RESEARCH ARTICLE

Interference of in vitro hemolysis complete blood count

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Background: Hemolysis may occur in vivo, under pathological conditions, or in vitro, related to pre-analytical errors. Hemolyzed samples may produce unreliable results, leading to errors in diagnostic and monitoring evaluations. This study aims to evaluate the interference of in vitro hemolysis on the interpretation of the parameters of the blood cell-counting performed by the impedance method.

Methods: Peripheral blood samples were collected in anticoagulant K2-EDTA and subsequently divided into three 1.0 mL aliquots. The first aliquot was not subjected to any intervention, and the second and third aliquots were passed 5 and 10 times through a small-gauge needle to produce scalar amounts of hemolysis. Hematological tests were performed by Hemacounter 60-RT 7600®.

Results: Comparison of the samples with different degrees of hemolysis showed a decrease in red blood cells count and hematocrit counts and increase in mean corpuscular hemoglobin concentration and platelet count in samples with a high degree of hemolysis. According to the accepted clinical point of view, the samples with a high degree of hemolysis exceeded the desirable bias, presenting decrease in red blood cells count, hematocrit and mean corpuscular volume, and increase in red cell distribution width, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet counts. However, samples with a mild degree of hemolysis showed only a slight increase in mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet count.

Conclusion: This study demonstrated that in vitro hemolysis can decrease the clinical and analytical reliability of the assessment of the blood count.

KEYWORDS

analytical error, blood cell count, clinical interference, hemolysis, impedance

1 | INTRODUCTION

Hemolysis is one of the most common causes of pre-analytical errors.¹ Defined as rupture of the red blood cell membrane with extravasation of hemoglobin and other intracellular components into the surrounding plasma, hemolysis can be detected visually during laboratory evaluation due to the pink to red plasma's coloration after sample centrifugation.² Hemolysis may occur in vivo, which is related to a clinical-pathological condition, or in vitro, related to pre-analytical errors.³

In vitro hemolysis usually results from the inadequate blood collection, involving factors such as the use of small-gauge needles, transfer of alcohol residue from the skin to the sample, difficulty in locating venous access, small and fragile veins that are easily traumatized, and attempts to unsatisfactory puncture.³ In addition, incorrect handling of samples such as insufficient filling of collection tube leading to excess anticoagulant, vigorous sample shaking, exposure to excessively hot or cold temperatures, and centrifugation at a very high speed for an extended period is also factors that may compromise the blood cells structural integrity.³

Inadequate blood sample analysis can lead to errors in diagnostic and monitoring evaluations, producing unreliable results that negatively impact the analytical quality and patient safety.⁴ Concurrently, hemolyzed samples frequently require the collection of a new clinical sample, delaying patient care.⁵

Previous studies have demonstrated that hemolysis may interfere with the methodology of several laboratory tests.^{2,3} Therefore, it is important for the clinical laboratory to perform studies to evaluate the interference of hemolysis for all laboratory test protocols.⁶

The literature presents a restricted number of studies about the influence of hemolysis on the complete blood count (CBC)⁷; moreover as far as we know, no data have been reported in using the impedance-based hematology analyzers. Therefore, the objective of this study was to evaluate the interference of in vitro hemolysis on the blood cell-counting parameters interpretation performed by the impedance method.

2 | MATERIALS AND METHODS

The volunteers of this research were recruited from the University Laboratory of Clinical Analyzes (LUAC), Ponta Grossa, Paraná. Participants were duly informed about the methods and objectives of this study and, after agreeing, signed an informed consent form. This study was approved by the Research Ethics Committee of the State University of Ponta Grossa (UEPG), N° 1.339.496. This research was conducted in accordance with the Declaration of Helsinki.

Biological venous blood samples were collected according to standard procedure, using a needle (0.70 × 30 mm gauge, 22G × 1 1/4) and disposable syringe (Becton Dickinson-Biosciences®, San Jose, California, USA). Blood samples were collected within 30 seconds of garroting, as well as the wait for the total evaporation of the 70% alcohol used in asepsis, and traumatic collections were excluded. The whole blood was placed in tube with anticoagulant K2-ethylenediaminetetraacetic acid (EDTA) and mixed gently.

Subsequently, the biological samples were placed for 15 minutes in a hematological homogenizer with a constant speed of 10 rpm. Immediately after homogenization, whole blood from each K₂-EDTA tube was divided into 3 aliquots of 1.0 mL each.

The first aliquot was not subjected to any intervention. While the second and third aliquots were subjected to mechanical hemolysis according to the Dimeski method: The anticoagulated total blood of the second aliquot was passed 5 times, whereas the third aliquot ten times through a small caliber needle (0.55 × 20 mm, 24G × 3/4) to elicit scalar amounts of hemolysis.⁸

All aliquots were analyzed on the Hemacounter 60-RT 7600® hematology counter (Hemogram, Brazil), by the impedance principle (colorimetry for hemoglobin measurement) to categorize and count the blood cells. All analyzes were performed with the same batch of reagents. The hematological parameters analyzed were as follows: red blood cell (RBC) count, hemoglobin, hematocrit, RDW (red cell distribution width), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration

(MCHC), total leukocytes, percentage counts of three subpopulations—lymphocytes, medium-sized cells (including monocytes, basophils, and eosinophils), granulocytes, and platelet counts.

Subsequently, the K₂-EDTA plasma was separated by centrifugation at 1200 g for 10 minutes, for determination of the degree of hemolysis according to free hemoglobin—Harboe method.⁹

The aliquots that passed through mechanical intervention were diluted 1/10 with saline solution. The principle of direct spectrophotometry was used by the absorption of oxyhemoglobin in the Soret band (415 nm) for the correction of absorption by interfering substances (lipids and bilirubin) with readings at 380 and 450 nm.¹⁰ The concentration of free hemoglobin was checked with absorbance determinations using the formula: $Hb (g/L) = [(167.2 * A_{415}) - (83.6 * A_{380}) - (83.6 * A_{450}) * 1/1000 * \text{dilution}]$ (A_{415} : absorbance at 415 nm, A_{380} : absorbance at 380 nm, and A_{450} : absorbance at 450 nm).

The result obtained is presented in g/L, being further divided by 10 to be expressed in g/dL. Then, the following formula was used to calculate the percentage of hemolysis¹¹: $\text{hemolysis degree (\%)} = [(100 - Ht) * (\text{free Hb}/\text{total Hb})]$, where Ht: hematocrit (in %), Hb: hemoglobin (in g/dL).

Later, the biological samples were divided according to the degree of hemolysis, in three groups: (i) group without hemolysis: samples without intervention; (ii) group of samples with hemolysis degree less than 5% (HD < 5%); and (iii) group of samples with hemolysis degree greater than 5% (HD > 5%).

In the statistical analysis, data normality was verified by the Shapiro-Wilk test. Once normality was confirmed, the data were presented as mean and standard deviation (mean ± SD) and the comparison of the means was performed by the Student's t test for paired samples. To assess the degree of agreement between the groups the Bland-Altman method was used; expressed as a percentage of the values in the axis $[(HD < 5\% \text{ or } HD > 5\% - \text{no hemolysis})/\text{mean\%}]$ vs the mean of the two measurements, with analysis of limits of agreement and their confidence intervals. Clinically acceptable limits were defined by the analytical quality specifications for desirable bias, for RBC ± 1.7%, hemoglobin ± 1.8%, hematocrit ± 1.7%, MCV ± 1.2%, MCH ± 1.4%, MCHC ± 0.8%, RDW ± 1.7%, leukocytes ± 5.6%, lymphocytes ± 7.4%, medium-sized cells ± 13.2%, granulocytes ± 9.1%, and platelets ± 5.9%.¹² Data were analyzed by the MedCalc® program, version 11.4.2.0 (MedCalc Software, Mariakerke, Belgium), and the level of significance was set at $P < .05$.

3 | RESULTS

Venous blood samples were collected from healthy subjects with an average age of 21.5 ± 0.7 years. Each sample was divided into three aliquots, with a total of 30 CBCs evaluated: 10 without hemolysis, 10 with mild degree of hemolysis (HD < 5%) and 10 with high degree of hemolysis (HD > 5%).

The difference between mild and high hemolysis groups was demonstrated by the free hemoglobin level and the degree of hemolysis, with a consequent significant increase ($P = .007$ and $P = .004$,

respectively) of the results for the HD > 5% group compared to the HD < 5% group (Table 1).

The CBC results for each study group are shown in Table 1. It was observed that some parameters of the CBC showed a statistically significant difference for the samples with a high degree of hemolysis in comparison with the sample group without hemolysis.

The HD > 5% group presented a significant decrease for the parameters, RBC count ($P = .035$), and hematocrit ($P = .014$) in relation to the group without hemolysis. At the same time, the HD > 5% group demonstrated a significant increase in MCHC results ($P = .003$) and platelet count ($P = .004$) in relation to the nonhemolyzed group (Table 1).

The parameters hemoglobin, RDW, MCV, MCH, leukocyte counts, lymphocytes, medium-size cells, and granulocytes did not present statistical difference between the analyzed groups (Table 1).

The results of the Bland-Altman interpretation for the RBC parameters are shown in Figure 1. The HD < 5% group did not present any parameters with a mean difference outside the acceptable clinical limit (Figure 1A, C, E, and G).

In the HD > 5% group, a significant bias of the acceptable clinical limits of $\pm 1.7\%$ was observed for the parameters: RBC count, hematocrit, and RDW (Figure 1B, F, H). However, no significant result was observed for the hemoglobin parameter which has an acceptable clinical limit of $\pm 1.8\%$ (Figure 1D).

The Bland-Altman graphs for the RBC indices are shown in Figure 2. The MCV indices showed clinical difference only in samples with a high degree of hemolysis, where a significant bias was observed in comparison with an acceptable clinical limit of $\pm 1.2\%$ (Figure 2B).

The clinical difference was also observed for the MCH and MCHC indices, which presented a significant bias in comparison with the acceptable clinical limit of $\pm 1.4\%$ and $\pm 0.8\%$; respectively, both in the group with mild degree of hemolysis and in the group with high degree of hemolysis (Figure 2C, D, E, F).

Figure 3 shows the Bland-Altman graphs for the leukogram parameters. No significant differences were identified because the parameters analyzed were within the acceptable limit of leukocytes $\pm 5.6\%$ (Figure 3A, B), lymphocytes $\pm 7.4\%$ (Figure 3C, D), medium-sized cells $\pm 13.2\%$ (Figure 3E, F), and granulocytes $\pm 9.1\%$ (Figure 3G, H).

The results of the Bland-Altman interpretation for platelet count are shown in Figure 4. The samples with degree of mild hemolysis, as well as the samples with degree of high hemolysis, presented a significant bias in comparison with clinical limit of 5.9% (Figure 4A, B).

4 | DISCUSSION

The results of this study demonstrated that samples with a high degree of hemolysis represent a problem for the clinical laboratory in the interpretation of the CBC, performed by the impedance method as it exhibited alterations in certain RBC parameters, indices and in platelet counts.

The results are similar to those found by Lippi et al⁷ for the Siemens Advia 2120 counter, showing a decrease in RBC and hematocrit counts and, on the other hand, an increase in MCHC and in platelet counts in samples with high degree of hemolysis.

TABLE 1 Mean $\pm$ standard deviation of the analyzed samples: without hemolysis, hemolysis degree less than 5% (HD < 5%), and hemolysis degree greater than 5% (HD > 5%)

Parameter	Without hemolysis (n = 10)	HD < 5% (n = 10)	HD > 5% (n = 10)
Free hemoglobin (g/dL)	-	5.98 $\pm$ 3.44	22.38 $\pm$ 12.09**
Hemolysis degree (%)	-	2.42 $\pm$ 1.40	9.75 $\pm$ 5.67**
RBC count (mol/L/ μ L)	4.79 $\pm$ 0.32	4.73 $\pm$ 0.27	4.49 $\pm$ 0.29*
Hemoglobin (g/dL)	14.39 $\pm$ 1.25	14.48 $\pm$ 0.75	13.67 $\pm$ 0.96
Hematocrit (%)	42.12 $\pm$ 3.16	41.79 $\pm$ 1.97	38.78 $\pm$ 3.07*
RDW	12.66 $\pm$ 0.39	12.55 $\pm$ 0.25	13.06 $\pm$ 0.68
MCV (fL)	87.85 $\pm$ 1.60	88.32 $\pm$ 1.05	86.29 $\pm$ 2.63
MCH (pg)	29.99 $\pm$ 0.90	30.59 $\pm$ 0.57	30.43 $\pm$ 0.90
MCHC (%)	34.14 $\pm$ 0.53	34.28 $\pm$ 0.57	35.43 $\pm$ 0.90*
Total leukocytes (10^3 cells/mL)	5.59 $\pm$ 1.36	5.39 $\pm$ 0.77	5.86 $\pm$ 1.71
Lymphocytes (%)	37.04 $\pm$ 7.05	36.97 $\pm$ 4.44	38.34 $\pm$ 8.75
Medium-sized cells (%)	7.05 $\pm$ 1.26	7.25 $\pm$ 1.17	7.01 $\pm$ 1.18
Granulocytes (%)	55.91 $\pm$ 7.43	55.77 $\pm$ 4.62	54.65 $\pm$ 9.51
Platelet counts (10^3 cells/mL)	247 $\pm$ 29.10	267 $\pm$ 41.64	393 $\pm$ 101.34*

Student's t test-not applicable.

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

*Statistically significant difference in relation to the group without hemolysis, $P < .05$.

**Statistically significant difference in relation to the HD group <5%, $P < .05$.

FIGURE 1 Bland-Altman graphs for RBC parameters: concordance analysis between samples without hemolysis and HD < 5% (A, C, E, and G); without hemolysis and HD > 5% (B, D, F, and H)

FIGURE 2 Bland-Altman graphs for RBC indices: concordance analysis between samples without hemolysis and HD < 5% (A, C, and E); without hemolysis and HD > 5% (B, D, and F)

The present study also evaluated the results according to the acceptable clinical point of view,¹² through the interpretation of the Bland-Altman graphs. The samples with a high degree of hemolysis exceeded the quality specifications for the desirable bias, presenting a decrease in RBC (4.7%), hematocrit (6.6%), MCV (0.6%), and an increase in the

parameters: RDW (1.3%), MCH (1.5%), MCHC (2.5%), and platelet count (36.7%). While samples with a mild degree of hemolysis had a modest increase in MCH (0.6%), MCHC (0.7%), and platelet count (1.4%).

Reduced RBC counts and hematocrit are clearly explained by hemolysis¹³ because these parameters directly evaluate the affected

FIGURE 3 Bland-Altman graphs for leukocyte parameters: concordance analysis between samples without hemolysis and HD < 5% (A, C, E, and G); without hemolysis and HD > 5% (B, D, F, and H). Lin, Lymphocytes; Mid, medium-sized cells; Gra, granulocytes

FIGURE 4 Bland-Altman graphs for platelet counts: concordance analysis between samples without hemolysis and HD < 5% (A); without hemolysis and HD > 5% (B)

cells. Regarding the evaluation of the Bland-Altman graphs, it was observed that the RCB had a bias of -6.4% (-22.8% to 10.0%) and the hematocrit a bias of -8.3% (-25.7% to 9.2%), exceeding the permitted specifications of $\pm 1.7\%$ for samples with a high degree of hemolysis. Thus, these parameters can be underestimated by up to 22.8% for RBC and up to 25.7% for the hematocrit or overestimated by up to 10.0% and 9.2% , respectively. These results are in agreement with previous data, which also showed a decrease in RBC count and hematocrit of -18.2% (-27.2% to -9.3%) and -20.6% (-33.3% to -7.9%), respectively.⁷

RBC fragmentation may lead to a modest reduction in MCV with increased RDW due to increased red cell volume variability, a result of high standard deviation;¹⁴ because the RDW is obtained by the relation of the distribution curve with a standard deviation, divided by the MCV.¹⁵

The RDW parameter presented a bias of 3.0% (-8.9% to 15%) and the MCV a bias of -1.8% (-8.9% to 5.3%) for the samples with a high degree of hemolysis, exceeding the allowed specifications of $\pm 1.7\%$ and $\pm 1.2\%$, respectively. However, Lippi et al⁷ demonstrated for the RDW a bias of 0.1% (0.9% - 1.0%), within the acceptable clinical limit; and a bias of -2.6% (-3.8% to -1.4%) for MCV, evaluated by the Siemens Advia 2120[®] equipment.

One possible explanation would be that the MCV measurement principle differs widely with the hematological analyzer used.¹⁵ In the present study, the volume is directly evaluated by impedance after isovolumetric diffusion, while in the Siemens Advia 2120, the MCV is directly measured by hydrodynamic focusing and optical dispersion.¹⁶ Therefore, the present study demonstrated that, by the impedance method, samples with a high degree of hemolysis may present a modest change in MCV (increase of 0.6%) with a consequent RDW increase of 1.3% , presenting a wide variation, in approximately 12% , evaluating the limits of agreement and their confidence intervals.

Therefore, it should be taken into account that a spurious change in one parameter often means that the validity of other parameters

needs to be analyzed.¹⁷ For example, in cases of hemolyzed samples with the presence of a stable hemoglobin concentration, decreased RBC and hematocrit, calculated parameters, such as MCH [(Hb/RBC) * 10] and MCHC [(Hb/Ht) * 100], may show a false increase.¹³ Thus, MCHC is an important RBC indices in the evaluation of the CBC, because it can alert the clinical laboratory to a spurious result.¹⁸

When evaluating the graphs, both indices presented a clinical difference, where a bias of 2.0% (-6.3% to 10.3%) for MCH and 1.5% (-3.3% to 6.3%) for MCHC were observed in the samples with mild degree of hemolysis and 1.5% (-6.6% to 9.5%) and 3.3% (-1.7% to 8.3%) for the samples with high hemolysis, respectively. In both situations, exceeding the allowed specifications of $\pm 1.4\%$ for MCH and $\pm 0.8\%$ for MCHC was observed. These results are in agreement with previous data demonstrating an increase in the indices for samples with hemolysis.⁷

The platelet count was another parameter that presented alteration from the clinical point of view according to the degrees of hemolysis evaluated in the study. This parameter presented a bias of 7.3% (-34.2% to 48.7%), for samples with a mild degree of hemolysis; and a bias of 42.6% (-16.8% to 101.9%), for samples with high hemolysis, both exceeding the allowed specifications of $\pm 5.9\%$. These results are similar to previous data showing increased platelet counts with the bias of 18.1% (4.2% to 32%) for samples with a mild degree of hemolysis and 40.6% (20.1% to 61.1%) for samples with a high degree of hemolysis.⁷

It is conceivable that cell debris and stroma resulting from erythrocyte breakdown can generate substantial analytical interference in platelet count.¹³ Because the counters that use the impedance method evaluate the cells by disrupting the electrical current generated by the cell volume and are unable to differentiate between erythrocytes and platelets, as they are analyzed in the same counting chamber of the equipment, according to its volume.¹⁹ Therefore, nonplatelet elements similar in size to platelets may result in inaccurate counting.¹⁹ Thus, a significant increase in the platelet count in samples with a high degree

of hemolysis is justified by the fact that this count is affected by the presence of RBC fragments.²⁰

The main difference of this study was evaluating hemolyzed samples in the impedance-based hematology analyzer. The results of this study showed different results when compared with other methodology, as the flow cytometry method. In this way, the clinical laboratory should have knowledge about technologies used in hematology analyzers and the possible changes in the results due to the different measurement principles in the hemolyzed samples.

In conclusion, the results of the present study clearly demonstrate that in vitro hemolysis, which frequently occurs in the pre-analytical phase of the laboratory routine, in the evaluation of the CBC performed by the impedance method, may decrease clinical and analytical reliability due to the influence on the interpretation of results.

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