A Second-Generation Blood Substitute (Perfluorodichlorooctane Emulsion) Generates Spurious Elevations in Platelet Counts from Automated Hematology Analyzers

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Perfluorocarbon emulsions (PFEs) appear as platelets in automated cell counters, which may affect samples from thrombocytopenic patients (less than 100,000/μL). Therefore, we mixed clinically relevant concentrations of perfluorodichlorooctane (Oxyfluor®; Hemagen, Inc., St. Louis, MO) *in vitro* with whole blood samples ranging from 0 to 150,000 platelets/μL and compared a new counter that uses optical platelet recognition (Abbott CellDyn 3200; Santa Clara, CA) with conventional electroimpedance-based counters (Abbott CellDyn 3500 and CellDyn 1700). We found that emulsion particles appear as small-sized platelets either in diluent or in blood. The emulsion results in a reproducible overestimate of the platelet counts, of greater importance as PFE concentration increases, and as the actual platelet count of the blood samples decreases. The new optical technology yields smaller overestimates but, even at low PFE concentrations, gives an unacceptable relative error at platelet counts near the transfusion thresholds recommended by the American Society of Anesthesiologists guidelines for blood component therapy. Unexpected interference in the leukocyte and erythrocyte channels is also reported. Experimental limitations preclude extrapolation of these findings to other automated cell counters, because differences in technology or software may affect their capacity to separate PFE particles from platelets. **Implications:** Perfluorocarbons are being investigated under conditions in which thrombocytopenia is likely to occur. In this *in vitro* study, we demonstrate significant overestimates in platelet counts from automated cell counters at clinically relevant perfluorocarbon concentrations in thrombocytopenic blood samples.

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Perfluorocarbon emulsions (PFEs) are oxygen-carrying solutions now in widespread clinical trials (1). PFEs may reduce requirements for allogeneic blood transfusion and find an application in cardiac surgery or in situations wherein tissue ischemia may occur. At our institution, a second generation PFE, Oxyfluor® (Hemagen, Inc., St. Louis, MO) has been in a Phase I trial for coronary artery bypass graft patients. During this trial, we observed a significant overestimate of the platelet count with impedance-type automated counters, particularly in thrombocytopenic patients. This suggested that PFEs might interfere with automated hematology analyzers. Plasma samples of patients receiving PFEs appear lipemic and, because of light-scattering effects, are known to cause interference in many colorimetric and spectrophotometric methods used in clinical laboratories. These compounds may have impact not only on hematology tests, but also on other laboratory-automated analyzers, including those used in chemistry study (2,3). Hematology tests are important in the care of patients likely to receive PFEs, but few studies have addressed the potential modification of these tests by oxygen-carrying solutions, and have only investigated normal blood samples (3–6).

In this study, we characterize the interference when Oxyfluor® is present at relevant concentrations in human blood. We also compare conventional electroimpedence-type analyzers with a new analyzer that uses optical platelet recognition, and we evaluate

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the consequences of this interference on thrombocytopenic human blood.

Methods

Perfluorochemical

Oxyfluor® is a PFE that contains egg yolk phospholipids and safflower oil as the emulsifying agents and perfluorodichlorooctane 40% by volume (PFDCO; Hemagen, Inc., St. Louis, MO).

Hematology Analyzers

The Abbott CellDyn 1700 (Santa Clara, CA) uses an impedance methodology to assess the platelet (PLT), red blood cell (RBC), white blood cell (WBC) counts, and a three-part leukocyte differential (LD) (7). The Abbott CellDyn 3500 uses both impedance (RBC, PLT, and WBC) and laser optical methodologies (WBC and LD) (8). The new Abbott CellDyn 3200 uses a laser optical methodology (WBC, LD, PLT, and RBC).

Patient Material

Fresh human whole blood, anticoagulated with K_3EDTA, came from samples received for routine analysis by our laboratory. Thrombocytopenic and normal blood samples were randomly selected. Our institutional human studies committee approved this study as part of the continuing clinical trial, for which informed consent was obtained from the subjects.

Methodology

Characterization of the Interference. Normal blood samples from five volunteers were analyzed in the CD3500, our institutional reference counter, to measure the actual PLT, WBC, and RBC counts. We obtained 1-mL aliquots of 1% and 8% PFDCO by mixing blood from each sample *in vitro* with Oxyfluor®. Concentrations of 1% have previously been measured in the blood of patients receiving 1.5 g/kg PFDCO (unpublished data), and concentrations of 8% have been measured in animals (9) given 7.2 g/kg, the maximum dosage recommended by the manufacturer. For each instrument, the differences between control, 1% PFE, and 8% PFE were analyzed by using an unpaired *t*-test with a Bonferroni correction (significance at $P < 0.01$). Using all instruments and for each aliquot, we performed five successive measurements plus five other measurements over 4 h, which represents the maximal time before blood samples are being processed at our institution. The coefficients of variability of these two series of measurements describe, respectively, the reproducibility and the stability of the interference.

Mechanism of the Interference. In all instruments, 6 aliquots containing 0%, 1%, 3%, 6%, 8%, or 16% PFDCO were analyzed in the CellDyn diluent (Isoton®: buffered saline) to investigate any dose effect of PFDCO alone on the hemocytometers. Using all instruments, we obtained histograms of normalized PLT number versus PLT volume distribution for measurements of 8% PFDCO alone in diluent, normal whole blood, and blood containing 1% and 8% PFDCO. This analysis was performed to compare how each analyzer separates PLT from PFDCO particles.

Impact of the Interference on Thrombocytopenic Blood. In all instruments, two series of 10 aliquots containing 1% and 8% PFDCO mixed *in vitro* with 10 random-selected thrombocytopenic samples (6,000–148,000 PLT/μL) were analyzed. Actual counts for the 10 thrombocytopenic samples were assessed with the CD3500, our institutional reference counter, before being mixed and were corrected for dilution. This evaluated the interference as a function of both the severity of the thrombocytopenia and the PFDCO concentration.

Results

Characterization of the Interference

The presence of PFDCO resulted in dose-dependent elevations in the PLT count with the impedance methodology (Table 1). The changes were significant only at 8% PFE ($P < 0.01$), but demonstrated a trend toward increase even at 1% PFE. There was no change in PLT count with the optical methodology. The successive measurements presented coefficients of variation smaller than 4.5% for the worst performance, not significantly different from control with 1% or 8% PFE, within the normal ranges of each instrument and accounting for reproducibility. The measurements over 4-h presented coefficients of variation smaller than 5.1% and were considered relatively stable, except for the CD1700 where 8% PFE seemed to cause significant variations in PLT counts ($P < 0.01$ for coefficient of variation 8% PFE vs control or vs 1%; Table 1). RBC counts were not affected with both methodologies, except in the CD1700, where high PFDCO concentrations (8%) caused unexpected decreases (Table 2). WBC counts were not affected with the optical methodology, but were overestimated by impedance methodologies, with significant increases at 8% PFDCO only ($P < 0.01$; Table 2).

Mechanism of the Interference

When PFDCO was alone in diluent, there was a strong linear correlation between measured PLT counts ($r^2 > 0.99$) or WBC counts ($r^2 > 0.98$), and PFDCO concentration with the same differences between the analyzers as described (Fig. 1). In all instruments, PFDCO alone produces the same signal as small PLTs (<10 pL,

Table 1. Platelet Counts from Hematology Analyzers When Perfluorodichlorooctane Is Mixed with Normal Blood Samples ($n = 5$)

		Control	PFE 1%	PFE 8%
Platelet counts (1,000/μL) (counts ± SD)				
CellDyn 1700	(Impedance)	226 ± 33	259 ± 39	488 ± 41*†
CellDyn 3500	(Impedance)	223 ± 31	253 ± 27	434 ± 28*†
CellDyn 3200	(Optical)	221 ± 31	216 ± 33	226 ± 22
Reproducibility (Coefficient of variation ± SEM)				
CellDyn 1700	(Impedance)	2.6 ± 0.4	2.5 ± 0.5	3.6 ± 0.8
CellDyn 3500	(Impedance)	4.5 ± 0.3	4.2 ± 1.5	3.1 ± 0.3
CellDyn 3200	(Optical)	3.0 ± 0.4	2.8 ± 0.5	2.4 ± 0.2
Stability over 4 h (Coefficient of variation ± SEM)				
CellDyn 1700	(Impedance)	4.5 ± 1.3	5.5 ± 1.1	33.4 ± 8.3*†
CellDyn 3500	(Impedance)	3.9 ± 1.0	5.1 ± 0.6	3.4 ± 0.4
CellDyn 3200	(Optical)	2.9 ± 0.2	3.8 ± 0.6	3.1 ± 0.6

CellDyn 1700, 3500, and 3200; Abbott, Santa Clara, CA.
Control = whole fresh blood, PFE 1% or 8% = whole fresh blood mixed with 1% or 8% PFE, PFE = perfluorocarbon emulsion, counts corrected for dilution.
* PFE 8% vs control ($P < 0.01$, unpaired t-test with Bonferroni correction).
† PFE 8% vs 1% ($P < 0.01$, unpaired t-test with Bonferroni correction).

Table 2. Red and White Blood Cells from Hematology Analyzers When Perfluorodichlorooctane Is Mixed with Normal Blood Samples ($n = 5$)

		Control	PFE 1%	PFE 8%
RBC counts (1,000/μL) (counts ± SD)				
CellDyn 1700	(Impedance)	4.6 ± 0.1	4.6 ± 0.1	3.5 ± 0.3*†
CellDyn 3500	(Impedance)	4.9 ± 0.1	4.8 ± 0.1	4.8 ± 0.1
CellDyn 3200	(Optical)	4.8 ± 0.1	4.7 ± 0.1	4.7 ± 0.1
WBC counts (1,000/μL) (counts ± SD)				
CellDyn 1700	(Impedance)	5.6 ± 0.5	5.7 ± 0.6	18.5 ± 5.2*†
CellDyn 3500	(Impedance)	6.3 ± 0.6	6.7 ± 0.6	11.9 ± 0.4*†
CellDyn 3500	(Optical)	6.5 ± 0.6	6.3 ± 0.6	6.2 ± 0.6
CellDyn 3200	(Optical)	5.8 ± 0.6	6.1 ± 0.6	5.9 ± 0.5

CellDyn 1700, 3500, 3200; Abbott, Santa Clara, CA.
Control = fresh whole blood, PFE 1% or 8% = fresh whole blood mixed with 1% or 8% PFE, PFE = perfluorocarbon emulsion, WBC = white blood cell, RBC = red blood cell; counts corrected for dilution.
* PFE 8% vs control ($P < 0.01$, unpaired t-test with Bonferroni correction).
† PFE 8% vs 1% ($P < 0.01$, unpaired t-test with Bonferroni correction).

normal PLT range 2–25 pL). As PFDCO concentration increases in blood, this signal overwhelms the PLT signal from normal blood faster in impedance instruments than in the optical counter, which performs better (Fig. 2). Similarly, impedence instruments included PFDCO as lymphocytes, which caused a decrease in the granulocytes part of the LD (data not shown).

Impact of the Interference

The emulsion always produced an overestimate of the PLT count, with increasing error as PFE concentration increased and as the actual platelet count of the blood samples decreased. Overestimates of PLT counts were smaller, although significant, using optical methodology (Table 3).

Discussion

We demonstrated a significant overestimate of the PLT count with automated hematology analyzers when PFDCO is present at relevant concentrations in human blood (Table 1). This overestimate is directly related to PFDCO concentration, as well as to the severity of the thrombocytopenia, but it is smaller using optical rather than impedance technology. Unexpectedly, we also found less significant dose-dependent overestimates of the WBCs with the impedance technology, and slight overestimates of the RBCs at large PFDCO concentrations in only one of the two impedence-based analyzers (Table 2).

These effects of the PFEs rely on the size of their particles, which range from 0.1 to 0.350 μM (10). All types of analyzers consider these particles as small PLTs (Fig. 2A), although with errors of different magnitude (Fig. 1A). Since the relationship between measured PLT counts versus PFEs alone demonstrated a good linear correlation ($r^2 = 0.99$), this could be potentially used to generate a correction factor for PLT count measurements in the presence of this agent using the PFE concentration in blood, which is easy to measure by centrifugation (11) or to estimate by calculation (12). However, when mixed

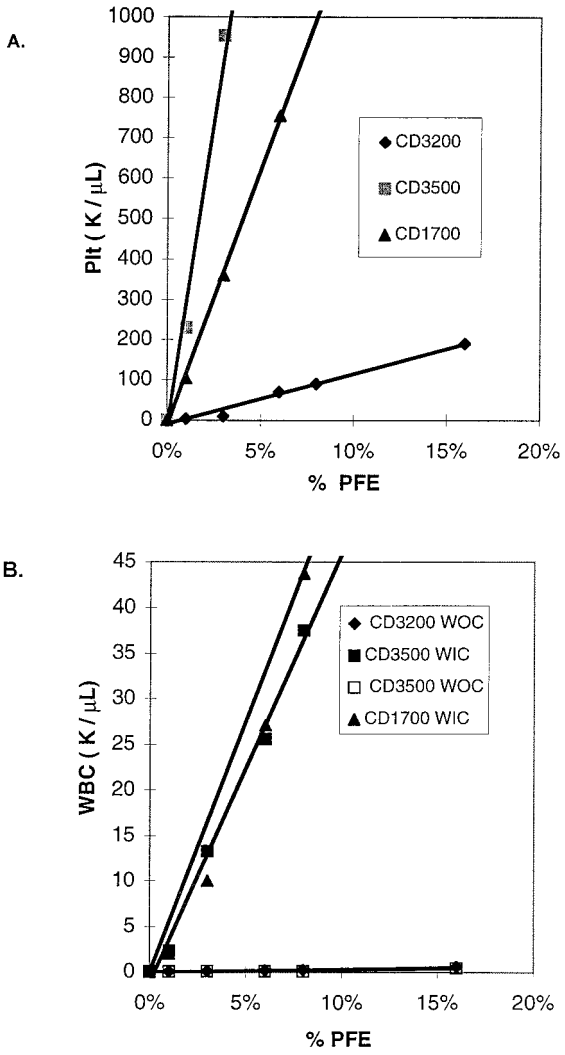

Figure 1. Dose-dependent effect of Oxyfluor® alone on hematology analyzers in the platelet channel (A) and in the white blood cell channel (B). A, The measured platelet count is a linear function of the percentage of perfluorocarbon emulsion in diluent ($r^2 > 0.99$ by linear regression for CD3200, CD1700, and CD3500). Results for CD3500 and CD1700 are outside scale range at concentration of perfluorocarbon emulsion > 6%. B, The measured white blood cell count is a linear function of the percentage of perfluorocarbon emulsion in diluent ($r^2 > 0.98$ by linear regression for CD3200, CD1700, and CD3500). CD3200 = CellDyn 3200, CD3500 = CellDyn 3500, CD1700 = CellDyn 1700 (Abbott, Santa Clara, CA), WOC = white blood cell optical count, WIC = white blood cell impedance count, WBC = white blood cell, Plt = platelet, PFE = perfluorocarbon emulsion, K/μL = 1,000/μL.

with blood, this dose-dependent relationship may not be linear, because PFE particles and PLT may interact. Furthermore, this correction factor would be instrument-specific, because each analyzer appears to have a different ability to differentiate PFDCO particles from PLT (Fig. 2, C and D). This ability varies mainly between optical and impedance instruments, although differences in the software algorithms used may also be a factor since two impedance analyzers from the same manufacturer separate PLTs differently from non-PLT signals (Fig. 1A). Both methodologies use either the change in an electrical current (impedance), either the light-scattering angles of a laser beam (optical) produced by each individual cell or other particles to measure their characteristics (8). Combining the information from up to four angles of light scatter, optical sensors can recognize intracellular organelles and offer a better cell discrimination than the single measurement of impedance variation.

These findings are apparently contradictory to the decrease in PLT count described by previous investigators using impedance analyzers and attributed to particle agglutination of an unspecified PFE with PLTs (5). This agglutination was possibly related to the large 80% PFE concentration used, which may explain differences from our study, as we used lower, but more clinically relevant, concentrations. Agglutination increased over time while the low coefficients of variation from our measurements over four hours (<5.1%) describe a rather stable phenomenon, occurring immediately after mixing and not disappearing at least for four hours—the estimated maximum latency before processing. We could not observe any particle size effects because all particles were probably trapped in the agglutinates. This size effect has already been described as reporting the tendency of impedance analyzers to falsely include acellular aggregates in their count (4).

The direct relationship between the error in PLT count versus the severity of thrombocytopenia may be problematic. Blood substitutes are currently being investigated in situations in which thrombocytopenia is likely to develop (hemorrhage, extracorporeal bypass) and in which PLT transfusion can reduce morbidity or even be life-saving. In settings such as cardiac surgery, identification of nonsurgical bleeding or PLT transfusion triggers should rely on testing PLT function as well as PLT count, because those procedures are known to affect both PLT quality and quantity (13). In addition, PFEs have been reported to dose-dependently inhibit PLT activation and aggregation (14). Unfortunately, none of the current point-of-care tests of PLT function (bleeding time, thromboelastography, and laboratory testing) achieves the necessary specificity, reliability, simplicity, or rapidity (15). Hence, the identification of PLT transfusion triggers relies on the clinician's experience and on PLT counts. The American Society of Anesthesiologists guidelines for blood component therapy recommend transfusion when the PLT count is below 50,000 or even 100,000/μL for interventions at risk for blood loss and/or PLT consumption (16). Relative errors for these two values are, respectively, 296% or 152% under the best conditions (i.e., optical methodology at 1% PFDCO) (Table 3). Therefore, significant thrombocytopenia in a patient receiving PFEs may go uncorrected if automated PLT counts are not questioned.

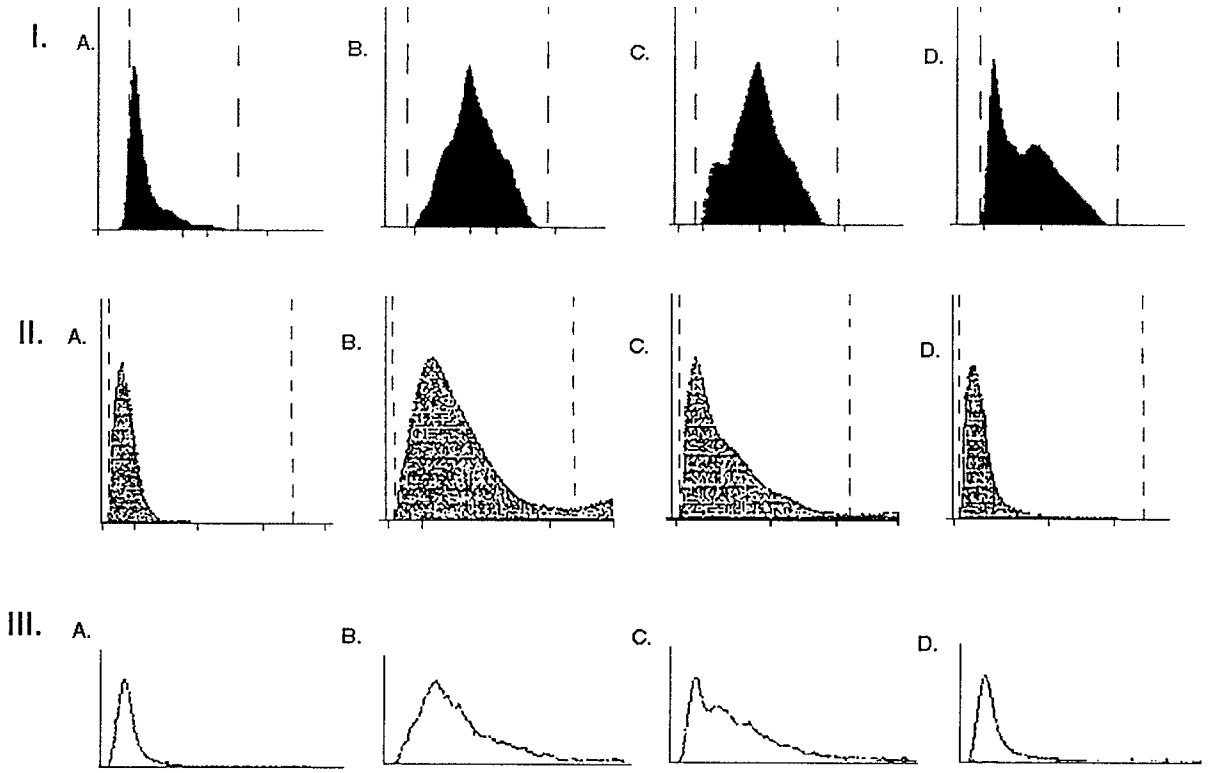

Figure 2. Histograms of the normalized number of platelets (Y axis) versus platelet size distribution (X axis) for the CellDyn 3200 (I), CellDyn 3500 (II), and CellDyn 1700 (III) (Abbott, Santa Clara, CA). A, Oxyfluor® 8% (Hemagen, Inc., St. Louis, MO) in diluent: all analyzers read a specific signal in the low range of platelet size. B, normal signal read for fresh whole blood. C, Oxyfluor® 1% in fresh whole blood: part of the signal considered by all analyzers comes from the particles of Perfluorocarbon emulsion. This part is smaller than the signal generated by the platelets in the CellDyn 3200, but larger in the two other analyzers. The CellDyn 3500 is barely able to differentiate the two signals, although the CellDyn 1700 still does to some degree. D, Oxyfluor® 8% in fresh whole blood: virtually all of the signal from the CellDyn 3500 and 1700 comes from the particles of Perfluorocarbon emulsion, whereas the signal from the CellDyn 3200 still contains a recognizable smaller part coming from the platelets.

This study has limitations that may moderate these conclusions. We have studied only one compound, and each of the substances that are currently under investigation may have different impacts on a variety of automated analyzers—not only hematological, but also others including chemistry studies. Another PFE, Perflubron® (Alliance Pharmaceutical Corp., San Diego, CA), may also present the same problem since it has a similar, although somewhat smaller, particle distribution size (0.1–0.250 μM) as PFDCO (17). However, the published results of Phase II clinical studies with intravascular Perflubron® do not report such interference at equivalent doses (1.5 g/kg), perhaps due to a more uniform particle size distribution and a better stability of the emulsion (18). Because all three studied analyzers from the same manufacturer reacted differently, we did not evaluate whether this effect would differ with other manufacturer's instruments. The current study was also limited to mixing PFE with blood in vitro, and it is unknown whether these elevations in blood cell counts may similarly occur with PFEs circulating *in vivo* for a few hours. The latter issues need to be addressed in patients receiving this and other similar agents by measuring the concentration of the respective agents, the PLT counts via several instruments and via manual counting to assess actual counts. Manual counting would be mandatory to confirm that the actual PLT count is substantially lower than that measured with the analyzers. We processed blood samples in the institutional reference automated analyzer (CD3500) before mixing them with PFEs and corrected these counts for dilution. We chose this approach because manual counting is time-consuming, and some reports suggest that it might not be as accurate as automated analysis when actual counts are low (19). The small number of analyzed human blood samples limits the degree of statistical significance of our conclusions, although the interference in the group of normal blood samples shows a small variability when compared with the importance of the errors (Table 1). As for the reported interference of PFDCO with thrombocytopenia, we chose to obtain thrombocytopenic samples by random selection as needed, rather than by dilution, which made it difficult to constitute a large pool of blood samples and prevented reliable statistical analysis. This should be

Table 3. Error in Measurement When Perfluorodichlorooctane Is Mixed with Thrombocytopenic Blood

	Platelet count (1,000/μL)							
	PFDCO 1%				PFDCO 8%			
		Measured by				Measured by		
Actual	Dilution-corrected	CD3200	CD3500	CD1700	Dilution-corrected	CD3200	CD3500	CD1700
9.4	9.2	175	1168	601	7.3	311	os	os
19.4	18.9	172	1141	615	15.5	318	os	os
34.2	33.3	191	1147	650	27.4	336	os	os
37.9	37.0	176	1183	627	30.3	334	os	os
46.4	45.2	179	1082	619	37.1	350	os	os
59.6	58.1	226	1262	775	47.7	405	os	os
76.0	74.1	215	1228	629	60.8	397	os	os
91.8	89.5	226	1151	648	73.4	409	os	os
120.0	117.0	256	1156	629	96.0	409	os	os
171.0	166.7	269	1184	741	136.8		os	os

CD3200, 3500, 1700; Abbott, Santa Clara, CA.

Actual counts were obtained from the CD3500 (reference analyzer) and are corrected for dilution. Errors—measured by CD3200, CD3500, and CD1700—represent an overestimation in all analyzers. Errors for the CD3500 and CD1700 at perfluorocarbon emulsion 8% exceeded the measurable scale. Errors for the CD3200 at perfluorocarbon emulsion 1% or even 8% are smaller than for the CD3500 and CD1700 at perfluorocarbon emulsion 1%. Perfluorocarbon emulsion 1% or 8% = perfluorocarbon emulsion at 1% or 8% in the samples.

CD3200 = CellDyn 3200, CD3500 = CellDyn 3500, CD1700 = CellDyn 1700, os = outside scale range, PFDCO = perfluorodichlorooctane.

done in further studies to present the variability inherent in the data and the statistical significance. Despite the limitations of this study, we have demonstrated that PFDCO may interfere significantly with automated hematological analyzers. This error must be considered when evaluating PLT counts in thrombocytopenic patients receiving PFEs, such as PFDCO.

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