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Evaluation of the hemostatic function of stored platelet concentrates using the platelet function analyzer (PFA-100™)

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ABSTRACT

Background and Objectives. **Progressive functional impairment is known to occur in platelet concentrates through the storage period. Standardized methods providing direct measurement of residual platelet function in stored platelets are lacking. The purpose of this study was to determine whether a new platelet function analyzer (PFA-100™) could provide standardized methods for assessing the hemostatic capacity of stored platelets.**

Design and Methods. **The PFA-100 was used to evaluate platelet function in stored platelets. The instrument can process citrated whole blood but it is unable to process platelet suspensions. Accordingly, the function of platelet concentrates should be measured following reconstitution of pseudo-whole blood. The analysis of the results included the *closure time* (sec) and a *predictive index*, an arithmetical index computed on the basis of the instrument's output data: the flow rate, the flow volume, the closure time.**

Results. **A final hematocrit of 58±2 and a final platelet concentration of $230\pm20\times10^9$/L were used as standardized operative conditions to measure the function of stored platelet concentrates. The closure time (PFA-CT) and the predictive index (PFA-PI) both resulted to be capable of discriminating platelet concentrates with maintained or impaired function. PFA-PI was more informative than PFA-CT in terms of description of the residual platelet function. Of the two agonists used, epinephrine (EPI) resulted to be particularly sensitive for the detection of initial platelet hyporeactivity, whereas adenosine 5'-diphosphate (ADP) was particularly useful for measuring the residual platelet reactivity.**

Interpretation and Conclusions. **PFA-CT and PFA-PI can be standardized; they provide new information about the hemostatic function of stored platelet concentrates and can be used to assess the quality of platelet concentrates.**
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Key words: platelets, platelet function, hemostatic platelet function, stored platelets, PFA-100

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Progressive loss of platelet viability and hemostatic function is known to occur during platelet storage. *In vitro* and *in vivo* studies have shown that platelet concentrates maintain an adequate function for at least five days, a longer storage period being discouraged because of the risk of bacterial growth.[1] A number of *in vitro* tests are available to assess platelet storage lesions.[2] These indirect tests measure soluble metabolites or morphologic properties. Conventional aggregometry, which seems to be a more direct approach to platelet function analysis, provides poorly reproducible results on manipulated and stored platelets. Too many variables affect the *in vivo* post-transfusion platelet recovery, and make such clinical evaluation an improper means to measure the hemostatic function of stored platelets. Reproducible and standardized means of providing direct and measurable evidence of the real hemostatic function of stored platelets have not been achieved. A new instrument for platelet function analysis has recently been introduced for clinical use. The instrument, PFA-100™ (Dade, Miami, FL, USA) is an evolution of a formerly available instrument Thrombostat 4000 (VDG von der Golz, Seean, Germany). PFA-100™ measures the *in vitro* bleeding time on citrate anticoagulated whole blood samples, and can discriminate hypoactive and hyperactive platelets from normoactive ones.[3] The aim of our work was to standardize operative conditions in order to allow PFA-100™ to be used for prompt measurement of the real hemostatic capacity of stored platelet concentrates.

Design and Methods

Preparation of platelet concentrates

Platelets were obtained from single buffy coat fractionation of whole blood (400-450 mL) donations collected in top-and-bottom bags containing CPDA as anticoagulant (Optipack, Baxter S.A., Mauerpas, France). The buffy coat fraction was obtained (Optipres II, Baxter S.A., Mauerpas, France) following high-spin centrifugation of whole blood at 5,000 g for 7 min (ACE=6.60e07). Platelets were then obtained as platelet-rich plasma fraction, following a low-spin centrifugation at 150 g for 14 min (ACE=4.57e06). Platelet suspensions were kept stored at 22°C, in a linear acceleration incubator (RTB4100, Bioitalia, Milan, Italy).

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Platelet sampling

During the storage period, the bag connected segment was kept clamped. Before sampling, the segment was unclamped and filled with platelet suspension by active stripping and counter-stripping of the segment. A terminal portion of the platelet suspension-containing segment was sealed and the platelets were transferred into a tube. Finally the segment was stripped again and clamped until the next sampling.

Platelet recovery, morphology, and swirling

Cytometric evaluations (Sysmex TOA T800, Sysmex, Kobe, Japan) were performed at every single step of the procedure. Swirling was scored as: level 4-excellent, level 3-good, level 2-fair, and level 1-poor or non-swirling, as reported by others.[4]

Platelet function analyzer

In vitro bleeding time was measured using the platelet function analyzer PFA-100™ (Dade, Miami, FL, USA). The instrument was originally designed for *in vitro* measurement of platelet function in citrate anticoagulated whole blood. The blood flows through a capillary to a nitro-cellulose membrane with a central microscopic aperture calibrated to 147 µm. The membrane is coated with 2 µg of fibrillar type I equine tendon collagen and 10 µg of epinephrine bitartrate (EPI) or 50 µg adenosine 5'-diphosphate (ADP). The blood flow is controlled by a microprocessor. During the flow, platelets adhere to collagen and become activated by EPI or by ADP, according to the disposable cartridge used. Adherent and activated cells form a platelet plug, progressively obstructing the membrane hole. The time necessary for blood to occlude the hole is a function of primary platelet hemostatic activity: this time, which mimics *in vivo* bleeding time, has been called *in vitro bleeding time* (IVBT) when used as an *in vitro* substitute of the *in vivo* bleeding time. In this study the assay was used to asses the function of stored platelets: for such use, the term PFA closure time (PFA-CT) seemed more appropriate than IVBT. The instrument provides two other additional data concerning the dynamics of the plug formation: the flow rate (µL/min), and the total flow volume (µL). If the time required for hole obstruction exceeds 300 sec, the instrument stops the test: in this case the report includes the flow rate, the total flow volume and the elapsed time up to 300 sec.

Whole blood reconstitution

The speed of the blood flow through the capillary and through the membrane aperture depends on the rheologic properties of whole blood as a combined function of red cell hematocrit and plasma viscosity. For this reason, prior to PFA-CT determination on platelet suspensions, a pseudo-whole blood must be reconstituted. Standard operative conditions were previously set up for quality control of platelet concentrates.[5] The relative volumes and concentrations of washed O group packed red cells, platelet suspension, and thawed AB group fresh frozen plasma were determined.

PFA-CT

Using standardized reconstituted pseudo-whole blood, PFA-CT was determined daily on thirteen platelet concentrates, from the day of preparation (day 0) through to the seventh storage day (day 7).

PFA-predictive index (PFA-PI).

PFA-CT is the end-point result of fluid mechanics and cell function-related variables. The informative value of the closure time may be supplemented by the relative account of the other variables influencing both the flow rate and the flow volume. In order to gain additional information about the real platelet function other than that supplied by the closure time, we set up a PFA-CT derived platelet function-describing index, the PFA predictive index (PFA-PI), calculated according to the following formula:

$$\text{PFA-PI} = \frac{[\text{total flow volume (µL)} : \text{flow rate (µL/min)}] \times \text{closure or elapsed time (sec)}}{100}$$

Results

Whole blood reconstitution

The standard operative conditions were assessed as reported in Table 1. PFA-CT was measured using a platelet concentration of $230\pm20\times10^9$/L and an hematocrit of 58±2. Fresh frozen plasma was added for two reasons: to provide washed red cells with a plasma-like viscosity biological medium with buffering capacity, and to supplement the non-cellular moiety with labile plasma factors possibly implicated in plug formation.

PFA-CT and PFA-PI

The cumulative results of the PFA-CT obtained on thirteen platelet concentrates evaluated each day for seven days are reported in Table 2. The range of the

Table 1. Standard operative conditions to assess platelet function on stored platelets using PFA-100™.

Washed O blood group red cells
Starting hematocrit: 79±3
Volume per test: 1300±200 µL
Final Hct: 58±2
Platelet suspensions
Starting count: $1136\pm204\times10^9$/L
Volume per test: 300±100 µL
Final count: $230\pm20\times10^9$/L
Fresh frozen (thawed) plasma
Volume per test: 150±50 µL

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Table 2. Descriptive results of the analyses performed each day on thirteen platelet concentrates stored for seven days

Days	*0*	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Swirling score range	4	4	4	3-4	2-4	2-3	1-3	1-3
CEPI								
PFA-CT								
median	142	183	242	201	300	300	300	300
mean	132	178	229	218	235	238	277	262
s.d.	37	54	79	69	81	75	40	60
range	92-216	88-254	100-300	88-300	113-300	119-300	201-300	163-300
PFA-PI								
median	3.97	5.75	10.15	6.74	11.65	11.28	12.72	13.00
mean	4.77	6.13	9.57	7.86	9.96	9.72	12.21	11.70
s.d.	1.96	3.45	4.72	3.62	5.17	5.01	2.99	3.99
range	2.28-9.10	2.06-15.05	2.62-14.69	2.10-13.45	2.53-16.32	2.58-16.26	7.54-15.37	4.56-15.63
CADP								
PFA-CT								
median	94	109	114	89	177	196	300	300
mean	95	105	109	151	175	210	268	234
s.d.	16	21	22	97	98	86	64	88
range	71-117	75-133	71-133	79-300	88-300	96-300	124-300	113-300
PFA-PI								
median	2.43	2.69	2.85	2.36	6.08	5.03	11.97	13.74
mean	2.44	2.65	2.89	5.48	6.94	8.39	11.47	9.98
s.d.	0.55	0.69	0.80	5.35	5.85	5.41	4.15	5.62
range	1.38-3.30	1.67-3.78	1.56-4.08	1.77-14.97	1.12-17.20	2.11-17.89	3.10-16.09	2.80-15.07

$$\text{PFA-PI} = \frac{[\text{total flow volume } (\mu L) : \text{flow rate } (\mu L/\text{min})] \times \text{closure or elapsed time (sec)}}{100}$$

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swirling score is reported together with the median, the mean, the standard deviation (s.d.), and the range of PFA-CT and of PFA-PI obtained using EPI and ADP-treated cartridges (CEPI and CADP). The results show the progressive decay of the swirling score, and the increase of the median and mean values for both the closure time and the computed index. More information about the function of the platelet concentrates is illustrated in Figures 1 (CEPI) and 2 (CADP). These figures provide various information. Firstly, with the exception of the closure time obtained using CEPI, the median value of closure time begins to increase after the third day of storage. Secondly, both the index and the closure time discriminate very clearly the concentrates that preserve their function from those which are going to lose it. Thirdly, evaluating only the closure time, which is scheduled by the manufacturer to a maximum of 300 sec, it is impossible to evaluate the residual platelet function for all those concentrates reaching the maximum level. The use of the computed index (PFA-PI) allows the evaluation of this residual function. Finally, it should be stressed that the swirling score did not fit perfectly with the *in vitro* bleeding time: all platelet concentrates with a low swirling score were proven to have lost their function, while some platelet concentrates with a good swirling score did not function accordingly when evaluated by PFA-CT and by PFA-PI.

Figure 3 shows the correlation of PFA-PI with PFA-CT using CADP (side A) and CEPI (side B). The concentrates which retained their function were clearly distinguishable from those with impaired function; this clear cut was particularly evident using CADP. The regression analysis of the computed index with the closure time obtained on the entire set of the results displayed a good correlation (solid line; side A: R = 0.94; side B: R = 0.91). The correlation obtained computing only the results which included a closure time lower than 220 sec was stronger (dotted line; side A: R = 0.98; side B: R = 0.97), while any correlation was lost for the data including closure times higher than 220 sec. For the platelet concentrates with a closure time of 300 sec the computed index, which represents the residual hemostatic function, ranged from 10.5 to 17.0 (side A), and from 10.0 to 16.5 (side B).

Discussion

A variety of *in vitro* assays have been described to assess the quality of platelet concentrates.[2,6,7] Many laboratories use some of these tests out of well defined standardized programs. Based on metabolic and morphologic grounds, most of these procedures give indi-

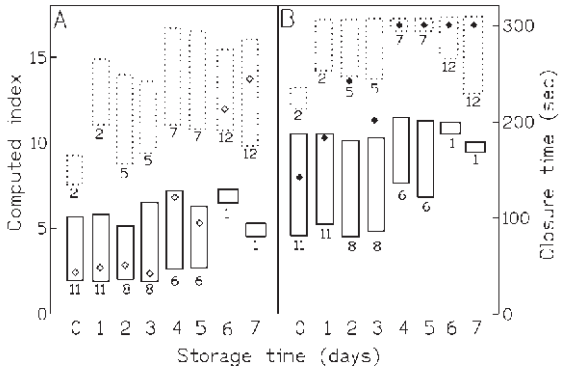

Figure 1. Function of the platelet concentrates during the storage period (days), as measured by PFA-100 using CEPI. Diamonds indicate the median value of the computed index, PFA-PI (unfilled ◇, side A), and of the closure time, PFA-CT (filled ◆, side B). Boxes indicate the range of PFA-PI (side A) and of PFA-CT (side B) of the platelet concentrates (indicated with the figures inside the graphic) with maintained (solid) or impaired (dotted) function.

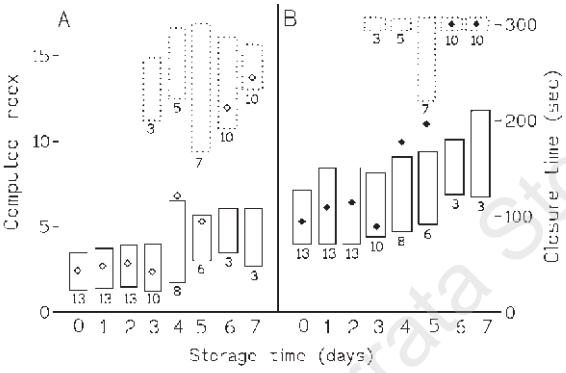

Figure 2. Function of the platelet concentrates during the storage period (days), as measured by PFA-100 using CADP. Diamonds indicate the median value of the computed index, PFA-PI (unfilled ◇, side A), and of the closure time, PFA-CT (filled ◆, side B). Boxes indicate the range of PFA-PI (side A) and of PFA-CT (side B) of the platelet concentrates (indicated with the figures inside the graphic) with maintained (solid) or impaired (dotted) function.

rect information about platelet function in platelet concentrates. *In vivo* assessment of the quality of transfused platelets is not feasible for practical and ethical reasons in humans, while a recently described animal model cannot be used extensively for large scale quality control of platelet products.[8] Among the theoretically available methods proposed to assess the quality of platelet concentrates, only a few produce results that correlate well with permanent platelet lesions, platelet hemostatic capacity, and *in vivo* viability; even fewer can be standardized for pH variation of the storage medium;[9,10] extent of shape change (ESC) and hypotonic shock response (HSR);[11] and platelet shape-dependent swirling.[4] Albeit used in some experimental condition as an additional parameter to evaluate platelet function of stored platelet concentrates,[12] conventional aggregometry is not considered either critical or useful in assessing new procedures or for routine quality control of platelet concentrates, because of the marked variability of such procedure and the lack of evidence that *in vitro* aggregometry correlates to *in vivo* viability.[13-15]

A new diagnostic tool recently made available for clinical investigations, the platelet function analyzer PFA-100™,[2,16] may have the prerequisites to change the perspectives and standardization of the quality control of platelet concentrates. The close correlation of the IVBT with *in vivo* platelet function has been stressed by recent clinical studies.[14,17-24] The low coefficient of variation (4-10%) of repeated analyses[3] was confirmed by preliminary tests performed by ourselves (data not shown). In a previous study performed by others, evaluation of the IVBT demonstrated a close correlation of the post-transfusion platelet function with the post-transfusion corrected count increment (CCI); this study used Thrombostat 4000/2 equipment, from which the PFA-100™ was derived.[25]

Taken together, all these data suggested the possible use of PFA-100™ to assess a standardized protocol for the quality control of platelet concentrates in terms of hemostatic capacity. The prerequisite of the sample needing whole blood-like viscosity was solved by reconstitution of pseudo-whole blood. Accordingly, washed red cells and fresh-frozen plasma were added to the platelet suspension at the volume/concentration ratio reported in Table 1. Keeping such conditions standardized, the results of the assay depended only on platelet function. The results obtained from thirteen platelet concentrates stored for seven days showed that the PFA-100™ could be used for a standardized program of quality control of platelet concentrates. Firstly, Figures 1 and 2 show that, in spite of swirling scored as 4+ (excellent), PFA-CT using CEPI demonstrated platelet hypofunction in some concentrates. However, in these concentrates PFA-CT using CADP did not evidence any platelet defect, ADP being a stronger agonist than EPI. Secondly, unlike CEPI that evidenced initial platelet hyporeactivity better than CADP, during the last days of storage CADP evidenced the residual platelet reactivity better than CEPI. Thirdly, the calculated index (PFA-PI) showed its theoretical and practical relevance. In fact the plug-dependent closure time is the net end-point result of a complex process subject to the following variables: blood viscosity (related to hematocrit and plasma viscosity); platelet concentration; platelet accessibility to physical surfaces (related to hematocrit and platelet concentration); number and function of platelet receptors for colla-

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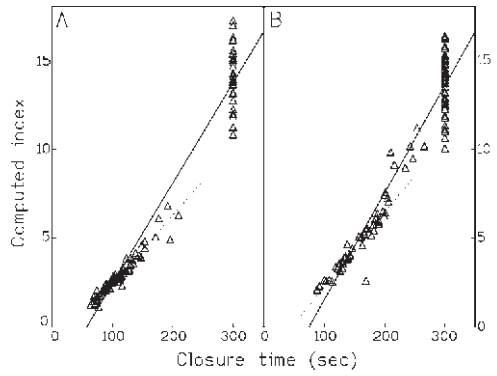

Figure 3. Correlation of the calculated index (PFA-PI) and the closure time (sec) (PFA-CT) obtained with CADP (side A) or CEPI (side B). Solid lines indicate the regression analyses of all data (Side A, R = 0.94; side B, R = 0.91); dotted lines indicate the regression analyses of the results with a closure time lower than 220 sec (side A, R = 0.98; side B, R = 0.97).

gen and agonists; pH of the liquid medium; global platelet function including the adhesion and aggregation phases. All these variables determine the flow rate variations within the capillary and through the membrane hole, the total flow volume through the membrane hole, and the closure time. The aim of the calculated index was to provide an arithmetical figure reflecting the platelet function state, technical variables apart. The comparison of side A and side B of Figures 1 and 2 show that the calculated index (side A) was much more informative than the closure time (side B), in terms of residual platelet function during the last 3-7 days of storage. These results are more evident in Figure 3: this shows that platelet concentrates considered as non-functional on the basis of closure time, could be considered as hyporeactive on the basis of the calculated index. As shown in side B of Figure 3 (CEPI), some platelet concentrates with an overlong closure time (300 sec) had a calculated index comparable to that of platelet concentrates with a closure time ranging between 200 and 250 sec. Side A of the same figure (CADP) also shows a variable calculated index, ranging from 10.5 to 17.0, for platelet concentrates with an overlong closure time. In the light of these results, we considered the calculated index (PFA-PI) more predictive of the residual platelet function than the closure time (PFA-CT).

The main conclusion of our study is that the PFA-100™ provides new information about the hemostatic function of stored platelet concentrates. The closure time and the calculated index provide different criteria for scoring the platelet function and defining scheduled values to validate (or invalidate) the stored platelet concentrates for clinical use.

PFA-CT and PFA-PI might seem like just two more tools to evaluate the quality of platelet units stored prior clinical use. In fact, the evidence described above indicates that PFA-CT and PFA-PI, by measuring platelet function directly, could be more valuable than indirect evidence, such as pH or swirling, in predicting the clinical outcome of a platelet transfusion. In this perspective, the laboratory evidence obtained measuring PFA-CT and PFA-PI should be cross-evaluated together with objective clinical parameters such as variation of transfusion requirement, post-transfusion corrected count increment, and *in vivo* and *in vitro* bleeding times.

Contributions and Acknowledgments

PB was responsible for the conception of study, interpretation of results and writing of the paper. AL collected data and performed the statistical analysis. LM carried out the laboratory tests. All the authors contributed to the drafting and critical revising of the article and approved its final version.

Disclosures

Conflict of interest: none.

Redundant publications: Yes ≥ 50%. The paper includes (>50%) and amplifies an abstract (ref. #5). The paper is worth of publication for the following reason: symposium abstracts are not necessarily peer-reviewed; the paper contains the extended methods, results, and discussion: it can be fully understand (criticized and ameliorated too); the abstract audience was mostly composed by blood bankers (international ISBT meeting); Haematologica is widely diffused among clinicians who are potentially interested in knowing that methods to control the hemostatic quality of platelet units are available, and may make pressure on Blood Bankers to provide patients with more valuable products.

Manuscript processing

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