

# Pathogen inactivation of double-dose buffy-coat platelet concentrates photochemically treated with amotosalen and UVA light: preservation of *in vitro* function

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## Vox Sanguinis

**Background** The INTERCEPT Blood System for Platelets (PLT) utilizes amotosalen (S-59) in combination with ultraviolet A (UVA) light to inactivate viruses, bacteria, protozoa and leucocytes that may contaminate PLT concentrates. However, limited data are available on the quality of INTERCEPT-treated double-dose (DD) buffy-coat (BC) PLT units allowing a single treatment procedure to produce two pathogen-inactivated PLT units for transfusion.

**Study Design and Methods** The objective of this study was to evaluate potential *in vitro* effects of the INTERCEPT treatment on pools of 7 BCs as compared to untreated units. Functional, phenotypic and mitochondrial properties of DD BC PLTs during storage over 7 days were studied.

**Results** For some parameters measured, small yet significant differences were observed including PLT count ( $P < 0.05$ ), pH,  $pCO_2$  and glucose concentration. Throughout storage, no significant differences were observed in ATP levels, ESC, HSR reactivity and CD62P expression. Similarly, no differences were observed in the expression of PAC-1, CD42b and PECAM-1 at any time-points. The mitochondrial membrane potential (MMP) determined by JC-1-labelling was well maintained until day 7 in all treated and untreated units (>90%). The release of sCD40L increased over time ( $P < 0.01$ ) in all units but without any significant differences between treated and untreated PLTs.

**Conclusion** Our data demonstrate that photochemical pathogen inactivation of DD-BC PLT concentrates with the INTERCEPT Blood System had no influence on the PLT *in vitro* quality over the 7 day of storage. However, whether *in vivo* efficacy of INTERCEPT-treated PLTs is affected may require clinical evaluation.

**Key words:** amotosalen, pathogen inactivation, platelets, UVA.

Received: 13 August 2014,  
revised 16 October 2014,  
accepted 13 November 2014

## Introduction

Transfusion of labile blood components can be lifesaving but also comes with potential risks. Significant progress has been made to reduce the risk of transfusion-transmitted infections (TTI), and this risk is lower than ever due to the improvements in blood collection and refinement of tests. Still these achievements are not enough. There-

fore, the transfusion medicine community is currently embracing further efforts leading to a more proactive approach to blood safety, namely pathogen inactivation (PI), to satisfy the need for safer blood products.

One such system, the INTERCEPT Blood System was developed to enhance transfusion safety by inactivating a broad spectrum of viruses, bacteria, protozoa, and white blood cells (WBCs) in platelet (PLT) units and in plasma [1, 2]. PI is accomplished by covalent modification of nucleic acids using photo-activation of amotosalen by ultraviolet A (UVA) light which prevents pathogens and leucocytes from replicating and functioning [3]. Thus, amotosalen ensures a high specificity for nucleic acid

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modification and a minimal impact to the efficacy of the treated blood products.

The various PI technologies and their effect on blood components differ significantly [4]. The current technologies apply light of different wavelengths with or without [5, 6] chemical compounds to target the genome of pathogens and leucocytes. The mechanism of action and specificity of a PI technology determine its impact on the blood component and affect the clinical safety and efficacy of treated products. Overall, it is important to limit the use of high-energy wavelengths [7] that can lead to the generation of reactive oxygen species (ROS) which are directly damaging to the PLTs [8, 9].

Several *in vitro* studies have subsequently assessed the quality of INTERCEPT-treated PLTs stored for up to 7 days after treatment [10, 11]. These results seem to be in line with *in vivo* results of INTERCEPT-treated PLTs obtained from clinical trials [12–14].

However, limited data are available on the quality of INTERCEPT-treated double-dose (DD) buffy-coat (BC) PLTs, allowing a single treatment procedure to produce two pathogen-inactivated PLT units for transfusion. Thus, conflicting data describing the effects on mitochondrial function after INTERCEPT treatment [15, 16] illustrates the need for further investigations.

Here, we report the potential *in vitro* effects of the INTERCEPT treatment on the functional, phenotypic and mitochondrial properties of DD-BC PLTs suspended in SSP+ as compared to untreated PLTs. All *in vitro* parameters were monitored over a 7-day storage period.

## Materials and methods

### Preparation and storage of platelet units

Whole blood (WB) units with a volume of  $450 \pm 45$  ml were collected and stored for at least 2 h on butanediol cooling plates to reduce the temperature to approximately 20°C before further processing. Buffy coats were separated on the day of collection by a hard-spin centrifugation (speed 2700 g for 10 min) and separation by Macopress Smart (MacoPharma, Mouvaux, France) following the blood bank standard operating procedures. After overnight storage (Fig. 1), seven ABO-matched buffy coats were selected from donors with an average PLT donor count of  $\geq 240 \times 10^9/l$ . Seven ABO-identical BCs were then pooled with 280 ml of SSP+ resulting in 65% SSP+ and 35% plasma (SSMASSP212U) using a Fenwal (code K4R7039) pooling set to create an ABO-identical DD primary pool. Two DD PLT units were then pooled and split to generate paired DD PLT units, with one DD PLT unit treated with the INTERCEPT System, whilst the other remained untreated (Fig. 1). Each DD PLT unit met the INTERCEPT processing requirements to undergo INTERCEPT dual treatment (Table 1).

### Treatment of platelet units with the INTERCEPT Blood System

Eight DD PLT units were designated to PI treatment with the INTERCEPT Blood System (Cerus Europe BV, Amerfoort, the Netherlands), whilst eight other identical DD

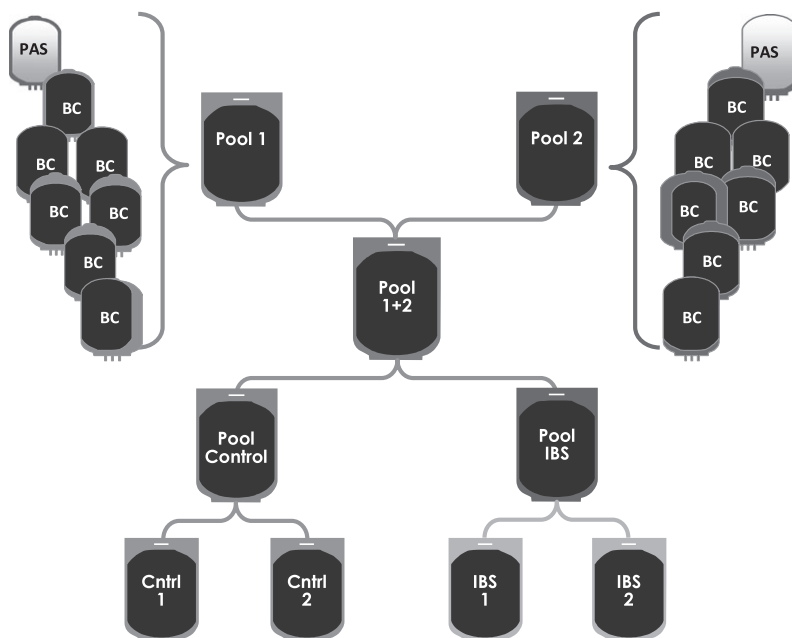


Fig. 1 Schematic overview of buffy-coat pooling.

**Table 1** *In vitro* characteristics of platelet components prior to INTERCEPT treatment

	DD INTERCEPT set input requirement	Validation of double-dose platelet concentrate (n = 9)	Final INTERCEPT of platelet concentrate (transfusable unit) (n = 8)
Volume (ml)	375–420	405 ± 11.3	198 ± 10.3
Platelet dose (10 <sup>11</sup> )	2.5–8.0	5.5 ± 0.3	2.4 ± 0.2
Plasma ratio (%)	32–47%	38 ± 1.1	–
RBC contamination (× 10 <sup>6</sup> /ml)	<4	Yellow, <4	Yellow, <4
CAD (h)	6–16	n/a	~14

CAD, Compound Adsorption Device; DD, double-dose

PLT units remained untreated to serve as controls. The INTERCEPT-treated components were exposed to 3 J/cm<sup>2</sup> UVA light in the presence of 150 µM amotosalen. Following illumination, the treated units were transferred by gravity flow into the CAD (Compound Adsorption Device) container in the integrated disposable set and incubated for 16 h on a flatbed agitator in a temperature-controlled incubator at 22 ± 2°C (model PC900i, Helmer, Noblesville, IN, USA). All DD PLT units (treated and untreated) were then split into two equal single-dose units and transferred to the associated PL2410 plastic container (Cerus Europe BV, Amersfoort, the Netherlands) and stored for 7 days at 22°C with agitation (PC900i, Helmer). Platelet samples (10 ml) were removed on days 2 (immediately following transfer from the CAD), 5 and 7 by sterile-docking a sample pouch (Fenwal Inc., Lake Zurich, IL, USA) and used to assess *in vitro* quality.

#### Analysis of cellular and metabolic, *in vitro* markers

Cellular *in vitro* parameters including recovery post-treatment of PLT counts (10<sup>9</sup>/l and 10<sup>9</sup>/unit) and mean PLT volume (MPV) using CA 620 Cellguard (Boule Medical, Stockholm, Sweden) were measured. The volume (ml) was calculated by weighing the contents of the storage bag, in grams, on a scale (Mettler PB 2000, Mettler-Toledo, Albstadt, Switzerland), and the result, in grams, was divided by 1.01 (1.01 g/ml is the density of the storage medium composed of approximately 65% SSP+ and 35% plasma). The extracellular lactate dehydrogenase (LDH) activity (% of total), a marker for cell disintegration, was measured with a spectrophotometric method (Sigma-Aldrich kit 063K6003; St Louis, MO, USA; Spectrophotometer Jenway 6500; Staffordshire, UK) [17].

The extracellular metabolic environment was studied by use of routine blood gas equipment (ABL 800, Radiometer, Copenhagen, Denmark) including pH (37°C), pCO<sub>2</sub> and pO<sub>2</sub> (kPa at 37°C), glucose (mmol/l) and lactate (mmol/l). Bicarbonate (mmol/l) was calculated based on the other measured variables. The pH of all samples was

measured at 37°C. Therefore, Rosenthal's factor of 0.0147 unit/1°C was used to correct pH to the temperature of sampling (22°C). This factor gives an approximation to the change in pH of the sample per degree centigrade when it is warmed anaerobically from the collecting temperature 22–37°C.

According to Bertolini and Murphy [18], the assessment of swirling was scored as 0, 1 and 2. The WBC on day 1 was determined with a Nageotte chamber and a microscope (Zeiss, standard, Chester, VA, USA) [19].

#### Analysis of *in vitro* functional, phenotypic and secretion markers

Hypotonic shock response reactivity (HSR) as well as the extent of shape change (ESC) measurements was performed using a dedicated microprocessor-based instrument (SPA 2000, Chronolog, Havertown, PA, USA) with the modifications of these tests described by VandenBroeke *et al.* [20]. The total adenosine triphosphate (ATP) concentration, (µmol/10<sup>11</sup> PLTs) was determined with a Luminometer (Orion Microplate Luminometer, Berthold Detection Systems GmbH, Pforzheim, Germany) on the basis of principles described by Lundin [21].

Expression of a conformational epitope on the GPIIb/IIIa complex of activated PLTs was assessed using the FITC-conjugated monoclonal antibody PAC-1, (IgM, Beckton Dickinson, San Jose, CA, USA). For this purpose, unfixed PLTs (10<sup>8</sup>/ml) were incubated with 20 µl ADP at 37°C for 15 min without stirring. The specificity of the PAC-1 binding was demonstrated by staining in the presence of RGDS [22]. Flow cytometric analyses for PAC-1, CD62P (a marker of activation), CD42b (a marker of adhesive capability) and PECAM-1 (PLT endothelial cell adhesion molecule 1) were performed using a FC500 cytometer; (Beckman Coulter, Villepinte, France). MLP Acquisition and MLP Analysis software packages (Beckman Coulter) were used for data acquisition and analysis, respectively. All methods including staining were performed as described [23, 24]. All

**Table 2** Metabolic and cellular analysis of PLTs ( $n = 8$ ) stored for 7 days in SSP+ platelet additive solution with or without INTERCEPT

Variables	Reference PLT			INTERCEPT DD		
	Day 2	Day 5	Day 7	Day 2	Day 5	Day 7
Platelet count ( $\times 10^9/l$ )	1220 $\pm$ 77	1206 $\pm$ 59	1205 $\pm$ 44	1175 $\pm$ 43	1154 $\pm$ 52	1143 $\pm$ 43 <sup>a</sup>
Platelet content ( $\times 10^9/Unit$ )	243 $\pm$ 18	240 $\pm$ 14	240 $\pm$ 11	232 $\pm$ 10	226 $\pm$ 9 <sup>a</sup>	224 $\pm$ 9 <sup>a</sup>
MPV (fl)	8.4 $\pm$ 0.3	8.6 $\pm$ 0.2	8.7 $\pm$ 0.2	8.5 $\pm$ 0.3	8.6 $\pm$ 0.2	8.8 $\pm$ 0.2
pH (22 <sup>o</sup> )	7.272 $\pm$ 0.025	7.374 $\pm$ 0.022	7.342 $\pm$ 0.025	7.085 $\pm$ 0.024 <sup>b</sup>	7.298 $\pm$ 0.029 <sup>b</sup>	7.247 $\pm$ 0.046 <sup>b</sup>
Glucose (mmol/l)	6.2 $\pm$ 0.2	4.4 $\pm$ 0.3	2.7 $\pm$ 0.3	5.8 $\pm$ 0.3 <sup>a</sup>	3.9 $\pm$ 0.4 <sup>a</sup>	2.2 $\pm$ 0.6 <sup>a</sup>
Lactate (mmol/l)	9.8 $\pm$ 0.4	13.0 $\pm$ 0.4	16.1 $\pm$ 0.4	10.1 $\pm$ 0.6	13.1 $\pm$ 0.5	16.3 $\pm$ 0.9
pCO <sub>2</sub> (kPa at 37°C)	3.44 $\pm$ 0.22	2.19 $\pm$ 0.17	1.99 $\pm$ 0.2	5.31 $\pm$ 0.2 <sup>b</sup>	2.04 $\pm$ 0.05	1.76 $\pm$ 0.1 <sup>a</sup>
pO <sub>2</sub> (kPa at 37°C)	25.0 $\pm$ 6.0	21.5 $\pm$ 0.7	20.0 $\pm$ 1.6	20.8 $\pm$ 0.9	21.6 $\pm$ 0.7	20.7 $\pm$ 1.3
Bicarbonate (mmol/l calculated)	6.8 $\pm$ 0.3	5.5 $\pm$ 0.5	4.7 $\pm$ 0.7	6.8 $\pm$ 0.2	4.3 $\pm$ 0.3 <sup>b</sup>	3.3 $\pm$ 0.4 <sup>b</sup>

Values are reported as mean  $\pm$  standard deviation (SD).

DD, double-dose; MPV, mean PLT volume.

<sup>a</sup> $P < 0.05$  vs. reference PLT.

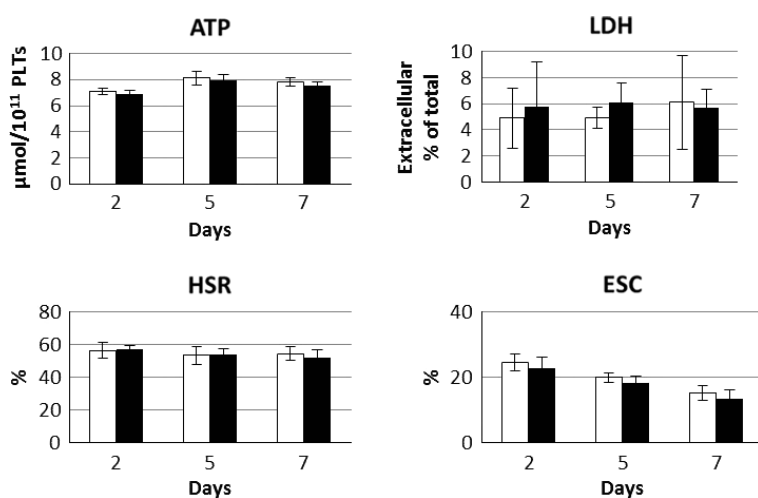
<sup>b</sup> $P < 0.01$  vs. reference PLT.

sCD40L concentrations were determined with commercial ELISA kits (Quantikine, CD40 Ligand Immunoassay DCDL40) in accordance with the manufacturer's (R&D Systems Inc., Minneapolis, MN, USA) recommendations. All measurements were performed in duplicate on the HT3 Microtiter Plate Reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at 466 nm, and the results for the sCD40L concentrations are given in pg/ml.

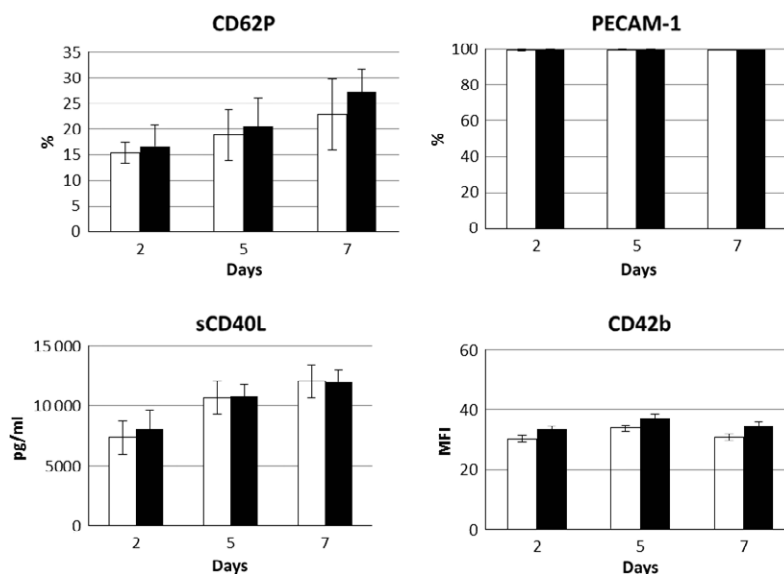
### Analysis of mitochondrial membrane potential (MMP)

Potential changes in the MMP, a marker of pro-apoptotic events and maintenance of oxidative phosphorylation capacity, were measured using the mitochondrial perme-

ability transition detection kit MitoPT JC-1 (Immuno-Chemistry Technologies, LCC, Bloomington, MN, USA). All samples from all groups ( $1 \cdot 10^6$  PLTs/ml) were stained with the MitoPT JC-1 dye reagent at 37°C for 15 min and analysed using flow cytometry (FC500; Beckman Coulter). MLP Acquisition and MLP Analysis software packages (Beckman Coulter) were used for data acquisition and analysis, respectively. Depolarized mitochondria (positive control) were prepared by incubating PLTs with 5  $\mu$ M CCCP (Carbonylcyanide *m*-chlorophenylhydrazone) for 30 min at 37°C. This proton gradient uncoupling agent quickly reduces the electrochemical potential across the inner mitochondrial membrane, resulting in a rapid intracellular mitochondrial depolarization event. Maintenance of MMP is expressed as JC-1-positive PLTs as described earlier [25].



**Fig. 2** *In vitro* functional and biochemical effects on PLTs ( $n = 8$ ) stored for 7 days in SSP + platelet additive solution of control (□) and PCT (■) PLTs on Day 2, 5 and 7 of storage. The bars represent the mean percentage  $\pm$  SD of positive PLTs ( $n = 8$ ).



**Fig. 3** Expression of the activation marker CD62P, PECAM-1 expression, release of sCD40L and expression of CD42b of control (□) and PCT (■) PLTs on Day 2, 5 and 7 of storage. The bars represent the mean percentage  $\pm$  SD of positive PLTs ( $n = 8$ ).

### Statistical analyses

The mean values and standard deviations ( $n = 8$ ) are usually given unless otherwise indicated. A repeated-measures ANOVA including *post hoc* test Bonferroni's adjustment was performed. The analyses were carried out using the STATISCA software, version 9 StatSoft, Inc. 1984–2007 (SPSS, Chicago, IL, USA).

### Results

*In vitro* quality parameters were measured on day 2 (following INTERCEPT treatment) and on day 5 and 7 of storage.

#### Effects on cellular, metabolic functional, phenotypic and secretion markers

During storage, there was a small yet significant difference in PLT counts ( $P < 0.05$  on day 7) and contents ( $P < 0.05$  on day 5 and day 7), this may be due to the manipulations involving CAD; meanwhile, no differences were observed in MPV between the two groups (Table 2). All PLTs maintained a pH at 22°C above 6.4; however, following treatment, the INTERCEPT PLTs had a consistently lower pH ( $P < 0.01$  (Table 2) than untreated units, but values were above a pH of 7).

We found that the glucose concentration ( $P < 0.05$  from Day 2) was higher in the untreated units as compared to the INTERCEPT-treated units (Table 2). However, the glucose consumption rate was equally consistent ( $0.05 \pm 0.0$  mmol/day/ $10^{11}$  PLTs) in the untreated units vs. ( $0.05 \pm 0.0$ ) in the treated units, as

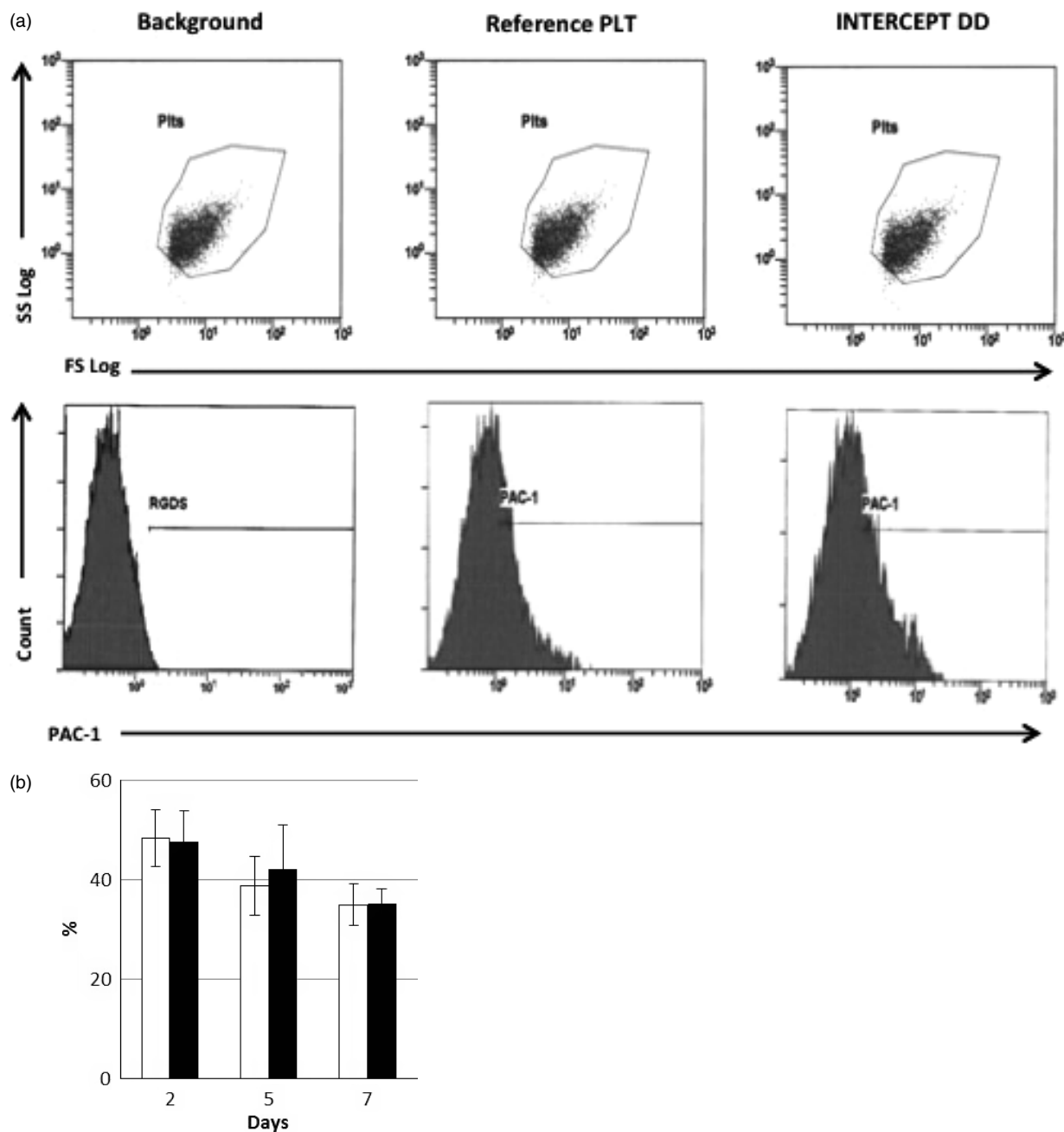
was the lactate production rate ( $0.09 \pm 0.01$  mmol/day/ $10^{11}$  PLTs) in the untreated units vs. ( $0.09 \pm 0.01$ ) in the treated units.

In addition, carbon dioxide ( $P < 0.01$  on day 2 and  $P < 0.05$  on day 7) and the calculated bicarbonate concentration ( $P < 0.01$  on day 2, 5 and 7) decreased more rapidly over time in the INTERCEPT units (Table 2). The ATP levels remained stable in all units, whilst the HSR reactivity and the response to ESC decreased over 7 days of storage; however, this was not significantly different between groups (Fig. 2). Subsequently, the percentage of the total extracellular LDH remained stable and at low levels in both INTERCEPT and untreated units (Fig. 2). Additionally, swirling remained at the highest level at all times in all INTERCEPT and untreated units (data not shown).

No statistically differences were observed in the expression levels of CD62P, PECAM-1 or CD42b at any time-point between groups (Fig. 3). Related to the increase in PLT activation, sCD40L accumulated during storage showing an increased release in all units but with no difference observed between INTERCEPT-treated vs. untreated units at any time-point (Fig. 3). Throughout storage, PAC-1 expression was equal between the two groups (Fig. 4a,b).

#### Effect on mitochondrial membrane potential

Throughout storage, the MMP (JC-1-positive PLTs) decreased slightly in all INTERCEPT-treated and untreated units but was maintained at similar high levels (NS) with  $>90\%$  maintenance of MMP on day 7 (Fig. 5a–d) as determined by equivalence in fold change.



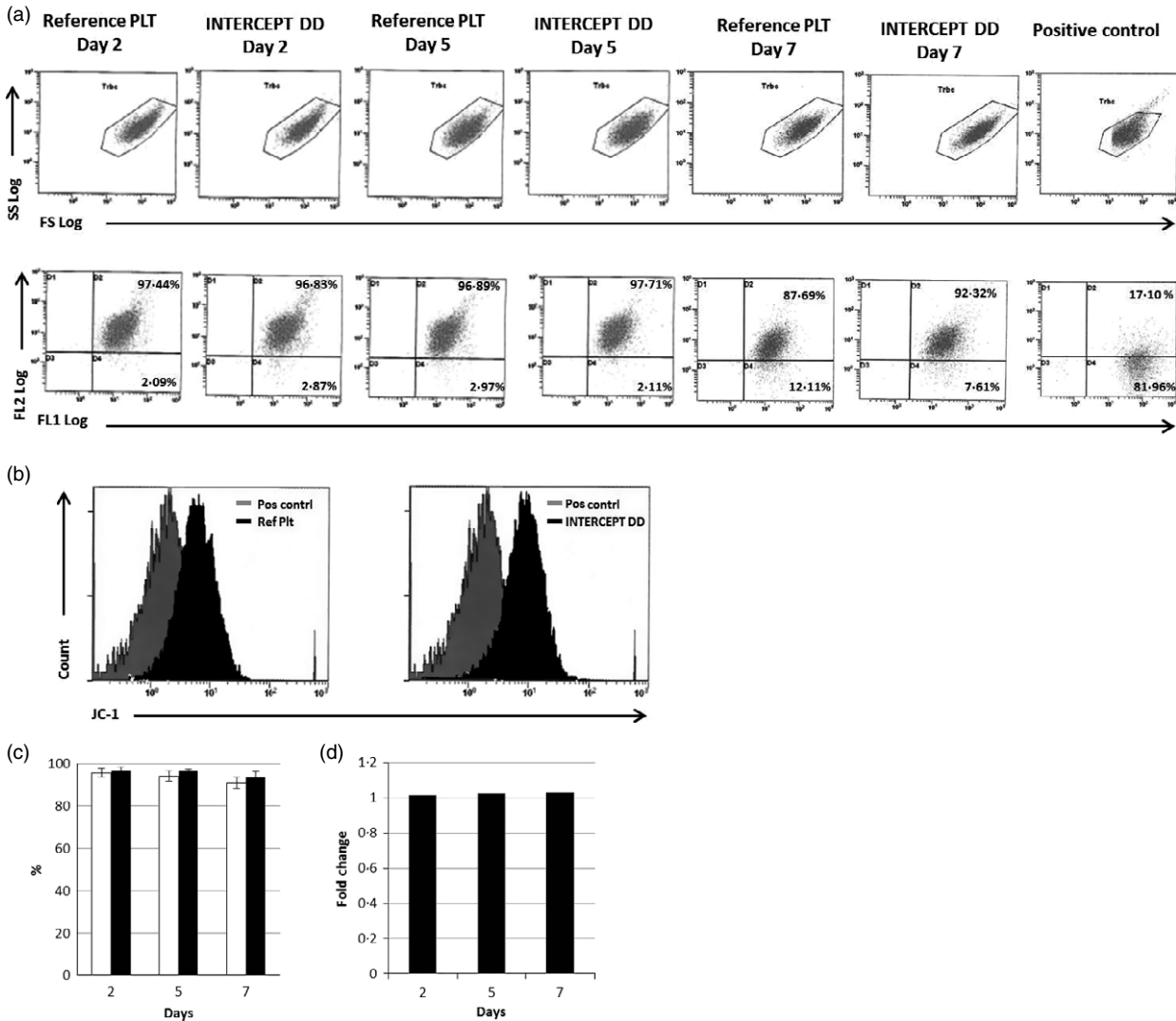
**Fig. 4** Expression of a conformational epitope on the GPIIb/IIIa complex of activated PLTs, as measured by surface expression of PAC-1. The expression of PAC-1 was measured by flow cytometry on unfixed PLTs after ADP stimulation. (a) Representative staining from one experiment at day 7. The specificity of the PAC-1 binding was demonstrated by staining in the presence of RGDS. (b) Expression of PAC-1 in control (□) and PCT (■) PLTs on Day 2, 5 and 7 of storage. The bars represent the mean percentage  $\pm$  SD of positive PLTs ( $n = 8$ ).

## Discussion

Pathogen inactivation of PLT concentrates has the potential to improve blood safety and possibly increase the shelf life of labile blood products, since it addresses the issues associated with bacterial contamination. This study

evaluated potential effects of the INTERCEPT treatment on *in vitro* functional, phenotypic and mitochondrial properties of DD-BC PLTs during storage in SSP+ for 7 days. Platelet quality was assessed using a wide range of *in vitro* assays designed to measure PLT metabolism, function and activation. Overall, INTERCEPT-treated





**Fig. 5** Maintenance of mitochondrial membrane potential (MMP) expressed as JC-1-positive PLTs. The expression of JC-1 was measured by flow cytometry on PLTs. (a) Representative quadrants derived from flow cytometric scatter plots at Day 2, 5 and 7 and the positive control. (b) Representative staining from one experiment at day 7. (c) Expression of JC-1 in control (□) and PCT (■) PLTs on Day 2, 5 and 7 of storage. The bars represent the mean percentage ± SD of positive PLTs ( $n = 8$ ). (d) Fold change in the maintenance of MMP was calculated from the means.

PLTs with subsequent storage remained largely unaffected.

Measurements of extracellular parameters offer, on one hand, valuable insights into the equilibrium among the cytosolic pathways and the mitochondrial oxidative pathways of PLTs during storage, as changes caused by PLTs may disturb metabolic homeostasis are linked to increased cytosolic glycolysis [25–27], PLT activation [25, 28–32], aggregation and release reactions [23, 25, 28, 33–36]. On the other hand, other physiological challenges, not related to increased glycolytic flux and accelerated cellular metabolism, may affect metabolic homeostasis.

In our study, we observed decreased pH and glucose concentration in the INTERCEPT-treated units. Although the pH declined following INTERCEPT treatment, it remained at a similar level [37] or higher than reported in previous studies [29, 38]. In contrast to the previous studies, this finding seems to be related to other physiological challenges, rather than an increase in the glycolytic flux caused by the UV-treatment. This suggestion is supported by the equivalence in the glucose consumption/lactate production rate as well as overall equivalence in the other biochemical markers over time between INTERCEPT-treated and untreated units. Moreover, the pH in all INTERCEPT-treated fractions at around 7 was well above the

Council of Europe recommendations (pH >6.4). This along with continued O<sub>2</sub> consumption and the ability to generate an equivalent concentration of ATP [39, 40] indicates that oxidative phosphorylation was likely maintained in these PLTs.

With exception of storage lesion and ageing effects which were detected for treated as well as untreated units, we observed no statistically significant increase in the activation levels as compared with the untreated units. Additionally, INTERCEPT treatment did not affect the ability of the PLTs to respond to agonists (ESC and PAC-1), indicating cellular responsiveness was maintained relative to their activation state. Furthermore, all HSR scores were well above the level for which poor *in vivo* viability has been predicted to occur [41]. Also the expression level of the GPIb-IX-V complex [42–44] and maintenance of the PECAM-1 (CD31) levels of tested PLTs [45, 46] strengthen the conclusion that cellular effects cannot be the cause for the extracellular differences observed.

Our results on the DD PLTs seem to agree with the functional integrity of the INTERCEPT-treated PLTs proven earlier in a series of *in vitro* assays [11, 47, 48] and clinical trials [12–14].

A large variety of factors is released from PLTs [49, 50] and is found present in PLT concentrates [25, 33, 51–54]. Because of the potentially harmful effects [35, 55–58] of PLT derived factors, it is of major importance that a PI technology does not exacerbate such negative events. The data presented in this study demonstrate accumulation of sCD40L in all PLT units during storage. This progressive increase in the activation levels per PLT population agrees with the previously observed relationship between activation levels and cytokine release [24 51, 53, 59]. However, this finding was not significantly different between INTERCEPT-treated vs. untreated units.

Previously published data suggest impaired mitochondria-based respiration and lower maintenance of cell quality in INTERCEPT-treated PLTs [16]. However, these results are not in line with our results. In our study, no shift towards anaerobic glycolysis was observed due to

depolarization of the PLT MMP resulting in lowering of ATP and less maintenance of cell quality. Our findings are instead in good accordance to the studies by Hechler *et al.* [15]. Moreover, our measurement of the JC-1 positive cells (>90% by means) is in agreement with our measured ATP values. Accordingly, we found no evidence for a decline in the ATP values, symptomatic of maintained mitochondrial function after INTERCEPT treatment. If mitochondrial function was affected, then the ATP levels would be expected to decline, since approximately 85% of the ATP values are suggested to be generated via the oxidative part of the metabolism [40].

Our results indicate no support for an inverse relationship between mitochondrial dysfunction and INTERCEPT treatment of PLTs. One reason for the conflicting data is likely the critical role for other factors influencing the PLTs during storage [26], which act in concert with the potential effects of INTERCEPT treatment [14, 48].

The constant quality of the INTERCEPT-treated PLTs over 7 day of storage in comparison with the untreated PLTs was somewhat surprising, given that PLTs, even under the best of circumstances exhibit a substantial amount of biochemical changes, as judged by the different *in vitro* variables. One possible explanation for the positive results could be that the manual preparation method was gentle [60] and optimized storage solutions [61, 62] and bags [26, 63] were used. These factors seem to play a major role regarding PLT quality associated with PI treatment.

In summary, our data demonstrate that photochemical PI of DD-BC PLT concentrates with INTERCEPT Blood System had no influence on the PLT *in vitro* quality over the 7 day of storage. However, whether *in vivo* efficacy of INTERCEPT-treated PLTs is affected may require further clinical evaluation.

## Conflict of interest

Cerus provided equipment and technical advice.

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