**In vitro** evaluation of pathogen-inactivated buffy coat-derived platelet concentrates during storage: psoralen-based photochemical treatment step-by-step

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**Background.** The Intercept Blood System™ (Cerus) is used to inactivate pathogens in platelet concentrates (PC). The aim of this study was to elucidate the extent to which the Intercept treatment modifies the functional properties of platelets.

**Material and methods.** A two-arm study was conducted initially to compare buffy coat-derived pathogen-inactivated PC to untreated PC (n=5) throughout storage. A four-arm study was then designed to evaluate the contribution of the compound adsorbing device (CAD) and ultraviolet (UV) illumination to the changes observed upon Intercept treatment. Intercept-treated PC, CAD-incubated PC, and UV-illuminated PC were compared to untreated PC (n=5). Functional characteristics were assessed using flow cytometry, hypotonic shock response (HSR), aggregation, adhesion assays and flow cytometry for the detection of CD62P, CD42b, GPIIb-IIIa, phosphatidylserine exposure and JC-1 aggregates.

**Results.** Compared to fresh platelets, end-of-storage platelets exhibited greater passive activation, disruption of the mitochondrial transmembrane potential ($\Delta V_m$), and phosphatidylserine exposure accompanied by a decreased capacity to respond to agonist-induced aggregation, lower HSR, and CD42b expression. The Intercept treatment resulted in significantly lower HSR and CD42b expression compared to controls on day 7, with no significant changes in CD62P, $\Delta V_m$, or phosphatidylserine exposure. GPIIbIIIa expression was significantly increased in Intercept-treated platelets throughout the storage period. The agonist-induced aggregation response was highly dependent on the type and concentration of agonist used, indicating a minor effect of the Intercept treatment. The CAD and UV steps alone had a negligible effect on platelet aggregation.

**Discussion.** The Intercept treatment moderately affects platelet function **in vitro**. CAD and UV illumination alone make negligible contributions to the changes in aggregation observed in Intercept-treated PC.

**Keywords:** Intercept Blood System™, pathogen-reduction technology, platelet storage lesion.

**Introduction**

The emergence of new pathogens and the risk of bacterial contamination of platelet concentrates (PC) remain major concerns when trying to guarantee the safety of platelet transfusions. Pathogen-reduction technologies have been developed over the last few decades to tackle these risks and include the Intercept Blood System™ (Cerus, Concord, CA, USA), the Mirasol technique (TerumoBCT, Lakewood, CA, USA), and THERAFLEX UV-Platelet (MacoPharma, Mouvaux, France). The Intercept technique utilises a combination of ultraviolet (UV) A light (320-400 nm) and amotosalen hydrochloride, an aminoethoxymethyl derivative of psoralen. Amotosalen intercalates into the helical regions of DNA and, upon UVA activation, cross-links to pyrimidine bases, thereby preventing the replication of susceptible pathogens. After illumination, residual amotosalen is removed by adsorption to guarantee a final concentration below 2 µM in PC.

Intercept treatment of PC has been shown to be efficient against a broad range of viruses, bacteria, and parasites, as well as contaminating leucocytes. To date, a number of clinical trials have compared the efficiency of Intercept-treated and conventional platelets. Four meta-analyses have been performed, each with different results. For example, in an Intercept-specific meta-analysis with reassignment of the reported bleeding scores into a single scale, Vamvakas showed that, compared to untreated platelets, Intercept-treated platelets resulted in lower corrected count increments and higher rates of mild and moderate bleeding. Conversely, Cid et al. found that
clinical trials of Intercept were too heterogeneous with regards to 1 hour corrected count increments to reach any conclusion, whereas the 24-hour corrected count increments were homogenously lower for Intercept-treated platelets. However, a reassessment of the bleeding rates did not show any difference between Intercept-treated and control platelets. Routine experience tends to indicate a lack of adverse events, no differences in platelet transfusion requirements, and a significantly lower incidence of acute transfusion reactions with Intercept-treated PC than with standard platelets or gamma-irradiated PC, as shown by haemovigilance programmes.

In addition, several groups have investigated the effect of Intercept treatment on in vitro platelet function. Because of differences in the preparation of PC, storage media, and the protocols used for in vitro assays, there are discrepancies in the published data. The studies showed metabolic changes, impaired mitochondrial function, accelerated passive activation, and altered in vitro platelet aggregation in response to agonist-induced activation in Intercept-treated platelets. Recently, the impact of Intercept treatment on platelets was assessed at the protein level using proteomic methods; the treatment induced an acceleration of storage lesions, even though the majority of proteins remained intact. Here, we evaluated the effect of the Intercept treatment on platelet function during storage. This study has the advantage of comparing PC prepared and analysed strictly in the same way. In addition, the influence of the different steps in Intercept treatment, particularly incubation with the compound adsorbing device (CAD), on the increase in storage lesions has been questioned; we, therefore, evaluated the contribution of incubation with the CAD and UV illumination alone to the changes in platelet aggregation observed in Intercept-treated PC.

**Materials and methods**

**Preparation of the platelet concentrates**

Whole blood was obtained from regular donors who gave their consent to the use of their blood components in research. Whole blood units (450±50 mL +63 mL citrate-phosphate-dextrose) were collected (day 0) in a quadruple bag system (NGR6428B, Fenwal, Lake Zurich, IL, USA) and kept at 22 °C overnight. After centrifugation of the whole blood at 3,500×g for 14 minutes, red blood cells and plasma were expressed on Optipress II (Fenwal). Five ABO-matcheduffy coats (60 mL, haematocrit 0.4, from donors who had not been taking non-steroidal anti-inflammatory drugs) were manually pooled with 280 mL of Intersol additive solution (Fenwal) and centrifuged at 500×g for 10 minutes. Platelets were expressed on a manual plasma extractor and filtered to remove leucocytes (R7013, Fenwal).

**Intercept process (study 1)**

In study 1, we compared platelets treated with the Intercept process to untreated platelets. On day 1, two ABO-matched PC were pooled and split into two identical products, each coming from 10 donors. One untreated PC was kept under agitation at 22 °C for up to 7 days after collection (container of filtration kit R7013, 1,300 mL, PL2410 plastic). The other paired PC was processed with the Intercept kit for large volumes (INT2203B, Cerus). A total of 17.5 mL amotosalen hydrochloride 3 mM solution was added sterilely to the PC and the mixture further illuminated with the Intercept illuminator (INT100, Cerus) at 3.9 J/cm². After illumination, the Intercept-treated PC was transferred to the CAD container and kept under agitation for 14 hours to remove residual amotosalen. The treated PC was then transferred to the final storage bag (1,300 mL, PL2410 plastic) under agitation at 22 °C.

**Intercept process step-by-step (study 2)**

In study 2, we aimed to evaluate the contribution of each step of the Intercept process, i.e. incubation with the CAD or UV illumination only, to the changes observed in Intercept-treated platelets. On day 1, three ABO-matched PC were pooled and split into four identical products. One untreated PC served as a control (untreated unit). The second PC was treated with the complete Intercept process as described above (Intercept unit). The third PC was directly incubated with the CAD for 14 hours under agitation at 22 °C and then transferred to the storage bag (CAD unit, no UV, no amotosalen). The fourth PC was treated with UVA only at 3.9 J/cm² in the absence of amotosalen and stored under agitation at 22 °C (UV unit, no amotosalen, no CAD).

**Agregometry**

Agregometry was performed with an APACT 4004 aggregometer (LABiTec, Ahrensburg, Germany). Briefly, 120×10⁶ platelets were centrifuged for 5 minutes at 500×g, suspended in 300 µL of AB group fresh-frozen plasma (FFP, previously centrifuged at 120,000×g to remove microparticles) and agitated for 5 minutes. The platelet suspension (250 µL) was transferred to a cuvette with 2 µL of 0.1 M calcium chloride and equilibrated for 2 minutes at 37 °C before being measured in the presence of: thrombin receptor activator peptide 6 (TRAP; Endotell [Allschwil, Switzerland], 8-60 µM), adenosine diphosphate (ADP; Sigma [Buchs, Switzerland], 2-7.5 µg/mL) and arachidonic acid (AA; Endotell, 0.38-0.7 mg/mL). Volumes of agonist ranged from 5 to 20 µL depending on the final concentration required. FFP-diluted platelets were set at 0% aggregation before addition of the agonist, and 300 µL AB-group FFP was set at 100% aggregation.
Hypotonic shock response

The hypotonic shock response (HSR) was measured with the aggregometer. Aliquots of platelets (3-5 mL) were diluted to 0.9×10^6 platelets/µL with AB-group FFP and agitated for 5 minutes at room temperature and then 250 µL of the diluted platelets were transferred to a cuvette. The diluted platelets were set at 0% aggregation, and distilled water set at 100% aggregation. Two hundred microlitres of distilled water or sodium chloride were injected 120 seconds after the start of monitoring, which continued for a total of 10 minutes.

Adhesion assay

The platelet adhesion assay was performed according to Bellavite et al. Briefly, platelets were centrifuged (1,000×g, 10 minutes) in the presence of 1 µM prostaglandin E1 (Sigma, P5515) and resuspended at 115 × 10^3 platelets/µL in HEPES-Tyrode's buffer containing 2 mM calcium chloride and 1 mM magnesium chloride. Platelets were allowed to adhere (1 hour, 37 °C) to fibrinogen-coated microplates (Maxisorp 96-well, VWR [Nyon, Switzerland], 735-0083). After incubation, the microplate was carefully washed twice with HEPES-Tyrod's buffer and the wells supplemented with 150 µL of 0.1M citrate buffer (pH 5.4) containing 5 mM p-nitrophenyl phosphate (Sigma, S0942) and 0.1% Triton X-100 (Fluka [Buchs, Switzerland], 93420). After incubation for 1 hour at 37 °C, the reaction was stopped with 100 µL of 2.5 M sodium hydroxide. The p-nitrophenol produced by the reaction was measured with a microplate reader (Biorad PR 2100, Cressier, Switzerland) at 405 nm. The number of platelets adhered to the wells was determined using a calibration curve from a standard platelet suspension.

Flow cytometry

Samples were analysed on a FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using CellQuest Pro software (BD Biosciences). The flow cytometer was calibrated with a bead kit (CalibRITE 3, BD Biosciences). PE-Cy™ mouse anti-human CD62P (cat. 551142), FITC annexin V (cat. 556420), FITC Pac-1 (cat. 340507), and PE mouse anti-human CD42b (cat. 555473) were provided by BD Biosciences. MitoProbe JC-1 (M34152) was purchased from Invitrogen.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Each experiment was performed on five different preparations of buffy coat-derived PC. Comparisons were made using analysis of variance (ANOVA) with repeated measures and post-hoc comparisons (R software, version 2.15.1). Differences were considered statistically significant when the p value was <0.05.

Results

Characteristics of the platelet concentrates

In study 1, all characteristics of the PC fulfilled the requirements of the Intercept treatment (Table I). In study 2, plasma and additive solution content, contaminating red blood cells and leucocyte counts were within the required ranges, but the volume per unit was below recommendations, which resulted in an increased amotosalen concentration compared to that in study 1 (approximately 21% higher than in study 1 and 7% higher than the maximum concentration recommended for Intercept treatment). Nonetheless, the platelet dose of all units was still above the lower limit of 2.5×10^11 platelets during early storage (day 2). The platelet dose decreased significantly in all end-of-storage PC in study 2 (Table I).

Table 1 - Characteristics of platelet concentrates (PC) (measured n=5).

<table>
<thead>
<tr>
<th>Specifications</th>
<th>Plasma content</th>
<th>Additive solution content</th>
<th>Volume/unit (mL)</th>
<th>Platelet dose (×10^11)</th>
<th>RBC</th>
<th>Leucocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>39%</td>
<td>61%</td>
<td>353±22</td>
<td>3.54±0.35</td>
<td>&lt;4×10^10/L</td>
<td>&lt;1.0×10^6/unit</td>
</tr>
<tr>
<td>Intercept</td>
<td>37%</td>
<td>63%†</td>
<td>353±15</td>
<td>3.40±0.35</td>
<td>3.46±0.36</td>
<td></td>
</tr>
<tr>
<td>Study 2</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>39%</td>
<td>61%</td>
<td>235±10</td>
<td>2.55±0.25</td>
<td>&lt;4×10^10/L</td>
<td>&lt;1.0×10^6/unit</td>
</tr>
<tr>
<td>Intercept</td>
<td>36.5%</td>
<td>63.5%†</td>
<td>279±41</td>
<td>2.89±0.28</td>
<td>2.69±0.29**</td>
<td></td>
</tr>
<tr>
<td>CAD only</td>
<td>39%</td>
<td>61%</td>
<td>239±10</td>
<td>2.61±0.31</td>
<td>2.48±0.29**</td>
<td></td>
</tr>
<tr>
<td>UV only</td>
<td>39%</td>
<td>61%</td>
<td>237±21</td>
<td>2.57±0.27</td>
<td>2.46±0.26**</td>
<td></td>
</tr>
</tbody>
</table>

(1) Two PC were pooled and split in two equally divided PC; (2) Three PC were pooled and split in four equally divided PC; † Intercept-treated PC contain amotosalen-hydrochloride solution in addition to the additive solution and plasma; * p<0.05, **p<0.001 for significant difference between day 2 and day 7. RBC: red blood cell; CAD: compound adsorbing device; UV: ultraviolet.
Platelet aggregation (study 1)

In study 1, platelet aggregation was evaluated in untreated and Intercept-treated PC in response to four agonists: TRAP (8-60 µM), ADP (8-60 µM), collagen (2-7.5 µg/mL), and AA (0.38-0.7 mg/mL; Figure 1A-D and Table II). The aggregation response increased as a function of the agonist concentration and reached a plateau upon TRAP, ADP, and collagen-induced activation, whereas the AA-activated platelets exhibited an on/off response. On day 7 of storage, the platelets exhibited a lower aggregation response in general compared to platelets on day 2. Untreated platelets tended to exhibit a better or similar aggregation response upon activation on day 2 compared to Intercept-treated platelets, except upon AA activation, when a significantly higher response was observed at low AA concentration. On day 7, Intercept-treated platelets had a significantly stronger response compared to controls after activation with ADP (20-60 µM of ADP) or AA (0.5 mg/mL of AA).

Platelet adhesion to fibrinogen (study 1)

On day 2, Intercept-treated and untreated platelets exhibited similar static adhesion to fibrinogen (Figure 2A). Throughout the storage period, the percentage of adhered platelets from Intercept-treated PC increased on days 4 and 7 compared to the percentage from control units. The expression of active GPIIb-IIIa (Figure 2B), as determined by the PAC-1 antibody, was stable throughout the storage period for untreated platelets, but a significant increase was observed on day 7 for Intercept-treated platelets. The Intercept treatment induced significantly higher GPIIb-IIIa expression on day 7 compared to that on control platelets.

Surface expression markers (study 1)

The CD62P (P-selectin) levels were significantly higher on day 7 of storage of all PC compared to the levels on day 2, with no significant differences upon Intercept treatment. Conversely, CD42b levels (GPIb alpha chain, von Willebrand factor receptor) tended to decrease...
**Table II** - Study 1: in vitro measures of Intercept-treated PC (n=5, each coming from 10 donors) in comparison to untreated PC (n=5, each coming from 10 donors).

<table>
<thead>
<tr>
<th></th>
<th>Aggregation response to TRAP (% of maximum aggregation)</th>
<th>Aggregation response to ADP (% of maximum aggregation)</th>
<th>Aggregation response to collagen (% of maximum aggregation)</th>
<th>HSR (%)</th>
<th>CD62 expression (%)</th>
<th>CD42 expression (%)</th>
<th>JC-1 aggregates (%)†</th>
<th>Annexin V expression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 7</td>
<td>10 µM</td>
<td>20 µM</td>
<td>40 µM</td>
<td>60 µM</td>
<td>10 µM</td>
<td>20 µM</td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>4.6±1.1</td>
<td></td>
<td>19.6±13.5</td>
<td>69.0±8.9</td>
<td>81.0±2.3</td>
<td>84.7±2.0</td>
<td>8.3±0.9</td>
<td>45.9±9.6***</td>
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</table>

*p<0.05,**p<0.01,***p<0.001 for difference between day 2 and day 7.
† JC-1 aggregates corresponds to FL1+/FL2+ platelets. PC: platelet concentrates; ADP: adenosine diphosphate; TRAP: thrombin receptor activator peptide 6; HSR: hypotonic shock response.

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**Characteristics of pathogen-inactivated PC**

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throughout the storage period for all PC. Intercept-treated PC had significantly reduced expression of CD42b on day 7 compared to that of controls.

Cell viability markers (study 1)

The HSR decreased significantly throughout the storage period for all units (Table II). Intercept-treated PC exhibited a significantly reduced HSR on day 7 compared to that of untreated PC. Annexin V, a marker of phosphatidylserine exposure, increased significantly during platelet storage. No significant differences were observed with Intercept treatment.

The percentage of JC-1 aggregates represents the percentage of platelets with functional transmembrane mitochondrial potential (ΔΨm) because JC-1 dye is distributed in the mitochondria as aggregate and in the cytosol as monomer. Approximately 5.1±1.9% and 16.0±4.8% of platelets had disrupted ΔΨm on days 2 and 7, respectively. Intercept treatment did not significantly alter the ΔΨm in platelets throughout 7 days of storage.

Intercept treatment step-by-step (study 2)

In study 2, we compared untreated platelets to platelets treated with the whole Intercept process, platelets incubated with the CAD only and platelets illuminated with UVA only. The platelet aggregation observed is illustrated in Figure 3. The agonist concentrations in study 2 were slightly different from the concentrations used in study 1, as another AB group FFP was used for aggregometry and required adjustments.

The CAD and UV treatments alone did not induce differences compared to untreated platelets, except for a lower response after CAD incubation on day 2 of storage and an increased response to UV illumination on day 7 of storage after ADP activation. Therefore, only the complete Intercept treatment (amotosalen+ UV+CAD) affects platelet aggregation.

Discussion

The main functions of platelets, such as a capacity to adhere to damaged endothelium, activation, platelet-to-platelet aggregation, and pro-coagulant activity, should be maintained following transfusion. Some groups have already examined the effect of pathogen inactivation processes on platelet function in vitro using buffy coat or apheresis-derived PC. There is a relatively clear consensus that Intercept-treated platelets have increased metabolism compared to untreated platelets, as indicated by a lower pH, lower glucose and bicarbonate levels, and higher lactate levels in the storage medium. Inactivated platelets also exhibit less shape change, reduced HSR, and greater spontaneous activation and lactate dehydrogenase release into the supernatant.

Platelet activation and aggregation are initiated by mediators such as ADP or thromboxane A2 (a product of AA), which are secreted or released from activated platelets, or thrombin, which is produced on the surface of activated platelets. Collagens are also important for adhesion and subsequent activation. Because all of these agonists act in a synergistic manner in platelets in vivo, we decided to perform aggregometry.
experiments with a panel of four agonists at different concentrations: TRAP, ADP, collagen and AA. There is general agreement that platelet responsiveness to agonist-induced aggregation declines throughout the storage period as part of the well-known platelet storage lesion\textsuperscript{49,50}. We also noted a lower response to aggregation in end-of-storage platelets. Even though in vitro findings indicate a decrease in aggregation during storage, Miyaji \textit{et al.} demonstrated that platelets can recover the ability to aggregate after transfusion\textsuperscript{51}. However, the effect of photochemical treatment on the aggregation properties of platelets is less clear. Hechler \textit{et al.} showed that photochemically treated and control platelets responded similarly to ADP-induced, collagen-induced, and thrombin-induced aggregation\textsuperscript{32}. Notably, their pre-analytic protocol consisted of several washes and resuspension of the platelets in Tyrode's buffer before any experiments. In contrast, others clearly showed a smaller aggregation response in photochemically treated platelets compared to controls at late storage times\textsuperscript{28,29,40,52}. We observed that differences in the aggregation response between photochemically treated platelets and controls are highly dependent on the type and concentration of agonists used for the assay. The contrast between the results published thus far can be attributed to inter-laboratory variability of the protocols used for platelet preparation and aggregometry (e.g., apheresis \textit{vs} buffy coat-derived PC, platelets washed in Tyrode's buffer \textit{vs} standard platelet-rich plasma), as well as the concentration of agonists used for activation. At the early storage time (i.e., day 2 post-collection), we observed no clear differences upon ADP-induced activation, whereas the Intercept-treated platelets tended to aggregate less upon TRAP- or collagen-induced activation. In contrast to published data, at the late storage time (i.e., day 7 post-collection) we observed that Intercept-treated platelets had a greater response than their untreated counterparts upon ADP stimulation. Thus far, we do not have a precise explanation for the better responsiveness of Intercept-treated platelets at the late storage time upon ADP activation, but it seems that the aggregation ability of Intercept-treated platelets is very similar to that of untreated platelets. To confirm these observations, the aggregometry experiments should be repeated with a higher number of replicates.

\textbf{Figure 3} - Aggregation response of platelets treated with the complete Intercept process or only partially on days 2 and 7 of storage (study 2). The first platelet concentrates (PC) remained untreated (Untreated unit). The second PC was treated with the complete Intercept process according to the manufacturer's recommendations (Intercept unit). The third PC was directly incubated with the compound adsorption device for 14 hours under agitation and transferred to the Intercept storage container (CAD unit). The fourth PC was illuminated with UVA at a dose of 3.9 J/cm\textsuperscript{2} in the absence of amorolfine (UV unit). Platelets (120×10\textsuperscript{6} platelets) were stimulated with (A) TRAP (40 µm), (B) ADP (40-80 µM on days 2 and 7), (C) collagen (5.5-7 µg/mL on days 2 and 7), and (D) arachidonic acid (0.09-0.19 mg/mL on days 2 and 7). Platelet aggregation was analysed as the mean maximum aggregation (±SD, n=5). The plain line (100%) represents the normalised value of the Untreated unit. Results obtained from the treated units were normalised against the control unit. *p<0.05, **p<0.01 between paired samples. TRAP: thrombin receptor activator peptide 6; ADP: adenosine diphosphate; CAD: compound adsorbing device; UV: ultraviolet; SD: standard deviation.
Platelet activation induces inside-out signalling leading to rapid conversion of the GPIIb-IIIa into its active conformation\(^5\), which promotes fibrinogen binding. We showed that, upon Intercept treatment, platelets exhibit a higher capacity to adhere to fibrinogen compared to untreated platelets. Similarly, Lozano *et al.* observed a slight increase in platelet coverage under flow conditions for Intercept-treated platelets compared to control platelets, although the difference did not reach statistical significance\(^3\). Our observation is in agreement with the expression of PAC-1, a marker of active GPIIb-IIIa, which is higher on the surface of Intercept-treated platelets. The higher expression of active GPIIb-IIIa in treated platelets may partly be the result of greater passive activation, as observed in Intercept-treated platelets based on the increase in P-selectin expression and a-granule secretion. Although not yet demonstrated with UVA irradiation, some groups have reported that UVB irradiation of platelets mediates platelet aggregation via protein kinase C signalling and subsequent activation of GPIIb-IIIa\(^4,5\). UVC irradiation also activates GPIIb-IIIa via a reduction of the disulphide bonds regulating integrin conformation\(^6\).

The HSR reflects platelet membrane integrity and normal energy metabolism in vitro, which has been correlated with in vivo platelet recovery\(^57\). We observed a significant decline throughout the storage period in all units, with a reduced HSR of Intercept-treated platelets on day 7. However, the measured HSR decreased to the level of that of fresh platelets (50-90%) and end-of-storage platelets (40-80%) and, therefore, remained in the acceptable range\(^3\).

The mitochondria-dependent apoptosis pathway is induced in platelets in response to either intrinsic chemical triggers or high shear stress\(^58,59\). Disruption of the $\Delta \Psi_m$, one of the intrinsic chemical triggers of apoptosis, is considered to be an early sign of apoptosis upstream of caspase activation, phosphatidylserine exposure, and terminal loss of platelet function. We observed a loss of the $\Delta \Psi_m$ in a relatively small fraction of platelets during storage. In agreement with other studies\(^31,32\), but in contrast with Picker *et al.*\(^29,42\), we did not observe an influence of Intercept treatment on $\Delta \Psi_m$.

The exposure of platelets to phosphatidylserine can be the result of: (i) a caspase-dependent apoptotic pathway independent of platelet activation, which may play a role in the clearance of ageing platelets\(^59,60\), or (ii) the conversion of activated platelets to a procoagulant state induced by strong agonists in combination with a sustained high intracellular concentration of Ca\(^{2+}\) and loss of mitochondrial membrane potential\(^61,62\). Thus far, the different mechanisms underlying the activation of procoagulant platelets and apoptotic platelets are not fully understood, as both present signs of apoptosis such as disruption of $\Delta \Psi_m$ or phosphatidylserine exposure\(^63\). In our study, we observed a small percentage of annexin V-positive platelets on day 2 of storage, the majority of which were activated based on P-selectin levels. Independently of Intercept treatment, end-of-storage platelets exhibited a significant increase in phosphatidylserine exposure and most of them exhibited markers of activation, but it remained relatively low (~10% in all units). Leytin *et al.* suggested a model of apoptosis in platelets that is sequential to activation and appears in long-term storage (>13 days)\(^58,64,65\). This concept is consistent with our findings that apoptosis markers appear late during storage.

The contribution of incubation with the CAD to greater passive platelet activation observed in photochemically treated PC has long been questioned. Some studies have reported higher initial platelet activation upon prolonged CAD exposure, even though no significant correlation has been found between CAD duration and initial platelet activation\(^29,44\). Our results indicate that incubation with the CAD, or UV illumination alone, does not contribute to, or at least plays a minor part, in the aggregatory changes observed with Intercept treatment.

**Conclusion**

In summary, compared to controls, PC treated for pathogen inactivation with the Intercept Blood System™ exhibit increased passive activation accompanied by moderate changes in adhesion and aggregation. Whether these *in vitro* changes translate into altered *in vivo* haemostatic function is still to be determined. Importantly, our experiments indicated that incubation with the CAD and illumination with UVA during Intercept treatment does not contribute to the changes in aggregation observed *in vitro* in Intercept-treated PC.

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**Source of support**

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**Authorship contributions**

MA and MP designed the experiments. MA, GS, JKG and DC performed the experiments. MA, MP, JDT and NL reviewed the results and the manuscript.
Characteristics of pathogen-inactivated PC

Conflict of interests

NL received conference honoraria on two occasions from Cerus, the provider of the Intercept Blood System™. JDT received an honorarium from TerumoBCT (European customer panel). The other Authors declare that they have no conflicts of interest.

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