Flow cytometry analysis of platelet populations: usefulness for monitoring the storage lesion in pooled buffy-coat platelet concentrates

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Background. Early detection of the platelet storage lesion is still a challenge in transfusion practice. Using flow cytometry, we evaluated the appearance of the storage lesion, based on the expression of platelet activation markers, in total platelets and platelet populations.

Materials and methods. Buffy-coat-derived platelet concentrates were stored under standard conditions for 5 days. The expression of activation antigens CD42b, CD36, CD62p and phosphatidylserine on total platelets and populations of small, medium-sized and large platelets was analysed by flow cytometry on storage days 1, 3 and 5.

Results. The activation/lesion on total platelets and each platelet population was detected on storage day 3, by the increased expression of CD36. On the same day, increased expression of CD42b and CD62p was detected, but only on large platelets. Small and medium-sized platelets had increased CD62p expression only on day 5. Externalisation of phosphatidylserine was not detected.

Discussion. Evaluation of the level of expression of various activation markers on different platelet populations could be an additional valid analysis in cell quality control of platelet concentrates, and in the assessment of novel approaches to platelet concentrate manipulation.

Keywords: platelet storage lesion, platelet glycoproteins, flow cytometry, buffy coat.

Introduction

Platelet transfusion therapy is widely used to prevent haemorrhage in patients with thrombocytopenia and platelet disorders occurring as the consequence of bone marrow failure in malignant or non-malignant haematological diseases, during radio- and chemotherapy for haematological malignancies and solid cancers, and in patients undergoing major surgical interventions[1]. Notwithstanding the great benefits, platelet transfusions are responsible for more than 25% of transfusion-related adverse events[2]. Platelet concentrates (PC) are currently prepared by two methods: (i) production of PC collected from single donors through an aphaeresis method, and (ii) from whole blood as platelet rich-plasma or buffy-coat-derived platelet concentrates (PC-BC). Once prepared, PC are stored in the liquid state at 22±2 °C, with permanent rotation or rocking. The average platelet life-span *in vivo* is 7-10 days[3] and accordingly, PC have a short shelf-life of maximally 5 or 7 days. The quality of PC is profoundly affected by the collection/processing methods, *ex vivo* manipulation (such as pathogen reduction), and by contaminating leucocytes. Besides, during storage the number of platelets decreases, their morphology changes and their functions become impaired. Damage accumulated during PC storage, *i.e.* the platelet storage lesion, compromises the haemostatic function of PC and is also responsible for transfusion-related adverse effects[2,4-6].

Early and precise detection of the platelet storage lesion is still a subject of great interest in transfusion practice. Various laboratory tests are used to monitoring the quality of stored platelets through analysis of their morphology, biochemistry and functions[4,7]. Although liquid stored platelets show evidence of activation, they are effective in stopping or preventing haemorrhage even when there is not an evident increase in their numbers in peripheral blood, highlighting that there is a lack of appropriate analytical techniques to study insufficiently defined functional characteristics of platelet[8]. Although it is not routinely used, flow cytometry analysis of the expression of major platelet glycoproteins (GPIb, GPIIb-III, GPIX) responsible for adhesive and cohesive functions of platelets, *i.e.* initial adhesion of platelets to the subendothelial matrix, platelet spreading on the subendothelium and aggregation[3,9], can be used to estimate these cells' haemostatic function[4,10]. Activated platelets release the content of their intracellular granules and so their

activation can be estimated by measuring the level of proteins released from the granules into the extracellular fluid[7]. At the same time, flow cytometry detection of intra- and extracellular antigens (CD63, CD62p, CD36, sCD40L) specific for platelets granules, and negatively charged phospholipids on the external surface of platelet membranes, can be an important additional tool for analysis of platelet activation[3,10]. Moreover, it has been shown that changes in the expression of platelet surface molecules detected by flow cytometry are correlated with the changes in platelet function parameters determined by standard laboratory tests, confirming that flow cytometry can be used in the quality control of PC[8,11-13].

Platelets are not an identical set of cells, but comprise several populations of different ages and in different functional states[3]. Platelet populations can be identified and analysed separately by flow cytometry[14]. In this study we analysed the impact of 5 days of liquid storage on the expression of major glycoproteins and activation markers by PC-BC platelet populations.

Material and methods

Preparation of buffy-coat-derived platelet concentrates

Blood samples were taken from seven randomly selected male, 19- to 27-year old, blood donors, with normal clinical data, who had not taken preparations of acetylsalicylic acid in the preceding 7 days. Blood units (450±45 mL) were collected into quadruple plastic bag systems (Terumo Corporation, Tokyo, Japan) with citrate-phosphate-dextrose, saline-adenine-glucose-mannitol. The time to collect each investigated unit was less than 6 minutes. The buffy coats were isolated from whole blood, no later than 2 hours after the collection, by 10 minutes of centrifugation at 3,890 *g* (Hettich-Roto Silenta RP, Hettich Tuttlingen, Germany) and additionally separated with an automatic cell processor EX-30 (Terumo Corporation). By further centrifugation at 377 *g* for 5 minutes and processing, PC were obtained from the buffy coats. On average, a PC-BC contained 50-60 mL of platelets suspended in autologous plasma, at a concentration of $471\pm274\times10^9$/L[15]. The PC-BC were kept for 5 days in a Cooled Orbital Incubator thermostat (Gallenkamp, Loughborough, United Kingdom) at the temperature of 22±2 °C with permanent horizontal shaking. Platelet samples were taken aseptically (with syringes) immediately after the PC-BC had been prepared (day 1), and on days 3 and 5 of storage.

Platelet count and platelet morphological score

The platelet counts were determined by an analyser (Technicon H-3 System, Technicon, Tarrytown, NY, USA), while the morphological properties of platelets were studied with a phase-contrast microscope (Polyvar, Austria). The values of the platelet morphological score (PMS) were determined according to the percentage of platelet having different shapes. In each PC-BC unit, 100 platelets were analysed. Numerical values assigned to different morphological types were: balloons=0, dendrites=1, spheres=2 and disks=4.

Hypotonic shock response test

Platelet viability was assessed by the hypotonic shock response (HSR) test as described by Odink and co-workers[16]. The whole-blood aggregometer model 560 (Chrono-Log Corporation, Havertown, PA, USA) was calibrated by measuring light transmittance with platelet-poor plasma. A gently mixed suspension of PC-BC in platelet-poor plasma (PPP, 200 µL) was prepared and stirred by a magnetic bar in two aggregometer cuvettes for 10 minutes. Next, 200 µL of distilled water were added to the first cuvette containing the PC-BC and PPP. After adding the water, the decrease of the optical density at 610 nm (OD_{610}) was followed and recorded until it reached its lowest level and then for the subsequent 10 minutes, as the OD_{610} rose again. An identical OD_{610} recording was made in parallel for the sample in the second cuvette, to which isotonic saline was added instead of water. The percentage of HSR was determined using the formula:

$$\%\,\mathrm{HSR} = \left(\frac{\mathrm{OD}_{610\ \text{post 2 min in water}} - \text{lowest } \mathrm{OD}_{610}}{\mathrm{OD}_{610\ \text{in isotonic saline}} - \text{lowest } \mathrm{OD}_{610}}\right)\times 100$$

Activity of lactate dehydrogenase

The cell lysis/damage in PC-BC during 5 days of liquid storage was estimated on the basis of the level of the extracellular activity of lactate dehydrogenase (LDH). In brief, supernatants of aseptically taken PC-BC samples were obtained by centrifugation for 10 minutes at 1,500 *g*. The level of LDH in the supernatants was determined on a Hitachi 912 automatic analyser (Roche Diagnostics, Mannheim, Germany) with the commercially available spectrophotometric Roche Hitachi-LDH Assay (Roche Diagnostics).

Platelet aggregation

Platelet aggregation was measured by Born's optical method[17], using the laboratory aggregometer (BCT-Behring Coagulation Timer; DADE-Behring, Marburg, Germany). The samples were warmed to 37 °C and stirred by a magnetic bar. The aggregometer was calibrated by measuring light transmittance through a turbid mixture of PC-BC re-suspended in autologous plasma at the concentration of 2.5×10^5/mL, and then by measuring light transmittance through PPP. Light transmittance

through the PC-BC suspension was marked as 0%, and that through PPP as 100%.

One of the following agonists was then added to the PC-BC samples: 20 µM adenosine diphosphate (ADP), 200 µg/mL collagen, 10 µM epinephrine (all from DADE-Behring) and 150 µg/mL ristocetin, (Helena Biosciences Europe, Sunderland, England). The aggregation monitoring time was 10 minutes. After adding an agonist for aggregation to the PC-BC sample, light transmittance increased because the suspension became less turbid as aggregation proceded. The maximum level of aggregation, expressed as % of aggregation, was determined using the formula:

$$\text{Platelet aggregation (\%)} = \frac{(\text{Light transmittance}_{to} - \text{Light transmittance}_{10\ min})}{(\text{Light transmittance}_{to} - \text{Light transmittance}_{PPP})}$$

Detection of soluble platelet release markers

Concentrations of transforming growth factor β (TGF- β), soluble P-selectin, soluble annexin, platelet factor 4 (PF4), and β-thromboglobulin (β-TG) in supernatants of PC-BC (obtained after the precipitation of platelets by centrifugation, and stored at –70 °C) were determined with commercially available enzyme linked immunosorbent assays (ELISA). The Quantikine Human TGF-β1 Immunoassay DB 100 (assay range: 31.20- 2,000 pg/mL) was obtained from R&D Systems, Minneapolis, MN, USA. The human soluble P-selectin ELISA (assay range: 2.19-140 ng/mL) was obtained from Bender MedSystems Diagnostics GmbH, Vienna, Austria. The Zymutest Annexin V (assay range: 0.1-120 ng/mL) was obtained from Hyphen BioMed, Andresy, France. Enzygnost PF4 (assay range: 2-60 ng/mL) was from Behringwerke AG, Marburg, Germany, while the Asserachrom β-Thromboglobulin test (assay range: 10-40 IU/mL) was produced by Diagnostica Stago, Asnieres Cedex, France. All analyses were performed in accordance with the manufacturers' instructions. Optical density was measured in a 96-well plate reader BioTek ELx800 (Absorbance reader, BioTek Instruments, Inc., Winooski, VT, USA).

Flow cytometric analysis

For analysis of the expression of platelet surface markers, we used the following fluorochrome (FITC or PE) conjugated monoclonal antibodies: BD Pharmingen CD42a (anti-GPIX/CD42a), (Becton Dickinson, San Jose, CA, USA), PM6/248 (anti-GPIIb-IIIa/CD41) (Serotec, Oxford, United Kingdom), CLB-MB45 (anti-GPIbα/CD42b), CLB-AK-6 (anti-GP140 P-selectin/CD62p), CLB-gran/12 (anti-GP53/CD63) and CLB-IVC7 (anti-GPIV/CD36) (CLB, Amsterdam, the Netherlands).

The samples of PC-BC were taken using a sterile syringe after 2 hours of standing on a shaker (day 1) and on the third and fifth day of storage. Cells were counted with an automatic blood cell counter and a volume containing 25×10^6 platelets was mixed with 200 µL of 1% paraformaldehyde for 15 minutes, for fixation. Fixed cells were re-suspended in 1 mL phosphate-buffered saline. Monoclonal antibodies were added in 100 µL of the fixed cell suspensions containing 2.5×10^5 fixed platelets, and incubated for 30 minutes, in the dark, at room temperature. Once the incubation had finished, platelets were washed by centrifugation, re-suspended in phosphate-buffered saline with 0.3 mM EDTA and 0.1% bovine serum albumin, and analysed using a flow cytometer (Epics XL flow cytometer, Coulter Corporation, Miami, FL, USA). The flow cytometer was subjected to the routine daily setup procedures, verification of its optical alignment together with fluidic stability (with FLOW-CHECK™ 770 fluorospheres) and standardisation of detector functions (with FLOW-SET™ 770 fluorospheres), all contained in the setup kit (PC7 setup kit, Beckman Coulter; Coulter Corporation). Instrument linearity was checked with an Immuno-Brite™ Standards kit (Beckman Coulter). Colour compensation was verified with the QuickCOMP kit (Beckman Coulter). Platelets were identified according to their forward scatter (FSC) and side scatter (SSC) characteristics. As described by Di Pumpo and co-workers[14], for every sample an electronic gate was drawn around total platelets and the large, medium-sized and small platelet populations. The results were shown as FSC/logSSC histograms and fluorescence intensity/cell count histograms. Values of mean fluorescence intensity (MFI, i.e. the number of the analysed molecules per cell) and the percentage of platelets expressing the analysed antigens were also given.

The externalisation of phosphatidylserine was analysed based on the binding of annexin V. Platelets were diluted ten-fold in fresh 10 mM HEPES buffer with 2.5 mM $CaCl_2$, immediately before the analysis. The diluted platelets (5 µL) were lightly mixed with 250 µL of annexin V-FITC solution (1 mg/mL Annexin V-Fluos, Roche Diag. Mannheim, Germany) in annexin V binding buffer (10mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). After in incubation for 5 minutes at room temperature in the dark, 250 µL of annexin V binding buffer were added, and the cells were analysed immediately. The results are presented as MFI values and as the percentage of positive platelets.

Statistical analysis

The results are presented as the mean±standard deviation (SD). The statistical significance of differences between two groups was determined by the paired two-tailed Student's *t*-test. All statistical analyses were

carried out using the OriginPro 8 programme (OriginLab Corporation, Northampton, MA, USA). Differences in p-values of less than 0.05 were considered statistically significant.

Results

Effect of 5 days of liquid storage on basic indices of platelet quality

Data in Table I show that the platelet count in the PC-BC analysed remained stable over the 5-day storage period. However, the decreasing PMS values indicate that the number of disc-shaped platelets progressively decreased. The level of extracellular LDH, a marker of platelet damage/lysis[9], was low on day 1, but increased on day 3, and remained unchanged until the end of storage. The results of the HSR test show that the percentage of PC-BC platelets with preserved capacity to recover shape after hypotonic stress decreased progressively during storage. Decreased platelet aggregation was detected on day 3, after which stayed aggregation remained unchanged until day 5.

Effect of 5 days of liquid storage on the concentration of soluble mediators in platelet concentrates

The levels of TGF-β and P-selectin in the PC-BC supernatants were unchanged during the 5-day storage period. Increased annexin and PF4 concentrations were detected on day 5. The concentration of β-TG increased on day 3 of storage, and continued to rise until the end of storage.

Flow cytometry follow-up of the influence of 5 days of liquid storage on platelets

As shown in Figure 1, flow cytometry was used to study the PC-BC during the entire storage period, focusing on three populations: small, medium-sized and large platelets. On day 1 the populations of small, medium-sized and large platelets accounted for 67±6%, 22±3% and 8±3% of the total number of platelets, respectively. After 3 days of storage we detected a significant decrease in the number of medium-sized platelets only. After 5 days of storage there were significant reductions in the numbers of medium-sized and large platelets, while the number of small platelets increased significantly.

The expression of major platelet glycoproteins (CD42a, CD41) and platelet activation markers (CD42b, CD36, CD62p, and externalised phosphatidylserine) was analysed. Representative fluorescence intensity/cell count histograms showing changes in the platelet expression of CD41 and CD62p during storage are shown in Figure 2.

Numeric flow cytometry data (% of positive cells and MFI values) for total platelets and the platelet populations are given in Table II and Table III, respectively.

The results show that the expression of analysed platelet surface molecules on total platelets, as a rule, reflects their expression on small platelets but differs from that on large platelets. Thus, the expression (MFI) of major platelet glycoprotein CD41 (GPIIb-IIIa) on

Table I - Influence of 5 days of liquid storage on in vitro parameters of platelet quality in buffy coat-derived platelet concentrates.

Day	Basic PC indices			
	Number (10^9/L)	*PMS*	*LDH (U/L)*	*HSR (%)*
1	471±274	364±24	139±26	69±15
3	421±230	288±17*	212±17*	49±12*
5	411±222	138±16†,‡	234±25†	36±11†,‡
Day	**Platelet aggregation (%)**			
	ADP	*Epinephrine*	*Collagen*	*Ristocetin*
1	71±14	63±19	67±17	85±7
3	33±10*	30±6*	35±15*	75±7*
5	31±9†	29±6†	27±10†	76±12†
Day	**Soluble mediators**			
	TGF-β (ng/mL)	*P selectin (ng/mL)*	*Annexin (ng/mL)*	*PF4 (μg/mL)*	*β-TG (IU/mL)*
1	2.0±0.3	29±7	0.7±0.2	128±35	151±81
3	3.3±1.5	31±12	1.4±0.5	438±244	760±295*
5	7.0±4.4	53±29	3.9±1.8†,‡	1,385±383†,‡	1,346±249†,‡

PMS: platelet morphological score; LDH: lactate dehydrogenase; HSR: hypotonic shock response; TGF-β: transforming growth factor β; PF4: platelet factor-4; β-TG: β thromboglobulin. The data are shown as the mean±SD of seven platelet concentrate units. Statistically significant difference ($p<0.05$) between: * the 1st and 3rd days; † the 1st and the 5th days; ‡ the 3rd and the 5th days.

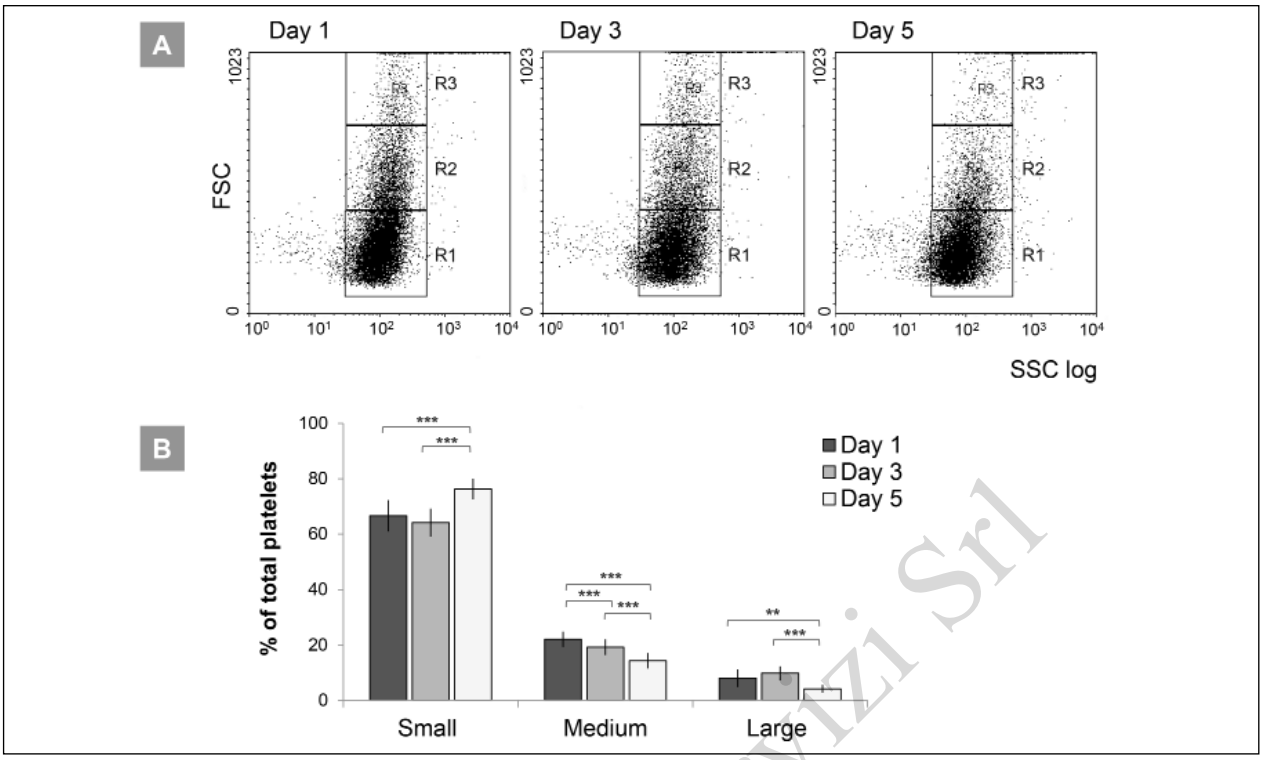

Figure 1 - The influence of 5 days of liquid storage on platelet subpopulations in buffy coat-derived platelet concentrates (PC-BC). (A) FCS/SSC diagrams of an individual PC-BC. (B) Cumulative results of seven PC-BC. Horizontal brackets indicate a statistically significant change in the percentage of platelets belonging to a particular platelet population; ***p<0.001; **p<0.01.

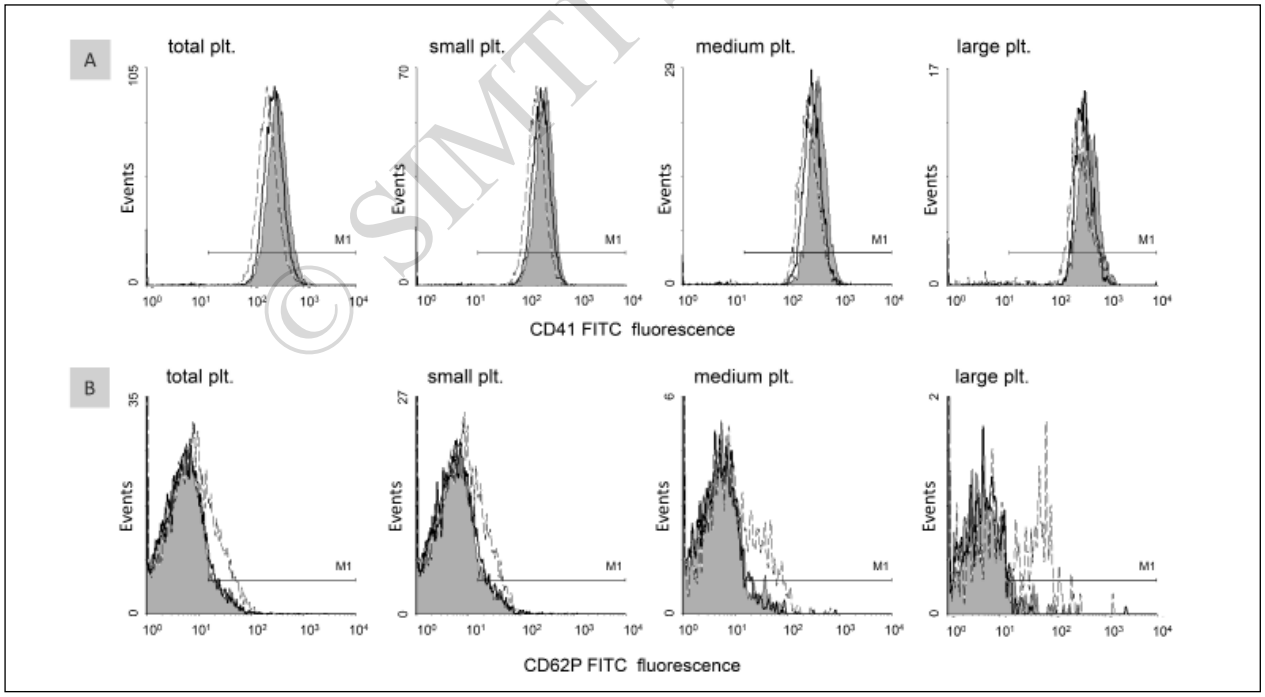

Figure 2 - The expression of platelet membrane antigens CD41 and CD62p in buffy coat-derived platelet concentrates (PC-BC) during 5 days of liquid storage - flow cytometry analysis.
Cell count/fluorescence intensity histograms are of one representative PC-BC. Grey plot: day 1; solid line: day 3; dashed line: day 5. The total platelets (plt) and platelet subpopulations with large, medium an small volume were identified based on FSC/log SSC values as it was shown in Figure 1.

Table II - Influence of 5 days of liquid storage on the membrane expression of major glycoproteins and activation markers on total platelets in buffy coat-derived platelet concentrates.

Marker	Day	Positive platelets (%)	MFI
CD41 / GPIIb-IIIa	1	99 ±1	27±3
	3	97±2§	23±3*
	5	98±1†	21±2 †,‡
CD42a / GPIX	1	99±1	6.2±0.7
	3	96±3*	8.0±1.2*
	5	97±2‖	8.1±0.8 †
CD42b / GPIbα	1	96±3	5.5±2.4
	3	95±3*	6.6±1.4
	5	96±1	6.4±0.9
CD36 / GPIV	1	91±4	9±2
	3	94±3*	13±4*
	5	94±2†	19±5†,‡
CD62p / P selectin / GP/140	1	7±2	2.4±0.5
	3	8±2	2.7±0.3
	5	15±5†,‡	2.5±0.3
Phosphatidylserine	1	4.4±0.9	60±23
	3	6.7±3.6	45±21
	5	5.5±2.0	40±19‡

The data are shown as the mean±SD of seven platelet concentrate units. Statistically significant difference ($p<0.05$) between: * the 1st and 3rd days, † the 1st and 5th days, and ‡ the 3rd and 5th days. Difference close to statistical significance ($0.05<p<0.06$) between: § the 1st and 3rd days and ‖ the 3rd and 5th days. MFI: mean fluorescence intensity.

total platelets decreased progressively during the 5 days of storage, mirrored by a progressive decrease of CD41 expression on small platelets. In contrast, the expression of CD41 on medium-sized and large platelets was decreased on day 3 but then remained unchanged until the end of storage on medium-sized platelets, but increased on large platelets, so that the MFI value of CD41 expressed on large platelets on day 5 of storage reached the level recorded on day 1.

The expression of CD42a (GPIX) on total platelets increased significantly on day 3, and stayed unchanged until day 5. The same pattern of CD42a expression was detected for small and medium-sized platelets, whereas on large platelets the expression continued to increase until the end of storage. The expression of CD42b (a major platelet glycoprotein but also an activation antigen) on total platelets, as well as on small and medium-sized platelets, remained unchanged during storage. However, CD42b expression on large platelets was increased on day 3, and remained at the same level until day 5.

When the percentages of platelets expressing major glycoproteins were analysed, it was found that the percentages of CD42a- and CD42b-positive total platelets were slightly, but significantly, reduced only on day 3, whereas on the day 5 the percentages had returned to their initial values. The same pattern of CD42b reactivity was shown for the populations of small and medium-sized platelets, while a decrease in the percentage of CD42b-positive large platelets was detected only on day 5. The CD42a reactivity for platelet populations differed from that of total platelets. Thus, a significantly reduced number of CD42a-positive small platelets was detected on day 3 and reduced numbers of CD42a-positive medium-sized and large platelets was detected on day 5. A decreased percentage of CD41-positive cells was detected among the total platelets and populations of medium-sized and large platelets. No significant change was detected in the percentage of CD41-positive small platelets during the storage conditions applied storage.

Platelet activation during the 5 days of storage was assessed based on the expression of CD36, CD62p activation markers and the externalisation of phosphatidylserine. The results show that the applied storage conditions induced no externalisation of phosphatidylserine on total platelets or on any of the platelet populations. No significant increase in the percentage of CD62p-positive total platelets was detected before day 5. The same pattern of expression was observed for small and medium-sized platelets, whereas an increased percentage of large CD62p-positive platelets was detectable already on day 3. The increased percentage of CD36-positive total platelets was detected on day 3, reflecting the increased CD36 positivity on small and medium-sized platelets. The percentage of CD36-positive cells among the population of large platelets did not change during storage. The level of CD36 expression (MFI) increased progressively during storage and the pattern of expression was the same for total platelets and the platelet populations.

Discussion

In the Institute for Transfusiology and Haemobiology of the Military Medical Academy, PC-BC are prepared as pools of six individual donations. In this study we analysed the changes in the expression of surface membrane markers on platelets from seven individual PC-BC during 5 days of liquid storage at 22±2 °C. Using laboratory tests recommended by the Food and Drug Administration[18,19] we showed that the values of basic parameters of platelet quality detected in this study

Table III - Influence of 5 days of liquid storage on the expression of major glycoproteins and activation markers on platelet populations in buffy coat-derived platelet concentrates.

Marker	Day	Positive platelets (%)			Mean fluorescence intensity		
		Small	*Medium*	*Large*	*Small*	*Medium*	*Large*
CD41 / GPIIb-IIIa	1	98±1	99±1	95±2	25±3	33±4	44±6
	3	96±3[§]	95±6	92±8	22±3[*]	27±4[*]	35±6[*]
	5	98±1	97±1[†]	91±4[†]	20±2[†,‡]	28±3[†]	45±9[‡]
CD42a / GPIX	1	98±1	98±1	96±2	5.9±0.7	7.3±0.8	9.1±1.0
	3	94±4[*]	94±7[§]	90±10	7.2±1.5[*]	9.6±1.7[*]	12.4±3.1[*]
	5	96±1[†]	96±1[†]	91±5[†]	7.8±0.5[†]	10.9±0.9[†]	17.0±2.7[†,‡]
CD42b /GPIbα	1	96±3	98±2	95±3	5.1±2.2	6.9±3.1	9.4±4.1
	3	94±4[*]	94±6[§]	92±8	6.1±1.3	8.6±1.8[§]	12.2±2.7[*]
	5	95±1	96 ±2	92±4[†]	6.1±0.9	9.0±1.2	13.8±1.9[†]
CD36 / GPIV	1	94±2	97±2	94±4	8±2	11±2	17±4
	3	95±3[*]	97±2[*]	96 ±2	11±3[*]	16±5[*]	24±7[*]
	5	96±1[†]	98±1[†]	97±2	16±4[†,‡]	23±6[†,‡]	46±11[†,‡]
CD62p / P selectin / GP 140	1	8±2	9 ±1	11±3	2.5±0.4	3.6±0.6	5.4±3.1
	3	9±3	9 ±3	8±2[*]	2.9±0.4[*]	4.2±1.7	5.6±2.8
	5	18±6[†,‡]	20±8[†,‡]	25±10[†,‡]	2.5±0.3[‡]	3.5±0.6	6.0±4.5
Phosphatidylserine	1	7.3±3.5	2.2±0.8	1.6±1.1	66±25	56±24	52±22
	3	9.8±5.4	3.9±2.2[§]	4.5±3.8	48±21	46±15	46±18
	5	8.7±4.7	3.0±1.6	2.6±2.0	42±21[‡]	50±20	76±29

The data are shown as the mean±SD of seven platelet concentrate units. Platelet populations were identified as shown in Figure 1. Statistically significant difference ($p<0.05$) between: ([*]) the 1st and 3rd days, ([†]) the 1st and 5th days, and ([‡]) the 3rd and 5th days. Difference close to statistical significance ($0.05<p<0.06$) between: ([§]) the 1st and 3rd days.

were within the norms proposed by Serbian National Regulations[20].

Flow cytometry is a sensitive method, used for single-cell level analysis of activation and reactivity of circulating platelets, for the diagnosis of inherited and acquired platelet disorders, and for monitoring the effects of antiplatelet agents[7]. Flow cytometry has also been considered as an appropriate method for evaluating the quality of PC after whole blood processing, filtering, liquid state storage, or cryopreservation[8,21-23]. When flow cytometry analysis of whole blood is performed, it is recommended that platelets are identified (*i.e.*, gated) based on their scatter properties and reactivity with antibodies to some pan-platelet glycoproteins[24]. Other blood cells are present in trace amounts in PC[15,25] and platelets could be gated based on their forward scatter and side scatter properties[4,26]. In our study we used this approach to identify platelets and then confirmed their identity by their expression of pan-platelet glycoproteins CD41 (GPIIb-IIIa) and CD42a (GPIX). Flow cytometric analysis of total platelets showed increased expression of the CD36 activation marker on day 3 of storage. The CD62p activation marker was detectable on day 5, whereas the externalisation of phosphatidylserine was not detected. Activation of platelets in our PC-BC on day 3 of storage was also confirmed by standard tests. Our results are in accordance with the results of other researchers showing that expression of platelet activation markers correlates with platelet activation parameters detected by standard laboratory tests, confirming that flow cytometry can be used as an additional method for the quality control of PC[8,11-13]. Furthermore, flow cytometry is less labour-intensive and less time-consuming than standard laboratory tests and our results and the results of our colleagues confirm that in certain conditions, flow cytometry might be a significant assurance for monitoring platelet storage lesion.

The quality of PC-BC can be influenced by many factors: the method used to separate the platelets from whole blood, temperature, transport time, bag disposal, bag agitation device, pre-storage, centrifugation, pooling, etc. According to Garraud and co-workers[2] there are 5 million combinations of individual parameters that can influence PC-BC quality. It is, therefore very

difficult to compare our results directly with those of other research groups[11,27-33] and we think that each blood transfusion centre should establish its own "in-house" reference range for the assessment of platelet storage lesion (as proposed by Daskalakis and co-workers[34] for the analysis of platelets of patients with bleeding diatheses).

Platelets are not homogenous cells, and based on their size, *i.e.* forward scatter properties, we identified, and separately analysed three populations of large, medium-sized and small platelets. This type of flow cytometric analysis of platelet populations was previously performed in a study of inherited platelet disorders[14]. The results of our study show that MFI values for all analysed surface molecules increased as follows: small platelets < medium platelets < large platelets. We assume that the number of molecules of the analysed markers per surface area is the same in all populations of platelets and that MFI values increase with the size of the platelets, so the results obtained were those expected. It has been suggested that MFI values could be expressed as a normalised fluorescence ratio to volume (*i.e.*, FSC value)[14] in order to eliminate the influence of platelet size on the overall fluorescence intensity. We did not express fluorescence intensity as the MFI/FCS ratio either for total platelets or for the platelet populations, because the influence of the volume on fluorescence intensity was minimised by determining, for each of the analysed PC, a specific FSC/SSC gating parameter of total platelets and platelet populations on day 1 and keeping it at the same level during analysis on storage days 3 and 5. By keeping the FCS constant we analysed platelets of exactly the same size during the entire storage period and recorded the MFI value that reflected the overall fluorescence intensity.

To our knowledge, our group and that of Södergren and co-workers[35] are the first to use this approach in the analysis of platelet storage lesion. According to Smyth (2014)[3], young platelets, recently released by megakaryocytes, are larger and denser, and undergo remodelling in the circulation, in part by shedding some of their surface components. The storage time-dependent decrease in the number of large platelets in PC-BC, shown in our study, confirms the *in vitro* ageing. Södergren and co-workers[35] showed that stored platelets (prepared by aphaeresis) undergo further fragmentation, but this phenomenon cannot be detected before the 12th day of storage. They also proposed recording fragmentation into platelet subpopulations as a potential marker for the platelet storage lesion. Our results show that, based on the expression of CD42b and CD62P on platelet populations, signs of activation can be detected much earlier. Thus, changing levels of CD42b and CD62P expression were not detected on small and medium-sized platelets. However, their increased expression and, therefore, activation was detected on large platelets on day 3 of storage.

In our study, we did not find increased expression of phosphatidylserine on stored platelets or their subpopulations, in accordance with the results of Södergren and co-workers[35], who did not find different expression of phosphatidylserine or lysosome-associated membrane protein-1 on populations of stored platelet (prepared by aphaeresis) in resting state but only after stimulation with different platelet agonists.

Conclusions

In the last few years there have been many attempts to analyse the platelet storage lesion by various highly sophisticated physicochemical methods[36-46]. Although these methods have provided very important data on platelet biology their use in routine haematological diagnosis or control of PC quality is still unlikely. Standard laboratory tests (platelet count, PMS, HSR, aggregometry, swirling, pH measurement, P_{O2}, P_{CO2}, extracellular LDH activity, soluble platelet release markers) and flow cytometry remain powerful and widely available techniques for platelet analysis[17]. We believe that our results and the results of other authors[14,35] confirm that flow cytometric analysis of platelet populations could be an additional method for routine quality control of liquid-stored platelets, but also for testing new additive solutions, cryoconservation protocols and cryoprotectants. This study shows that expression of activation markers (CD42b and CD62P) in stored PC can be detected only in the population of large platelets. Expression of activation markers analysed in this study and expression of other markers such as CD63, activated CD41 (GPIIb-IIIa), and the mediator of vascular inflammation sCD40L[47] in specific platelet populations were shown to be useful in early/timely detection of the platelet storage lesion and prevention of serious adverse transfusion effects including transfusion-related acute lung injury. Further studies are warranted to investigate a possible correlation between flow cytometric analysis of platelet populations, PMS and the haemostatic response of the platelet transfusion recipient.

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Authorship contributions

DVu designed the research, performed experiments, analysed data, and prepared the manuscript; VI analysed data, prepared and wrote the manuscript; DVo and VS performed experiments and analysed data; MT critically read the manuscript; BB oversaw the project and assisted with preparation of the manuscript.

The Authors declare no conflicts of interest.

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