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Changes in platelet morphology and function during 24 hours of storage

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Abstract

For *in vitro* studies assessing the interaction of platelets with implant materials, common and standardized protocols for the preparation of platelet rich plasma (PRP) are lacking, which may lead to non-matching results due to the diversity of applied protocols. Particularly, the aging of platelets during prolonged preparation and storage times is discussed to lead to an underestimation of the material thrombogenicity. Here, we study the influence of whole blood- and PRP-storage times on changes in platelet morphology and function.

Blood from apparently healthy subjects was collected according to a standardized protocol and examined immediately after blood collection, four hours and twenty four hours later. The capability of platelets to adhere and form stable aggregates (PFA100, closure time) was examined in sodium citrate anticoagulated whole blood (WB) using the agonists equine type I collagen and epinephrine bitartrate (collagen/epinephrine) as well as equine type I collagen and adenosine-5'-diphosphate (collagen/ADP). Circulating platelets were quantified at each time point. Morphology of platelets and platelet aggregates were visualized microscopically and measured using an electric field multi-channel counting system (CASY). The percentage of activated platelets was assessed by means of P-selectin (CD62P) expression of circulating platelets. Furthermore, platelet factor 4 (PF4) release was measured in platelet poor plasma (PPP) at each time point.

Whole blood PFA100 closure times increased after stimulation with collagen/ADP and collagen/epinephrine. Twenty four hours after blood collection, both parameters were prolonged pathologically above the upper limit of the reference range. Numbers of circulating platelets, measured in PRP, decreased after four hours, but no longer after twenty four hours. Mean platelet volumes (MPV) and platelet large cell ratios (P-LCR, 12 fL – 40 fL) decreased over time. Immediately after blood collection, no debris or platelet aggregates could be visualized microscopically. After four hours, first debris and very small aggregates occurred.

After 24 hours, platelet aggregates and also debris progressively increased. In accordance to this, the CASY system revealed an increase of platelet aggregates (up to 90 µm diameter) with increasing storage time. The percentage of CD62P positive platelets and PF4 increased significantly with storage time in resting PRP. When soluble ADP was added to stored PRP samples, the number of activatable platelets decreased significantly over storage time.

The present study reveals the importance of a consequent standardization in the preparation of WB and PRP. Platelet morphology and function, particularly platelet reactivity to adherent or soluble agonists in their surrounding milieu, changed rapidly outside the vascular system. This knowledge is of crucial interest, particularly in the field of biomaterial development for cardiovascular applications, and may help to define common standards in the *in vitro* hemocompatibility testing of biomaterials.

Keywords

platelet, platelet function, platelet rich plasma, whole blood, platelet aging, platelet storage, hemocompatibility, biomaterials

1. Introduction

The contribution of platelets to hemostasis lies in the secretion of activators for further recruitment of platelets, the formation of a primary hemostatic plug, and the provision of a surface for the plasmatic coagulation to proceed [44]. The spontaneous hyperaggregability of platelets is described to be a risk factor for major adverse cardiovascular events and occlusion syndromes [1,2,15,28,38]. On the other hand, there are a lot of diseases associated with non-functional or partly non-functional platelets, which need to be tested using different activators (adenosine 5'-diphosphate, arachidonic acid, thrombin, collagen, ristocetin, etc.) depending on the respective defects [18]. Moreover, the testing of a thrombosis risk differs significantly from the question whether a bleeding risk exists. Therefore, a lot of function tests have been developed for special purposes [16].

Blood samples drawn in primary healthcare are often sent to a laboratory for analysis so that the measurement of platelet aggregation is carried out after a certain period of time. As platelets outside the body can age rapidly [20] with changes in morphology and function, this storage time is an inherent problem of platelet function testing. Also for studies assessing the interaction between platelets and implant materials aged platelets can lead to an underestimation of the thrombogenicity of the material, because the platelets are not able to react with their full response or only a part of the platelets are able to react while others are already activated and degranulated. Such platelets lose their pro-coagulant activity *in vitro* [25] and can no longer contribute to the adhesion or aggregation process.

The evaluation of platelet adhesion to surfaces foreign to the body is complex, poorly standardized and time consuming. Among others, a prolonged storage time can have a strong influence on the results of platelet interactions with implant materials. Currently, only scarce data are available about the influence of the storage time on platelet aggregation - a process named “platelet storage lesion” [26,45] - and most of those studies were carried out only after short storage times of up to two hours [19,20,22,42]. In the present study, the association between storage time and integrity and function of platelets was assessed over a time period of 24 hours.

2. Material and Methods

2.1 Study design

The study was designed and performed according to the current guidelines of the British committee for standards in hematology (BCSH) as well as of the International Society on Thrombosis and Haemostasis (ISTH) [7,31,33,34]. According to the criteria of the Nordkem-workshop, blood was taken from apparently healthy subjects, who received no platelet function inhibitors or other pharmaceuticals for at least 10 days [2]. Blood was obtained from the cubital vein by an experienced phlebotomist using a standardized atraumatic protocol, and was collected in S-Monovettes® (Sarstedt, Germany) filled with sodium citrate (final concentration 0.106 mol·L^{-1}) or ethylenediaminetetraacetic acid (EDTA, for hemogram) as anticoagulant in the morning (between 8:30 and 09:00 a.m.). Immediately after blood collection, tubes were slowly agitated to ensure an appropriate mixing of anticoagulant and blood (and discarded if there was any evidence of clotting).

The study protocol received an approval by the institutional committee of the Charité University Medicine Berlin and was in accordance with the ethical guidelines of the journal.

2.2 Blood sample preparation

Platelet poor plasma (PPP) was obtained by centrifugation of whole blood (or platelet rich plasma) at 2000 *g* for 20 minutes. To obtain platelet rich plasma, blood was centrifuged at 140 *g* for 20 minutes. Platelet rich plasma (PRP) was carefully collected and transferred into fresh polypropylene tubes (Biochrom-Merk Millipore, Berlin, Germany). Tubes were kept capped and stored at room temperature.

The platelet density was adjusted to 2 x 10^5 platelets·μL^{-1} using platelet poor plasma. Prior to any further testing, PRP was allowed to rest for 30 minutes at room temperature under gentle agitation (10 rpm, VWR Rocking Platform, VWR, Darmstadt, Germany).

2.3 Inclusion and Exclusion criteria

Blood pre-analytics were carried out to exclude subjects exhibiting early inflammatory processes and abnormalities in platelet count or function. The following parameters were tested and proven to be within the respective reference ranges. Hemogram values, including numbers of red and white blood cells, hemoglobin, and hematocrit, were tested from EDTA

anticoagulated whole blood (Sysmex XS-800i, Norderstedt, Germany). Platelet function was assessed with the PFA-100® platelet function analyzer (Siemens Healthcare Diagnostics, Marburg, Germany). As agonists to assess the reactivity of platelets in sodium citrate anticoagulated whole blood, equine type I collagen and epinephrine bitartrate as well as equine type I collagen and adenosine-5'-diphosphate (ADP) were utilized [10,11]. C-reactive protein (CRP) levels were tested from EDTA anticoagulated whole blood utilizing a semi-quantitative quick test (IMACO, Lüdersdorf, Germany). As agonist for platelet activation in PRP, ADP in a concentration of 20 µM was used.

2.4 Measuring Methods

For the assessment of platelet aggregation under high shear rates, mimicking *in vivo* flow conditions, the PFA-100® was utilized [29]. Blood is aspirated at a shear rate of 5000 - 6000 s^{-1} through a glass capillary with a diameter of 200 µm into a membrane pore (diameter 150 µm) coated with collagen (2 µg type I collagen) and either epinephrine (EPI: 10 µg epinephrine-bitartrate) or ADP (50 µg) as platelet activator. Platelets adhere to the collagen-coated pore wall, become activated and aggregate among each other, forming a platelet plug, which finally occludes the pore and stops the blood flow, which is measured as the closure time. Measurements were performed in duplicate and mean values were calculated. The reference range of the closure time is – in case of collagen/ epinephrine activation between 80 – 160 seconds, for the collagen/ADP activation between 68 and 121 s [27].

Platelets were counted using following techniques: hematology analyzer (Sysmex XS-800i, SYSMEX Deutschland, Norderstedt, Germany), CASY® (Roche Innovatis AG, Bielefeld, Germany), and flow cytometry (MACSQuant®, Miltenyi Biotec, Bergisch Gladbach, Germany). The CASY technology for measuring platelet volume [32] is based on an electric field multi-channel cell counting system with a pulse area analysis. The cells are suspended in an electrolyte and aspirated through a measuring pore (120 µm) with constant shear rate. During the measurement process, a pulsed low voltage field with 1 MHz is applied to the measuring pore. The electrolyte-filled measuring pore represents a defined electrical resistance. Since intact cells can generally be considered isolators, an increased level of resistance is achieved over the measuring pore. This resistance is a dimension for the volume of the cells.

To microscopically visualize the platelets, 10 µL PRP was then filled in a Neubauer chamber. Images were acquired with an inverted microscope (Axiovert 40C, Zeiss, Jena, Germany). All

samples were photographed 3 minutes after filling to allow sedimentation of the platelets to the chamber bottom. This was done in an identical time table for all specimens.

Flow cytometric analyses were carried out with formaldehyde fixed platelets on a MACSQuant® analyzer (Miltenyi Biotec) [4]. Platelets were stained for the GPIb/IX platelet membrane glycoprotein (anti-CD42a-FITC, Becton Dickinson Bioscience, San José, USA) and the glycoprotein P-selectin (anti-CD62P-PE, Immunotech, Beckman Coulter, Marseille, France) as marker for alpha-granules release upon platelet activation [9].

Platelet factor 4 (PF4) concentrations in PPP were measured using a PF4 human ELISA kit (Cloud-Clone Corp., Houston, USA) according to the manufacturer´s guidelines.

2.5 Statistics

The results are presented as mean value ± standard deviation (MV ± SD). The Gaussian distribution was tested using the Kolmogorov-Smirnov test. Changes between time points were evaluated by ANOVA followed by Bonferroni's multiple comparisons test (in case of Gaussian distribution) or by Friedman test followed by Dunn's multiple comparisons test (in case the samples were not Gaussian distributed). GraphPad Prism was used for statistical analysis (version 6.02, GraphPad Software Inc., San Diego, USA).

3. Results

3.1 Study group

Six apparently healthy subjects were included. None of them suffered from hypertension, diabetes mellitus or lipid metabolism disorder. None was on platelet function inhibitor or other medication at the time of examination. One out of the six participants was current a smoker. Table 1 summarizes demographical and laboratory data. All laboratory variables from all participants were within the reference ranges, none of the apparently healthy test subjects had to be excluded from the study.

Table 1. Demographical and laboratory data of the blood donors (*: indicate smokers, hemogram reference values are provided by SYSMEX; reference values for PFA100 – closure times are provided by Siemens Healthcare Diagnostics).

	Donors							Reference values	
	1	2	3	4	5*	6	MV±SD	female (f)	male (m)
Demographic data									
Sex	f	m	f	m	f	m			
Age	28	22	47	32	42	34	34 ± 9		
Weight (kg)	52	77	59	79.5	58	80	68 ± 13		
Height (cm)	160	175	159	183	165	180	170 ± 10		
BMI ($kg \cdot m^{-2}$)	20.3	25.1	23.3	23.7	21.3	24.7	23.1 ± 1.9	18.50 – 24.99 [37]	
Hemogram									
WBC ($1x10^3 \cdot \mu L^{-1}$)	7.97	6.03	4.88	4.28	6.71	6.76	6.11 ± 1.35	3.9 – 10.0	4.2 – 9.1
RBC ($1x10^6 \cdot \mu L^{-1}$)	4.82	5.33	4.64	4.68	4.36	5.18	4.84 ± 0.36	3.9 – 5.2	4.6 – 6.1
HGB ($mmol \cdot L^{-1}$)	8.9	9.85	8.9	8.95	8.5	8.85	8.99 ± 0.45	7.5 – 9.9	8.7 – 11.2
HCT (%)	40.5	44.3	41.5	39.1	40.2	40.2	41.0 ± 1.8	34.1 – 44.9	40.1 – 51.0
MCV (fL)	83.9	83.1	8.6	83.6	91.3	77.5	84.8 ± 5.0	79.4 – 94.8	79.0 – 92.2
PLT ($1x10^3 \cdot \mu L^{-1}$)	201	186	147	266	256	265	220 ± 50	182 - 369	163 - 337
MPV (fL)	10.5	10.1	11.8	10.8	10.6	9.8	10.6 ± 0.7	7 - 13	7 - 13
PFA100 – closure times									
Col/EPI (s)	118	142	100	151	101	130	124 ± 21	84 - 160	
Col/ADP (s)	97	99	78	90	79	95	90 ± 9	68 - 121	

3.2 Whole blood closure time

There seemed to be an influence of the storage time on the PFA100-closure time after collagen/ADP activation, though not significant (Friedman test, Dunn´s multiple comparison test, $p>0.05$, see Figure 1). However, 24 h after blood collection, closure times were significantly prolonged and above the upper limit of the reference range. Similar results were obtained when whole blood was stimulated with collagen/epinephrine. After 24 h of storage, closure times were pathologically prolonged, whereas, after 4 h values were elevated but not significantly different to the resting whole blood (Friedman test, Dunn´s multiple comparison test, $p>0.05$, see Figure 1). It has to be noted that in cases, where closure times were prolonged and exceeded the maximum measuring time, values were calculated as 240 s, in order to enable a comparison with the other measurements.

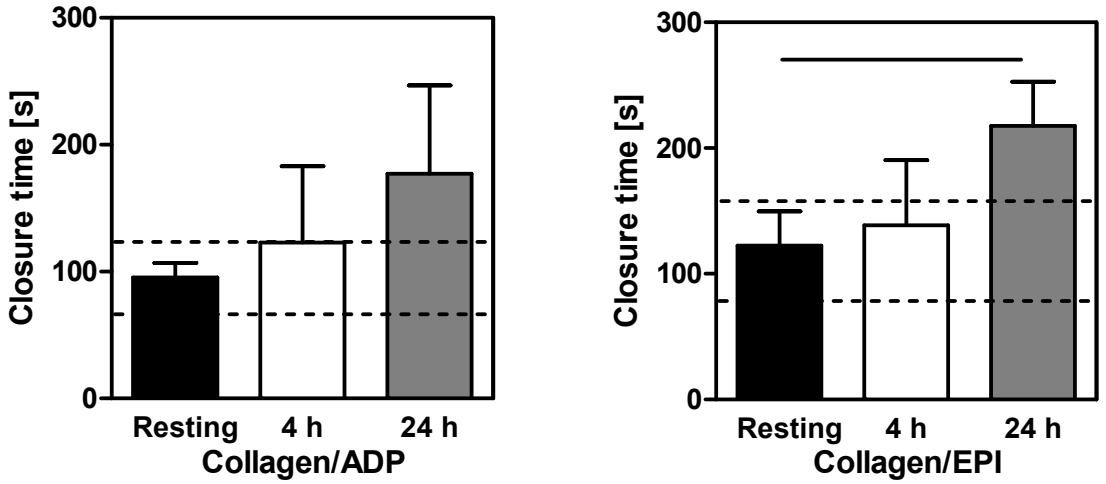

Figure 1. PFA100 closure times after stimulating sodium citrate (105-109 mm/L final concentration) anticoagulated whole blood with collagen/ADP (left) or collagen/epinephrine (right) immediately after blood collection (resting), 4 h or 24 h of whole blood storage at room temperature. Dashed lines indicate the lower and the upper limit of the reference range. In cases where closure times were prolonged and exceeded the maximum measuring time of 240 s (three measurements for collagen/ADP and four measurements for collagen/EPI), values were calculated as 240 s, in order to enable a comparison with the other measurements (n=6 donors, measurements in duplicate, bars indicate $p<0.05$; Friedman test, Dunn's multiple comparisons test).

3.3 Platelet numbers and morphology in platelet rich plasma

The number of platelets – measured in an automated platelet counter (SYSMEX) – slightly changed over storage time (Figure 2). The decrease was significant after 4 h (ANOVA, Dunn´s multiple comparison test, $p<0.05$) but no longer significantly decreased after 24 h (ANOVA, Dunn´s multiple comparison test, $p<0.05$). Particularly, mean platelet volume (MPV) and the large platelets, quantified as platelet large cell ratio (P-LCR: 12 fL – 40 fL) decreased over time in the SYSMEX measurements (Figure 2). Measurements of resting PRP in the CASY system (using a 120 µm diameter capillary) revealed similar changes in the numbers of circulating platelets compared to the SYSMEX (Figure 2). However, different trends were observed for the mean volume and the number of events larger 5.04 µm, which can be considered as platelet aggregates (Figure 3). As shown in Figure 2, both parameters increased with storage time and were significantly different after 24 h compared to the resting PRP ($p<0.05$).

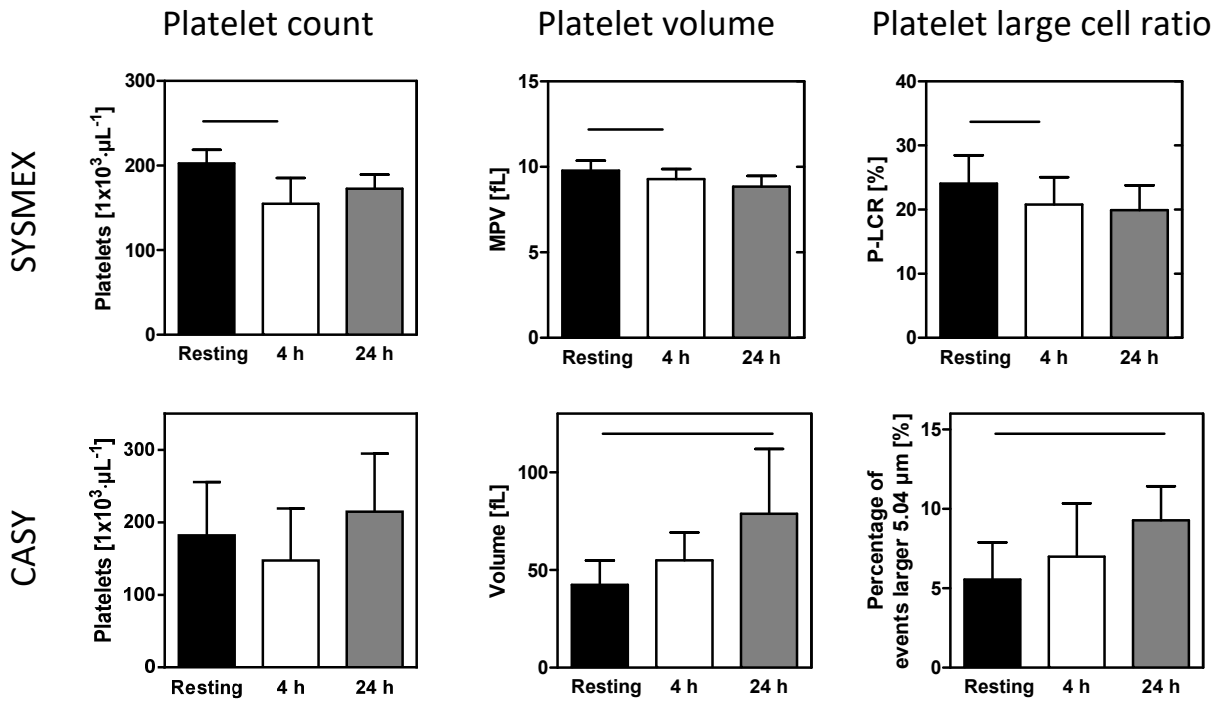

Figure 2. Platelet number, mean platelet volume (MPV) and platelet large cell ratio (P-LCR) of platelets and platelet aggregates measured in platelet rich plasma immediately after blood collection (resting), 4 h and 24 h of storage. Upper row represents measurements in the SYSMEX system (n=6 donors, measurements in duplicate), lower row represents measurements in the CASY system (n=4 donors, measurements in duplicate, bars indicate $p<0.05$; Friedman test, ANOVA, Dunn´s multiple comparison test).

Platelet shape was observed by phase contrast microscopy. As shown in Figure 4, small and large platelet aggregates were increasingly present after 4 and 24 h storage (Figure 4 B and C) and could be clearly distinguished from the platelets with discoid shape that are predominantly observed immediately after blood collection (Figure 4 A). Furthermore, platelet fragements with impaired membrane integrity (trypan blue positive platelets) and debris were observed (Figure 4 D).

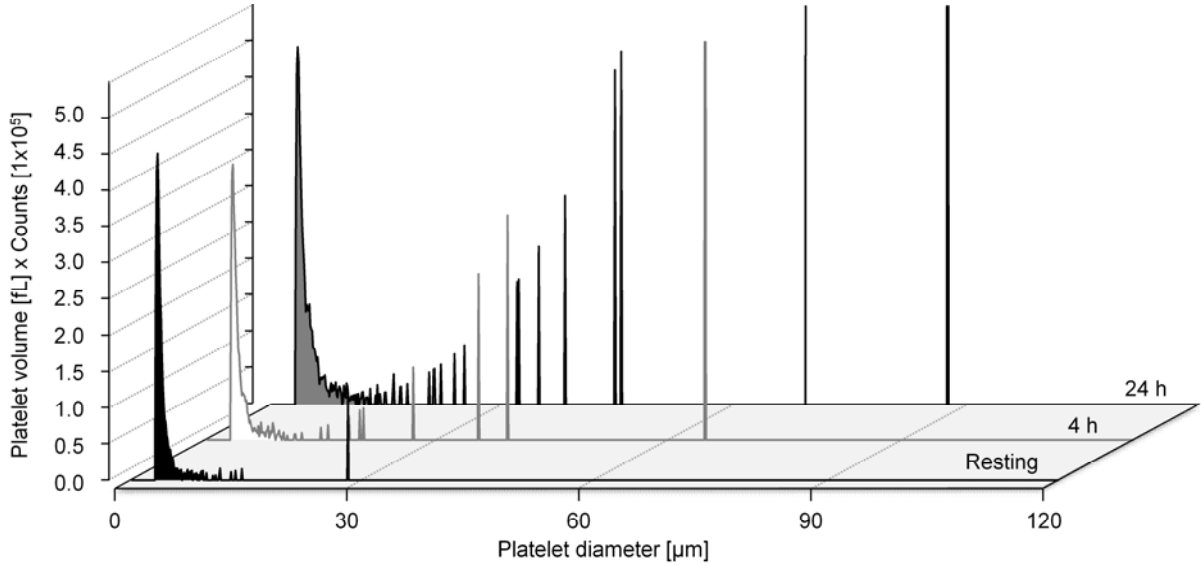

Figure 3. Representative area and volume distribution curves of circulating platelets and platelet aggregates in platelet rich plasma after different storage times. Black filling = immediately after blood collection (resting), white filling = 4 h, gray filling = 24 h.

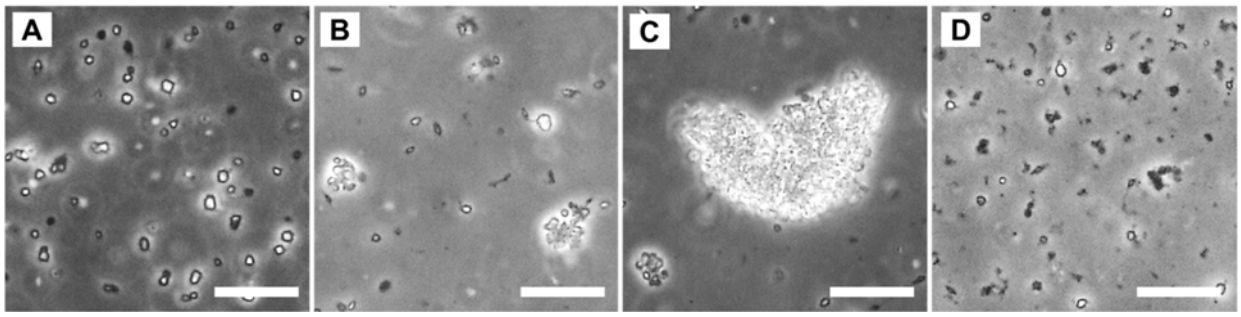

Figure 4. Representative phase contrast images showing platelet morphologies after different storage times of platelet rich plasma. A) platelets with discoid morphology immediately after blood collection (resting), B) and C) small and large platelet aggregate formation as well as D) debris after 4 h and 24 h of storage (Scale bar represents 25 µm).

3.4 Platelet aggregation, membrane receptors and soluble platelet factors

Forward scattered light can be quantified proportional to cell size, so that relative distributions of the platelet sizes could be obtained by flow cytometry. Figure 5 A shows a typical example of the platelet size distribution before and after stimulating the PRP with ADP (20 µM). After activation, there was a clear right shift with a steep increase of sizes indicating platelet aggregates (this could not be demonstrated completely because the range of platelet dimensions had been exceeded). Aggregation of platelets after ADP stimulation decreased and size distribution curves of activated and not activated platelets equalized with storage time.

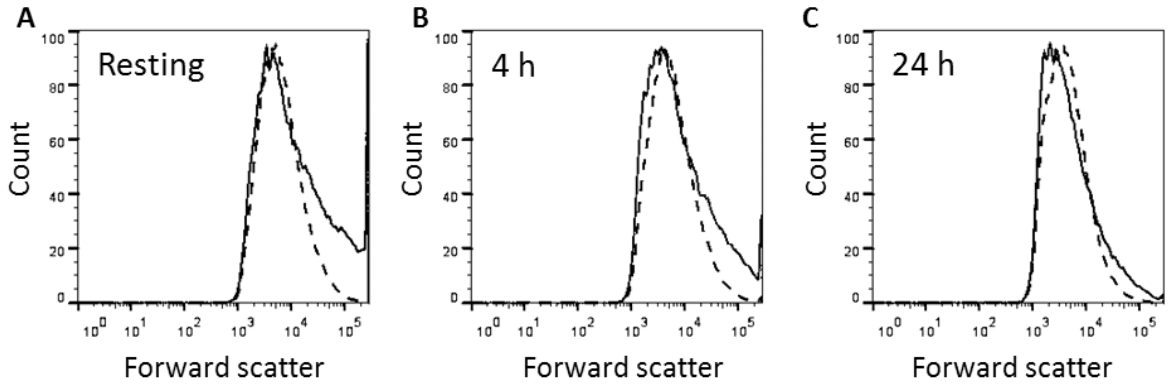

Figure 5. Representative histograms of flow cytometric analysis of platelet size in platelet rich plasma. A) immediately after blood collection, B) 4 h and C) 24 h later. Dashed line represents resting platelet rich plasma; solid line represents platelet rich plasma stimulated with 20 µM ADP. The results are presented as histogram plots of forward scatter intensity.

Numbers of CD42a positive platelets ($CD42a^+$) decreased after 4 h of storage but were similar after 24 h compared to the numbers obtained in resting PRP. These results are in line with the SYSMEX and CASY measurements. The number of platelets able to internalize the GPIb/IX receptor upon ADP stimulation – given as $CD42a^+$ $\Delta PRP\text{-}PRP_{stim}$ – decreased continuously and significantly (ANOVA, $p<0.01$) with storage time from 53,669 $CD42a^+$ platelets·μL^{-1} ± 22,071 $CD42a^+$ platelets·μL^{-1} in resting PRP to 28,354 $CD42a^+$ platelets·μL^{-1} ± 17,560 $CD42a^+$ platelets·μL^{-1} after 4 h ($p<0.05$ compared to resting PRP) and 23,227 $CD42a^+$ platelets·μL^{-1} ± 13,445 $CD42a^+$ platelets·μL^{-1} after 24 h ($p<0.01$ compared to resting PRP, Figure 6).

Numbers of activated platelets - measured as P-selectin ($CD62P^+$) expression - increased over time significantly in the stored samples (repeated measures one way ANOVA: $p=0.05$, Figure 6). While immediately after blood collection 8.2% ± 3.5% of the platelets expressed CD62P, the proportion increased to 26.3% ± 9.1% after 4 h ($p<0.01$ compared to resting PRP) and 38.3% ± 13.1% after 24 h ($p<0.01$ compared to resting PRP).

The number of platelets able to express CD62P upon ADP (20 µM) stimulation – given as $CD62P^+$ ΔPRP_{stim}-PRP – decreased continuously and significantly (ANOVA, $p<0.01$) with storage time. Values ranged from 35.1% ± 10.3% $CD62P^+$ platelets in resting PRP to 20.2% ± 8.0% $CD62P^+$ platelets after 4 h ($p<0.01$ compared to resting PRP) and 13.7% ± 8.4% $CD62^+$ after 24 h ($p<0.05$ compared to 4 h and $p<0.001$ compared to resting PRP).

In PPP, immediately sourced after blood donation, a very low PF4 concentration of 11,638 $pg \cdot mL^{-1}$ ± 7,168 $pg \cdot mL^{-1}$ was measured. In stored PRP, the PF4 concentration increased from 33,062 $pg \cdot mL^{-1}$ ± 21,466 $pg \cdot mL^{-1}$ at baseline (resting), to 57,030 $pg \cdot mL^{-1}$ ± 28,232 $pg \cdot mL^{-1}$ after 4 h and 226,668 $pg \cdot mL^{-1}$ ± 244,305 $pg \cdot mL^{-1}$ after 24 h of storage. After ADP stimulation of the PRP, all measured PF4 values were lower compared to the respective values under storage conditions. Differences between the stored and ADP activated PRP – given as PF4 $pg \cdot mL^{-1}$ $\Delta PRP\text{-}PRP_{stim}$ – increased with time, markedly after 24 h. Values ranged from 8,164 $pg \cdot mL^{-1}$ ± 9,935 $pg \cdot mL^{-1}$ at baseline (resting), to 14,797 $pg \cdot mL^{-1}$ ± 12,030 $pg \cdot mL^{-1}$ after 4 h and 143,549 $pg \cdot mL^{-1}$ ± 210,719 $pg \cdot mL^{-1}$ at 24 h of storage.

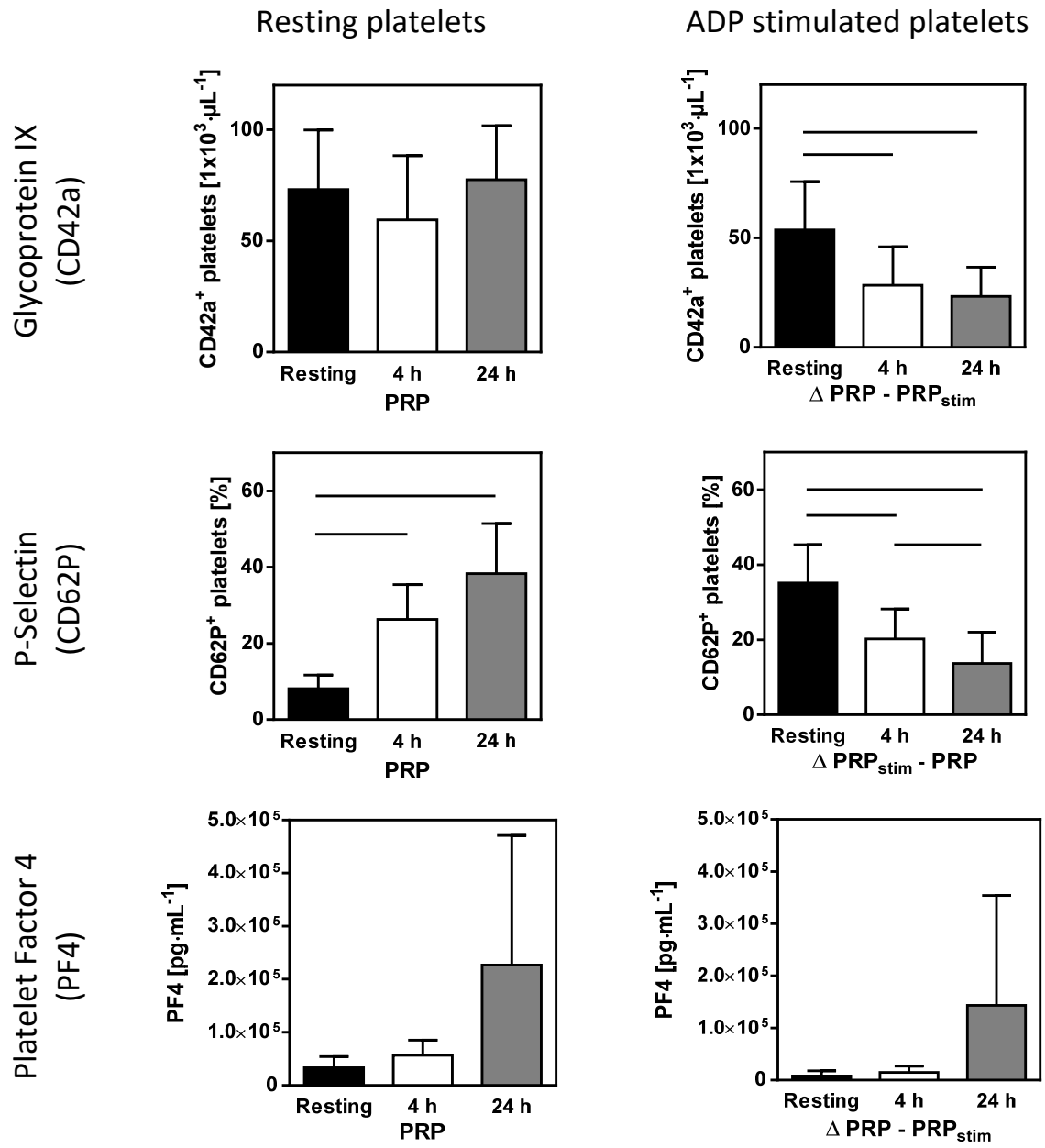

Figure 6. Expression profiles of platelet surface receptors CD42a and CD62P as well as amounts of soluble PF4, measured in platelet rich plasma immediately after blood collection (resting), 4 h and 24 h of storage (left column). Values in the right column represent measurements of ADP stimulated PRP samples at the respective time points (n=6 donors for glycoprotein IX and P-selectin, n=3 donors for PF4, bars indicate $p<0.05$; Friedman test, Dunn's multiple comparisons test).

4. Discussion

The study revealed that it is extremely important to use freshly prepared platelets for hemocompatibility testing since they age rapidly outside the vascular system losing their reactivity to soluble agonists in their surrounding milieu. In addition, platelets are sensitive to manipulation, and are prone to artifactual *in vitro* activation [24], which makes it difficult to compare results of different studies because no standardized and generally accepted protocol exists, particularly for hemocompatibility testing of biomaterials. As there are so many different platelet functions, an all-in-one method suitable for every platelet function study is hardly conceivable today. In addition, testing of platelet function requires a high degree of experience as well as expertise to perform and interpret. To get valid results, a lot of preconditions need to be fulfilled.

In the framework of this study, the influence of storage time on the morphology and function of platelets was analyzed. Immediately after blood collection, trisodium citrate-anticoagulated whole blood or PRP were separated and stored up to 24 h. Stored whole blood lost - with increasing duration of storage - its ability to close the capillary in the PFA100 device (Figure 1), which indicates a loss of platelet function, particularly, the adhesion and aggregation at high shear rates.

Studies in PRP revealed that the number of platelets decreased significantly after 4 hours due to the formation of platelet aggregates (Figure 2), which could be demonstrated by the CASY measurements and the microscopic analysis. Here, an increasing number of platelet aggregates up to 90 µm diameter occurred within the 24 hours storage time (Figures 3 and 4). This observation was further confirmed by the SYSMEX measurements since platelet volume and the ratio of large platelets (platelet volume: 12 fL – 40 fL) decreased with increasing storage time (Figure 2). Different groups could show that especially the larger platelets are metabolically active, aggregate preferably and have a higher thrombotic potential than smaller platelets [5,35,42]. However, in automated hematology analyzers, such as the applied SYSMEX, single circulating platelets or small platelet aggregates are measured up to a volume of 40 fL. Platelet aggregates that exceed this upper volume limit cannot be determined and, consequently, these parameters decrease when larger platelet aggregates occur as depicted above. The number of single platelets in the SYSMEX and CASY measurements did not further decrease after 24 h, which might be due to the lesion of stored platelets and the subsequent generation of cell debris or platelet-derived microparticles (PMP) - a heterogeneous population

of platelet membrane-derived vesicles (<1 µm) - which can be counted falsely as small platelets [41]. The occurrence of this phenomenon is well described for platelet concentrates, which are stored over a more prolonged time period, but seemed to be also relevant in the here applied time frame since cell debris or PMPs can clearly be seen microscopically after 24 h (Figure 4). Further proof is given by the volume distribution curves of the CASY measurements. Particularly for events up to 5 µm diameter, a clear increase after 24 h of storage can be observed, compared to the resting values and the measurements after 4 h (Figure 3).

Numbers of activated platelets and soluble platelet activators - measured as P-selectin (CD62P) and glycoprotein IX (CD42a) expression as well as PF4 generation - increased over time in the stored samples (Figure 6). This appears to be the consequence of an ongoing activation process in the stored PRP that leads, not only to the activation of platelets and the secretion of soluble agonists, but also to the formation of e.g. $CD62P^+$ PMPs [17,43]. These results are in good agreement with the above described morphological data and reports concerning the PMP generation of platelets in platelet concentrates, the so-called platelet storage lesion [8,39,45]. Moreover, our data reveal a progressively reduced number of platelets that can be activated by ADP (CD62P, CD42a) in the PRP sample. Furthermore, the increase of platelet size and formation of platelet aggregates after ADP activation was progressively diminished within the storage time as shown by the flow cytometry data (Figure 5). These results from the PRP studies are in line with the measurements in whole blood. Here, the loss of platelet function led to strongly increased closure times that correspond to a pathologically prolonged bleeding time *in vivo* [13].

The activation of platelets is further associated with the release of various platelet agonists from different granules, among others e.g. thrombin, ADP, thromboxane, serotonin, thrombospondin and platelet activating factors [21,36]. These molecules can induce a progressing activation of further platelets, which could be documented by the measurement of PF4 since the relative concentration of PF4 in platelets exceeds that of plasma by 280,000-fold [6,12,14,23]. Figure 6 shows the tremendous increase of PF4 after 24 h of storage, which is in very good agreement with the above discussed data, particularly the increase of $CD62P^+$ platelets and the generation of PMPs at this time point. The observed reduction in PF4 after activation with ADP is most probably a result of the increased binding of PF4 to the β2 glycoprotein I (β2GPI) on the membrane of activatable platelets [6,40].

5. Conclusion

In conclusion, the study showed that an increasing number of platelets became activated during 24 h of storage and could no longer be induced to adhere or to aggregate appropriately [30]. At the same time, platelets dissolved and cell debris as well as microparticles occurred. Within the analyzed storage time of 24 h, a significantly smaller number of platelets remained that could be activated and reacted on the soluble agonist ADP. However, even after 4 h of storage, a clear influence on all analyzed parameters, particularly on platelet function, was shown. Based on these findings, we recommend that hemocompatibility studies using human platelet rich plasma should be completed within 4 h after blood taking. In view of the need for better standardized and appropriately scheduled hemocompatibility experiments [3], we are convinced that this will enable a more reliable and reproducible testing, which may further help to prevent underestimation of the thrombogenicity of a biomaterial.

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