara berurutan >11.000-20.000 sel/μL, >20.000-30.000 sel/μL, >30.000-40.000 sel/μL, >40.000-50.000 sel/μL, >50.000-100.000 sel/μL, >100.000-200.000 sel/μL, >200.000-300.000 sel/μL, >400.000 sel/μL. Kenasaban rerata selisih hemoglobin dengan kelompok jumlah leukosit menunjukkan makin tinggi leukosit maka makin besar rerata selisih hemoglobin dengan kenasaban positif sedang. Semakin tinggi jumlah leukosit makin tinggi persentase hemoglobin tinggi palsu dan rerata selisih hemoglobin makin besar. Leukosit minimal penyebab hemoglobin tinggi palsu pada keganasan hematologi yaitu 56.745 sel/μL yang memiliki kepekaan 89,4% dan kekhasan 100%, dengan koefisien κ 0,592.

Kata kunci: *Leukositosis, hemoglobin tinggi palsu, keganasan hematologi*

ABSTRACT

Hematology malignancies patients often show symptoms of anemia with leukocytosis in laboratory results. Hemoglobin is one of the parameters determining anemia, but the examination is disturbed by high leukocyte counts which lead to false high hemoglobin. This study was conducted to find the lowest leukocyte number that caused false high hemoglobin in hematology malignancy. Patient's EDTA blood samples of hematology malignancy were examined at the Clinical Pathology Laboratory in the Soetomo Hospital, April-May 2016. Observational analysis study was done by comparing between hemoglobin without centrifugation and hemoglobin after centrifugation and removing the buffy coat. There were 93 samples with 43% leukemia, 16.12% lymphomas and 38% suspected leukemia. Mean delta hemoglobin was 0.06±0.089; 0.025±0.05; 0.1±0.081; 0.15±0.07; 0.81±0.63; 0.94±1.13; 1.13±0.718; 1.6±0.818 g/dL with an increasing number of leukocytes in a sequential group >11,000-20,000 cells/μL, >20,000-30,000 cells/μL, >30,000-40,000 cells/μL, >40,000-50,000 cells/μL, >50,000-100,000 cells/μL, >100,000-200,000 cells/μL, >200,000-300,000 cells/μL, >400,000 cells/uL. Correlation of mean delta hemoglobin with increased number of leukocytes showed that the higher leukocytes the higher mean delta hemoglobin with moderate positive correlation. The higher leukocyte number the larger percentage of false high hemoglobin and mean delta hemoglobin. The lowest number of leukocytes that caused false high hemoglobin in hematology malignancies was 56,745 cells/μL with a 89.4% sensitivity and 100% specificity, with κ coefficient of 0.592.

Key words: Leukocytosis, false high hemoglobin, hematology malignancies

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INTRODUCTION

Hematology malignancy are one of the non-infectious diseases causing mortality. The data from inpatient department of Indonesian hospitals in 2008 showed that from 100% of non-infectious diseases in Indonesia, 7.3% (3,189 cases) were leukemia and 6.5% (2,862 cases) were Non-Hodgkin Lymphoma, these were hematologic malignancies that emerges.[1] Based on Riskesdas 2013 hematology malignancies odd ratio in Indonesia was 0.9 (range 0.6 to 1.4 with a 95% CI/Confidence Interval).[2] Symptoms in hematology malignancies are anemia and increased number of leukocytes (leukocytosis). In acute leukemia 50% leukocytes count increased >10,000–100,000 cells/μL, 25% leukocyte count >100,000 cells/μL and 25% leukocyte count could be normal or decreased. In early stages of chronic leukemia the leukocyte count was 20,000-50,000 cells/μL and in late stages it can be hyperleukocytosis >100,000 cells/μL. Anemia is also a common symptom in hematology malignancies, it is an important diagnosis related to the clinician's decision to give blood transfusion or not.[3–5]

Hemoglobin level is one of the parameters for diagnosing anemia that can be examined by spectrophotometry method. In theory, a high leukocyte count interferes with the hemoglobin examination in this method. High leukocyte count can cause turbidity because leukocytes do not lyse. Patient hematology malignancies with high leukocyte count can caused false high hemoglobin result, so one should be aware concerning anemia diagnosis in hematology malignancies.[6–8]

The number of leukocytes that interfere hemoglobin examination with spectrophotometry method suggested differences in the leukocytes number. Some references stated that leukocyte count >100,000 cells/μL caused the above.[6] Some journals stated leukocytes count >50,000 cells/μL can cause false high hemoglobin.[7,12] Some stated >60,000 cells/μL while other stated >30,000 cells/μL.[7,12,13] Study in leukocyte numbers that interfere hemoglobin level on hematology malignancy patients is still limited. The aim of the study was to analyze the minimal number of leukocytes that can cause false high hemoglobin in hematology malignancies.

METHODS

This study was an observational analytical study with cross-sectional design, using EDTA blood samples from hematology malignancies patients who regularly examined blood at the Clinical Pathology Laboratory in the Dr. Soetomo Hospital during April to May 2016. This study used an automated hematology analyzer Sysmex XN-1000® as the instrument to measure hemoglobin levels and leukocyte counts. Inclusion criterias for this study were EDTA blood with a diagnosis of hematology malignancies, leukocyte count >11,000 cells/μL and age ≥21 years. Exclusion criterias were platelet count >700,000 cells/μL, lipemia and hyperchylomicronemia samples (plasma visually appeared turbid, with triglyceride levels ≥1 g/dL), sample patients with diagnosis of Multiple Myeloma and Waldenstrom Macroglobulinemia (containing abnormal plasma protein), hemolyzed samples (plasma visually is homogenous red, with plasma hemoglobin level >1 g/dL), bilirubinemia (plasma visually looks yellow/jaundiced with a bilirubin level of >30 mg/dL), erythrocyte number after treatment more than ±10% from the initial count.[6–9,14]

Lines of inquiry from EDTA blood with a diagnosis of hematology malignancies were examine for exclusion criteria. Samples that included in the inclusion criteria were directly examined for hemoglobin level and leukocyte count. The sample was then centrifuged at 3000 rpm for 10 minutes, it resulted a three layers of plasma, buffy coat and red blood cells. The buffy coat was removed with pasteur pipette manually as much as possible, after the buffy coat removed, the sample was homogenise and examined for hemoglobin level. The aim of centrifugation was to concentrate leukocytes in the buffy coat layer between plasma and erythrocytes. Removing buffy coat tend to eliminate the factor that interferes hemoglobin examination. Concentrated leukocytes at the buffy coat layer, was removed as much as possible without removing the erythrocytes. Hemoglobin levels were compared between hemoglobin from samples without centrifugation and hemoglobin from samples with centrifugation and removed buffy coat, if there was a difference between hemoglobin level this was a false high hemoglobin. Data were statistically analyzed with Kolmogorov-Smirnov test, Spearman correlation test and Receiver Operator Curve (ROC) with SPSS 20.0 version.

RESULTS AND DISCUSSION

This study collected data from 150 samples, there were some excluded samples such as leukocytes <11,000 cells/μL as much as 2.6% samples, 2% with platelets as >700,000 cells/μL, 33.33% with erythrocytes count after treatment was more than ±10%. Thirty-three point three percent samples were

excluded because the removal procedure was done manually with Pasteur pipette, so the erythrocyte and plasma were accidentally discarded.[1,2] Leukemia is a major hematology malignancy in Indonesia.[3] Samples with leukemia diagnosis in this study were as much as 43%, suspected leukemia 40.86% and 16.12% lymphomas. These data showed that leukemia incidence corresponded with Rahman study in Bangladesh and data from inpatient department of Indonesian hospitals in 2008. Rahman obtained 112 (0.77%) patient samples with leukemia and 3 samples (0.02%) with leukemoid reaction from 14,500 total samples. Leukemia in males was more higher than females, Rahman showed samples of males (58%) and females (42%), even Bakta stated that leukemia incidence in males was higher than females. In this study, the proportion of males was 71 samples (76.35%) and females was 22 samples (23.65%). The onset of acute leukemia was in young adults, this was supported by a study result with the age between 21-35 years old. The incidence of chronic leukemia at all ages and data from this study showed 21-63 years old, consistent with Bakta.[3,11] Characteristics of the samples are shown in Table 1.

The data were analyzed for false high hemoglobin by grouping the number of leukocytes. Grouping leukocytes was based on the grouping of leukocyte numbers in Rahman study, who stated that leukocyte counts >11,000 cells/μL may give false high hemoglobin. Examination of hemoglobin by SLS-Hb method, the reagent lysed the erythrocytes but leukocytes do not lyse, so the higher leukocytes the more turbid the solution. Turbidity, resulting the light beam was being scattered/blocked/dispersed by the unlysed leukocytes, only few light passed the cuvette. Hemoglobin is measured by absorbance, meaning that the light that passed the cuvette are measured by the detector. Few light was received by the detector, it was caused by turbidity, assuming a high absorbancy, so that the computer displayed a false high hemoglobin level.[11,16]

Table 1. Characteristics of the samples

Characteristics of the samples	Total
Sex (93 sample)	
– Males	71 (76.35%)
– Females	22 (23.65%)
Age (years)	
– Mean	30.77±10.86
– Age range	21-76
Diagnosis	
– Suspect leukemia	38 (40.86%)
– Acute myeloid leukemia	22 (23.65%)
– Chronic myeloid leukemia	16 (17.20%)
– Non-Hodgkin lymphoma	14 (15.05%)
– Chronic lymphocytic leukemia	2 (2.15%)
– Hodgkin lymphoma	1 (1.07%)
False high hemoglobin	
– Positive result	85
– Negative result	8

Kolmogorov-Smirnov test was used to show correlation between delta hemoglobin level with the number of leukocyte, the test showed moderate positive correlation (0.583). Delta hemoglobin level was from hemoglobin without centrifugation minus hemoglobin after centrifugation and removed buffy coat. Number of leukocytes was taken from first examination samples without removal buffy coat. A positive correlation between leukocyte count and mean delta hemoglobin can be seen in figure 1. This correlation result showed in a scatter graph, a positive correlation could be seen between the number of leukocytes (horizontal axis) and the mean delta hemoglobin (vertical axis). This meant that the higher leukocytes the higher mean delta hemoglobin. This was demonstrated by the increasing mean delta hemoglobin from each group number of leukocytes 0.06±0.089; 0.025±0.05; 0.1±0.081; 0.15±0.07; 0.81±0.63; 0.94±1.13; 1.13±0.718; 1.6±0.818 g/dL (Table 2). Rahman obtained the same results with this study, the higher leukocyte count the higher false high hemoglobin levels. Mean delta hemoglobin in his study was 0.2 g/dL, 0.3 g/dL, 0.45 g/dL, 0.6 g/dL, 1.2 g/dL, 2.5 g/dL, 3.6 g/dL, 4.7 g/dL, 5.8 g/dL. He concluded that increased leukocytes of 50,000 cells/μL showed a mean delta hemoglobin of 0.556 g/dL. There were differences in the amount of mean delta hemoglobin between this study and Rahman, although both were increased as well. This may be caused by some differences although the same method was carried out (SLS-Hb and HiCN). Rahman used semi-automatic spectrophotometer, 530 nm wavelength, comparing hemoglobin level with and without centrifugation to remove the sediment, sample volume 20 μL diluted with reagent until final dilution 1:251 and no diagnosis criteria of samples.[11] The difference was that in this study used automated spectrophotometer with a 540 nm wavelength, comparing the levels of hemoglobin level without centrifugation and hemoglobin level after centrifugation and removing the buffy coat, sample volume was 0.4 μL diluted by reagent until a final dilution 1:747 and diagnosis criteria from hematology malignancies samples.[12,14]

Table 2. Mean delta hemoglobin without and with centrifugation and buffy coat removal in leukocytes count grouping

Leukocytes count	Mean delta hemoglobin±SD
>11,000-20,000	0.06±0.089
>20,000-30,000	0.025±0.05
>30,000-40,000	0.10±0.081
>40,000-50,000	0.15±0.070
>50,000-100,000	0.81±0.635
>100,000-200,000	0.4±1.131
>200,000-300,000	1.06±0.437
>300,000-400,000	1.31±0.718
>400,000	1.60±0.818
Mean delta hemoglobin	**0.85±0.816**

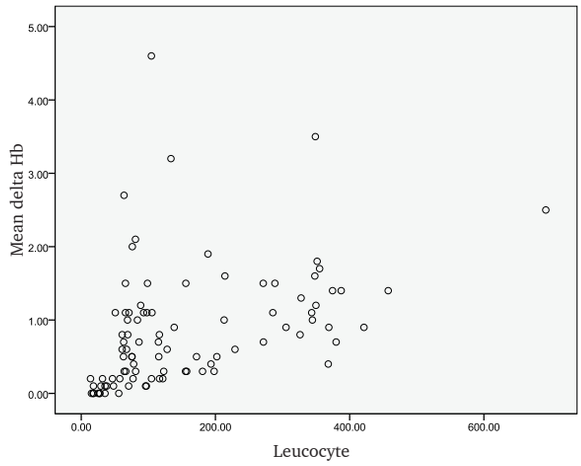

Figure 1. Correlation of leukocytes with mean delta hemoglobin scatter graph

There was some scatter outside the scatter group, this was called outliers, this might be other factors that affect hemoglobin levels besides leukocytes, like erythrocyte numbers and plasma. These outliers should be controlled, so the result can be standardized. Procedure of manually removing leukocytes with Pasteur pipette caused this outlier. Manual procedure resulted in erythrocyte and plasma were discarded, this manual procedure was less standardized. If plasma was discarded, but not erythrocytes, the hemoglobin level will increase, whereas if erythrocytes was discarded, the hemoglobin will be decreased.[4,15]

The ROC result showed an Area Under Curve (AUC) of 0.966 (Figure 2). The minimal number of leukocytes

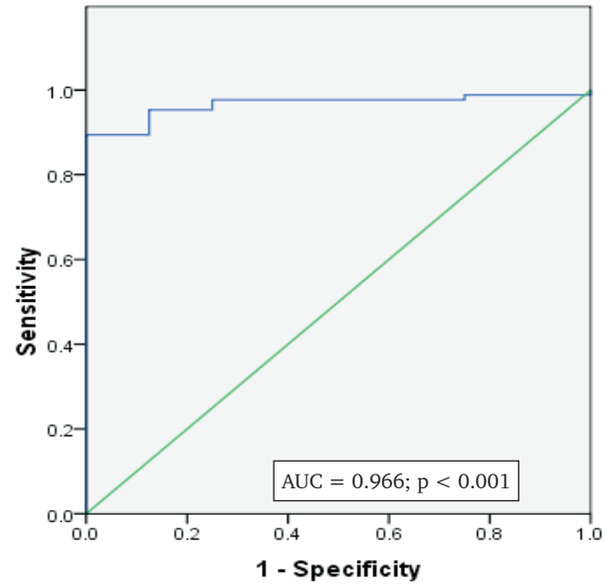

Figure 2. ROC curve from leukocytes and false high hemoglobin

that caused false high hemoglobin was 56,745 cells/μL, with a 89.4% sensitivity and 100% specificity. Agreement between false high hemoglobin and leukocyte count by ROC obtained kappa coefficient (κ) 0.592 (moderate) with a significancy of <0.001. This study result resembled some journals such as Sanberg, Ward, Lippi and Guidi and Mc Pherson and Pincus. These journals stated that false high hemoglobin became significant at a leukocyte count of more than 50,000 cells/μL. Disagreement about number of leukocytes that caused false high hemoglobin can be due to many factors. Hemoglobin level was determined by the concentration of hemoglobin in each erythrocyte, red blood cell count and hematocrit. Factors that interfere with hemoglobin examination were lipemia and hyperchylomicronemia, increased leukocyte counts, high platelet counts (>700,000 cells/μL), abnormal immunoglobulins, severe hemolysis, HbCO (carboxyhemoglobin), hyperbilirubinemia and erythrocytes that were not lysed. In addition, differences in method, instrument and sample criteria can also influence. Zandecki stated that there was no clear cut off number of leukocyte count to cause false high hemoglobin, but one should be aware when leukocyte count is >50,000 cells/μL or >100,000 cells/μL. One automated hematology analyzer stated that a limit of leukocyte count >250,000 cells/μL did not interfere hemoglobin examination with spectrophotometer method, because all leukocytes are lysed prior to hemoglobin examination.[14,15,14]

This study has some weaknesses, the manual procedure in removing buffy coat, this made the

procedure was not standardized, when leukocytes were withdrawn, erythrocytes and plasma were wasted accidentally as well. The denser the buffy coat, the more difficult removing leukocytes and more erythrocytes and plasma were discarded. The denser buffy coat caused that the margin of buffy coat and erythrocyte was blurred and less plasma obtained. Plasma volume or hematocrit was not controlled yet, so this can disturb the results and obtain outlier data. Another limitation was that this study used leukocytosis samples only. Carboxyhemoglobin >10% and erythrocytes containing Hb C that were not lysed can contribute to turbidity, these factors can not be excluded in this study because they were not examined or measured.[11,15,16]

CONCLUSION AND SUGGESTION

The correlation between delta hemoglobin (from hemoglobin without centrifugation and hemoglobin after centrifugation with removal buffy coat) with the number of leukocyte, showed a moderate positive correlation (0.583). It showed that the higher the leukocyte count the higher the mean delta hemoglobin. The minimal leukocyte number that caused false high hemoglobin was 56,745 cells/μL, with a 89.4% sensitivity, 100% specificity and kappa coefficient (κ) 0.592. The number of leukocytes was one factor that could interfere hemoglobin examination in samples with hematology malignancies, although there were other factors that affected hemoglobin level. Hemoglobin level from samples with leukocyte $\geq$56,745 cell/μL should be corrected by removal of the buffy coat after 3000 rpm centrifugation. This study can be continued with controlling the number of red blood cell, plasma volume and hematocrite before and after treatment.

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