Evaluation of platelet activation in leukocyte-depleted platelet concentrates during storage

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ABSTRACT

Structural and functional changes in platelets during storage can lead to the loss of platelet reactivity and response. Our aim was to evaluate leukocyte-depleted platelet concentrates on storage days 0, 3 and 5, obtained by in-line filtration. In non-filtered platelet concentrates (NF-PC) group, 180 whole blood units were collected with quadruple blood bags and then compared to another group of 180 whole blood units (leukocyte-depleted platelet concentrates [LD-PC]), collected in Imuflex Whole Blood Filter Saving Platelets (WB-SP) bags with an integrated leukoreduction filter, with regard to the platelet quality and characteristics. The efficacy of the two techniques for platelet concentrate preparation was evaluated by white blood cell (WBC) and platelet count on day 0. The partial pressure of oxygen (pO_2), pH, platelets positive for P-selectin (CD62P), CD63, cluster of differentiation 42b (CD42b), phosphatidylserine (PS), and mitochondrial membrane potential (MMP) were analyzed during the storage in both groups. A significantly lower WBC count and higher platelet count was observed in LD-PC compared to NF-PC group, indicating the overall efficacy of the first technique. During the 5-day storage, pH and pO_2 decreased in both groups. In LD-PC group, higher pH, increased pO_2 and decreased platelet surface expression of CD62P, CD63 and PS were observed compared to NF-PC group. In both groups, the percentage of CD42b positive platelets and MMP did not change significantly during the 5-day period. The assessment of different markers of platelet activation may be an effective tool in evaluating the quality of platelets during storage. A better understanding of platelet activation may provide new insights for developing a novel therapeutic approach in the manipulation of platelet aggregation.

KEY WORDS: Platelet storage; platelet activation markers; leukocyte depletion; in-line filtration
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INTRODUCTION

Platelet transfusion is an important step in the treatment of thrombocytopenic patients who suffer from severe bleeding. Although platelet transfusions are extensively used across the world, improving their safety and efficacy remains the primary goal in patients with high risk of bleeding. For optimal results, platelet activation is minimized prior to transfusion, to prevent deleterious changes in platelets [1,2]. During the storage (usually 4-7 days), different biochemical, morphological and functional changes of platelets, known as platelet storage lesion (PSL), may occur, as well as platelet activation and secretion of proinflammatory factors [3]. These changes lead to decreased efficacy of platelet transfusion and may contribute to the development of adverse reactions in the recipients [4].

Platelet concentrates (PCs) can be obtained by apheresis, or derived from the platelet-rich-plasma (PRP) or buffy-coat from the whole blood, with the third method being more common in Europe [5]. The quality of PCs may be impaired during the preparation and storage, as well as by the contamination of white blood cells (WBCs). In addition, due to the risk of bacterial contamination, platelet storage is often limited to a maximum of 5 days [6]. Previous studies also indicated that leukocytes in PCs could mediate adverse effects such as human leukocyte antigen (HLA) alloimmunization, graft-versus-host disease, and febrile reactions [7]. Moreover, functional changes of platelets have been associated with elevated basal levels of platelet activation [8].

Different methods for pathogen reduction and leukocyte depletion in PCs produce platelets that vary greatly in their quality. Thus, a proper and early detection of PSL in platelets remains one of the most important aspects of platelet

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transfusion [9]. In this study, we evaluated the efficacy of an in-line filtration method for obtaining PCs compared to the standard method. The platelet quality and characteristics were analyzed during 5-day storage.

MATERIALS AND METHODS

Preparation and storage of PCs

Whole blood (450 ± 45 ml), obtained from donors who had not taken any antiplatelet drugs for two weeks prior to donation, was stored 2-3 hours at room temperature (day 0) prior to further processing. All samples showed no reactivity for infectious disease markers (i.e. acquired immune deficiency syndrome [AIDS], syphilis, and hepatitis B and C infection), using serological screening tests (Biokit S.A, Llissá de Munt, Spain and Ortho Clinical Diagnostics, Inc., Raritan, NJ, USA). The collected blood units were randomly assigned to two groups: non-filtered platelet concentrates (NF-PC group) and leukocyte-depleted platelet concentrates (LD-PC group). In NF-PC group, 180 whole blood units were collected with quadruple blood bags (MacoPharma, Tourcoing, France), while in LD-PC group, another 180 whole blood units were collected in Imuflex Whole Blood Filter Saving Platelets (WB-SP) bags (Terumo Medical, Tokyo, Japan) with an integrated leukoreduction filter [10]. Both types of bags contained 63 ml of citrate phosphate dextrose (CPD) and 100 ml of saline-adenine-glucose-mannitol (SAGM) solutions.

The preparation of NF-PCs started within 6 hours following the collection of whole blood units. Thirty PCs were prepared from the 180 whole blood units. Each blood unit was centrifuged in a Cryofuge 8500i centrifuge (Heraeus, Langenselbold, Germany) at 3,890 × g for 10 minutes, at 20 ± 2°C and further processed using a T-ACE II$^+$ Automatic Component Extractor (Terumo Medical, Tokyo, Japan). The separated buffy coat was left at 20 ± 2°C for 2 hours and then centrifuged again at 310 × g for 7 minutes, at 20 ± 2°C. Using the sterile connection system Terumo Sterile Tubing Welder SC-201A [TSCD] (Terumo Medical, Tokyo, Japan), six NF-PCs were pooled together and stored in a horizontal shaker (60 cycles/minute, Teknolabo A.S.S.I. S.r.l., Italy) at 20 ± 2°C for 5 days.

The whole blood from additional 180 donors was collected in Imuflex WB-SP bags for the preparation of 30 LD-PCs. The centrifugation, pooling and storage procedures were performed as described for NF-PCs.

Parameter analysis

The cell count in PCs was performed using a Beckman Coulter AcT Diff Hematology Analyzer (Beckman-Coulter, Fullerton, CA, USA). The contamination of WBCs in NF-PCs and LD-PCs was determined after the samples were diluted in Turk solution (1:10), by manual cell counting in a Nageotte hemocytometer (Hausser Scientific, Horsham, PA, USA), as described previously [11].

The partial pressure of oxygen (pO_2) was determined using Cobas b 221 blood gas system (Roche Diagnostics, Rotkreuz, Switzerland), immediately after the preparation of PCs (day 0), as well as on storage day 3 and 5. Extracellular pH was evaluated on day 0, 3 and 5 using a FP20 pH meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland).

The percentages of platelets positive for P-selectin (CD62P; BD Biosciences, USA), surface glycoprotein gp53 (CD63; BD Biosciences, USA), platelet glycoprotein Ib alpha chain [GP Ib] (CD42b; BD Biosciences, USA), and phosphatidylserine [PS] (annexin V binding; BD Biosciences, USA) on day 0, 3 and 5 were evaluated by flow cytometry analysis, as described previously [12]. In brief, freshly collected platelets were diluted in phosphate-buffered saline (PBS) and adjusted to the final concentration of 1×10^6 cells/ml. The cells (1×10^6 per tube) were incubated with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (anti-CD62P, anti-CD63, and anti-CD42b) and annexin V, according to the manufacturer's instructions. Control cells were incubated with FITC-conjugated IgG isotype control and run in parallel as negative controls. The labeled cells were fixed in 1% paraformaldehyde for 1 hour and 10000 cells/sample were analyzed on a Coulter Epics XL M CL flow cytometer (Beckman Coulter, Krefeld, Germany). For the flow cytometry analysis, a gate was set around the platelets, as described previously [13].

Changes in the mitochondrial membrane potential (MMP) in platelets were evaluated using the lipophilic cation Rhodamine 123, as previously described [14]. The fluorescence of Rhodamine 123 in the cells was determined by flow cytometry. For each sample, basal intensity values were subtracted from those obtained after different treatments and the results were presented as the ratio of mean fluorescence intensity.

Statistical analysis

SPSS for Windows, Version 15.0. (SPSS Inc., Chicago, Il, US) was used for statistical analysis. The results are presented as mean ± standard deviation (SD). Differences between groups were determined using Student's *t*-test, Mauchly's sphericity test, or analysis of variance (ANOVA) with repeated measurements, combined with Bonferroni's post hoc test for multiple comparisons. A p value <0.05 was considered significant.

RESULTS

To evaluate the efficacy of the filter-based method for leukocyte depletion, we determined the total WBC and platelet count in NF-PCs and LD-PCs on day 0. A significantly lower

WBC count (t = 16.693, p < 0.001) and higher platelet count (t = 7.862, p < 0.001) was observed in LD-PC (WBC count: 0.04 ± 0.02 × 106/unit; platelet count: 88.52 ± 24.31) compared to NF-PC group (WBC count: 5.18 ± 1.5 × 106/unit; platelet count: 49.27 ± 11.46), indicating the overall efficacy of the first technique. Furthermore, no significant changes were observed in the volumes of LD-PCs (64.97 ± 19.22) and NF-PCs [63.21 ± 17.34] (Table 1). During the 5-day storage, we observed decreased overall pO_2 (for LD-PC χ^2 = 311.006, p < 0.001; for NF-PC χ^2 = 223.522, p < 0.001) and pH (for LD-PC χ^2 = 306.046, p < 0.001; for NF-PC χ^2 = 194.425, p < 0.001) in both groups (Figures 1 and 2, respectively). Nevertheless, on day 3 and 5, the level of pO_2 was significantly higher in LD-PC compared to NF-PC group [for day 3 t = 8.544, p < 0.05; for day 5 t = 9.312, p < 0.05] (Figure 1).

The level of platelet activation during the 5-day storage was evaluated by determining the percentage of platelets positive for several platelet activation markers, using flow cytometry analysis. As shown in Table 2, the percentage of platelets expressing CD62P was significantly lower in LD-PC compared to NF-PC group on day 0 (t = 10.642, p < 0.05), 3 (t = 12.754, p < 0.05) and 5 (t = 10.724, p < 0.05). Similarly, a significantly lower percentage of CD63-positive platelets was observed in LD-PC compared to NF-PC group on day 0 (t = 8.226, p < 0.05), 3 (t = 7.852, p < 0.05) and 5 (t = 10.664, p < 0.05). We also observed a decrease in the percentage of CD42b-positive platelets during the 5-day storage in both groups, however, these changes were not statistically significant. In addition, the number of platelets expressing PS on the surface was significantly higher in NF-PC compared to LD-PC group, on day 0 (t = 7.342, p < 0.05), day 3 (t = 8.724, p < 0.05), and day 5 [t = 7.295, p < 0.05] (Table 2). In both groups, MMP did not change significantly during the 5-day period (p > 0.05, Figure 3).

DISCUSSION

In the present study, we evaluated the efficacy of integrated leukoreduction filters for obtaining LD-PCs and analyzed the changes in the percentage of activated platelets during 5-day storage, compared to NF-PCs. We found that the number of WBCs in LD-PCs was significantly decreased compared to NF-PCs. The total number of WBCs was below 5 × 10^4 leukocytes/unit in the PCs obtained using the Imuflex WB-SP bags with integrated leukoreduction filters, which is below the limit recommended for WBC count in PCs to avoid adverse effects of platelet transfusion [7]. Furthermore, pH and pO_2 decreased

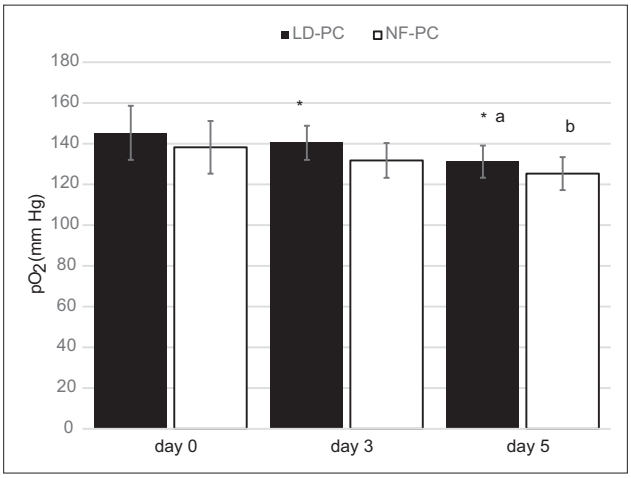

FIGURE 1. Levels of partial pressure of oxygen (pO_2) during 5-day storage in non-filtered (NF-PCs) and leukocyte-depleted platelet concentrates (LD-PCs). A decreased overall pO_2 was observed in both groups over the 5-day period ($^a p$<0.001 for LD-PC and $^b p$<0.001 for NF-PC). Nevertheless, on day 3 and 5, the level of pO_2 was significantly higher in LD-PC compared to NF-PC group (*p<0.05).

TABLE 1. WBC, platelet count, and volume of platelet concentrates (PCs) obtained with two different methods

Day	LD-PC	NF-PC
Leukocytes (10^6/unit)		
0	0.04±0.02 ***	5.18±1.5
Platelets (10^9/unit)		
0	88.52±24.31***	49.27±11.46
Volume of PCs (ml/unit)		
0	64.97±19.22	63.21±17.34

Results are presented as mean±standard deviation (SD) from 30 platelet concentrates in each group. WBC: White blood cells; LD-PC: Leukocyte-depleted platelet concentrates; NF-PC: Non-filtered platelet concentrates. ***p<0.001 compared to NF-PCs

TABLE 2. Expression of activation markers on platelet surface during 5-day storage in non-filtered and leukocyte-depleted platelet concentrates

Day	LD-PC	NF-PC
Platelets positive for CD62P (%)		
0	5.1±1.5*	10.2±2.1
3	12.4±4.2*	31.7±10.6
5	29.3±9.7*	45.2±12.1
Platelets positive for CD63 (%)		
0	3.2±1.1*	7.3±2.4
3	7.4±2.4*	14.7±5.2
5	12.9±3.7*	29.6±8.7
Platelets positive for CD42b (%)		
0	96.9±2.3	95.5±2.8
3	93.5±3.2	91.2±3.1
5	92.7±4.7	89.7±4.6
Platelets positive for phosphatidylserine (%)		
0	1.1±0.3*	2.3±0.7
3	3.3±1.2*	7.2±2.4
5	11.5±2.1*	17.5±2.1

Results are presented as mean±standard deviation (SD) from 30 PCs in each group. LD-PC: Leukocyte-depleted platelet concentrates; NF-PC: Non-filtered platelet concentrates; CD62P: P-selectin; CD63: GP53 glycoprotein; CD42b: Cluster of Differentiation 42b. *p<0.05 compared to NF-PCs

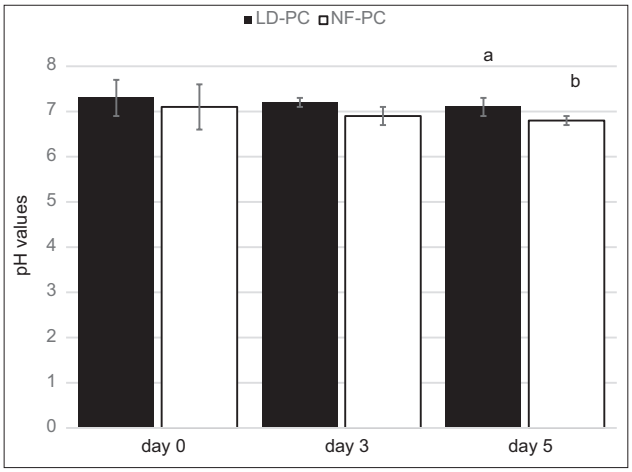

FIGURE 2. Levels of pH during 5-day storage in non-filtered (NF-PCs) and leukocyte-depleted platelet concentrates (LD-PCs). Over the 5-day storage a decreased overall pH was observed in both groups ([a]$p<0.001$ for LD-PC group; [b]$p<0.001$ for NF-PC group).

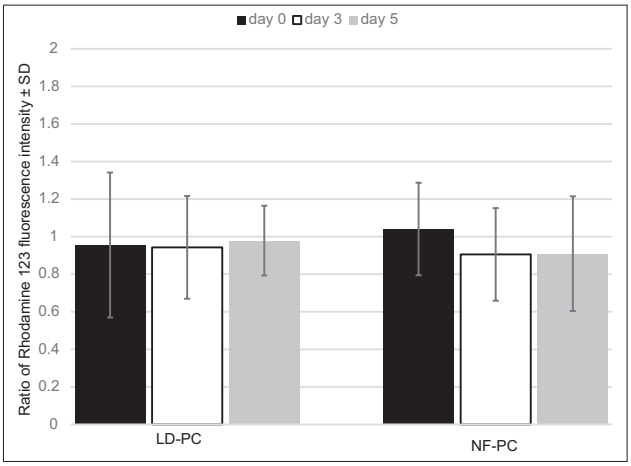

FIGURE 3. Changes in the mitochondrial membrane potential (MMP) during 5-day storage in non-filtered (NF-PCs) and leukocyte-depleted platelet concentrates (LD-PCs). In both groups, the MMP did not change significantly during the 5-day period. Results are presented as ratio of fluorescence intensity ± standard deviation (SD) of NF-PCs on day 0.

in both groups during the 5-day storage, with lower values for both parameters in NF-PC compared to LD-PC group, indicating that cells were more metabolically active in NF-PC group. As previously indicated, irreversible damage occurs in platelets below pH 6 [15]. Although we observed pH above 6 in both of our groups, on day 5, pH was 6.8 in NF-PC group which is the value of pH when platelets undergo morphological changes, such as from disk- to spherical-shaped platelets [16].

The increased oxygen consumption by the activated platelets, observed in our study, correlates with previous reports [17,18]. Energy production in platelets is based on the two major metabolic pathways, anaerobic glycolysis and oxidative phosphorylation [19]. In stored platelets, increased glycolysis leads to the accumulation of lactic acid and results in lower pH [20]. Furthermore, elevated oxygen consumption by platelets may be associated with mitochondrial dysfunction and increased mitochondrial membrane permeability to cytochrome c [21]. Overall, our results indicate decreased metabolic activity of LD-PCs compared to NF-PCs.

Flow cytometry is considered the gold standard for the evaluation of platelet activation [22]. Using flow cytometry, we showed that the percentage of platelets expressing CD62P and CD63 was significantly higher in NF-PC compared to LD-PC group. These findings are in line with earlier reports, which indicated that increased CD62P and CD63 expression in platelets is associated with PSL [1,23]. P-selectin (CD62P) is located in the membrane of α-granules in platelets, while CD63 is present in the membrane of dense granules [24]. Upon platelet activation, these proteins are translocated to the platelet surface, but the effect of this process on transfusion is still not clarified [23]. Generally, increased expression of platelet activation markers is observed in PCs without leukocyte depletion, due to the presence of WBCs and cytokines [25]. Although the number of CD42b positive platelets slightly decreased over the 5-day period, we did not observe a significant difference between the two groups. CD42b is a receptor for von Willebrand factor (VWF); the binding of CD42b to VWF initiates platelet deposition, and the complex is cleaved upon platelet activation [26]. In some studies, stabile CD42b expression was demonstrated during platelet storage [9,27], while in other studies, decreased CD42b expression was observed [28,29]. These differences in CD42b expression in platelets may be due to the differences in storage time, e.g., in our study the storage time was 5 days, while in other studies it was 12 and 14 days [28,29]. In addition, PS exposure, another marker of platelet activation and apoptosis [19], was significantly increased in NF-PC group, which is in agreement with previous studies [30,31]. However, the significance of this process remains to be clarified [9]. We also evaluated MMP in the two groups, as platelet apoptosis was previously associated with the loss of MMP and intrinsic apoptotic pathway [32]. The MMP remained stable in both of our groups over the 5-day storage. Nevertheless, despite the stability of MMP in our PCs, increased PS exposure alone remains an indicator of apoptosis in platelets, as shown in a previous study [33]. In agreement with our observations, another study indicated that, after stimulation of platelets with thrombin, platelet activation occurred before apoptotic changes [34].

One of the limitations of our study is that we did not analyze soluble mediators in PCs, which may provide an insight into the role of cytokines in platelet activation. Another limitation of our study is the relatively small number of analyzed PCs.

In summary, we showed that WB-SP bags with an integrated leukoreduction filter is an effective method for obtaining LD-PC. The removal of WBCs from PCs decreases platelet activation during storage. Proper detection of PSL is a crucial step in preventing adverse effects of transfusion.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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