Annexin V Release and Transmembrane Mitochondrial Potential during Storage of Apheresis-Derived Platelets Treated for Pathogen Reduction

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Key Words

Pathogen reduction technology · Platelet in vitro function · Endogenous annexin V · Transmembrane mitochondrial potential · INTERCEPT BLOOD SYSTEM · MIRASOL-PRT

Summary

Background: In vitro function of stored platelet (PLT) concentrates was analyzed after applying two different techniques of pathogen reduction technology (PRT) treatment, which could increase cellular injury during processing and storage. **Methods:** Nine triple-dose PLT apheresis donations were split into 27 single units designated to riboflavin-UVB (M) or psoralen-UVA (I) treatment or remained untreated (C). Throughout 8 days of storage, samples were analyzed for annexin V release, the mitochondrial transmembrane potential ($\Delta\psi$) and some classical markers of PLT quality (pH, LDH release, hypotonic shock response (HSR)). **Results:** PLT count and LDH release of all units maintained initial ranges. All units exhibited a decrease in pH and HSR and an increase in annexin V release and $\Delta\psi$ disruption. Notably, throughout the entire storage period, annexin V release remained lowest in M units. Throughout 7 days of storage, M units remained comparable to C units ($p > 0.05$), whereas inferior values were observed with I units. Here, differences to C units reached significance by day 1 (pH: $p < 0.0001$), day 5 (annexin V release: $p < 0.014$), and day 7 (HSR, $\Delta\psi$: $p \leq 0.003$). After PRT treatment, annexin V release and $\Delta\psi$ disruption were significantly ($p < 0.001$) correlated with pH and HSR. **Conclusion:** During storage, all units showed a decrease in HSR and an increase in acidity, annexin V release and $\Delta\psi$ disruption. While M units remained comparable to C units, I units demonstrated significantly inferior values during terminal storage. This could have resulted from differences in PRT treatment or simply be due to differences in storage media and should be analyzed for clinical relevance in future investigations.

Schlüsselwörter

Pathogeninaktivierung · Thrombozytäre In-vitro-Funktion · Endogenes Annexin V · Transmembranes mitochondriales Potential · INTERCEPT BLOOD SYSTEM · MIRASOL-PRT

Zusammenfassung

Hintergrund: Die In-vitro-Funktion gelagerter Thrombozytenkonzentrate wurde nach Anwendung zweier verschiedener Techniken zur Pathogeninaktivierung (PRT) untersucht. **Design:** Neun Dreifachapheresespenden wurden in 27 Einzeleinheiten geteilt. Eine Einheit wurde mit Riboflavin-UVB (M) und die andere mit Psoralen-UVA (I) behandelt. Die dritte blieb unbehandelt (C) und diente als Kontrolle. Während einer Lagerung von 8 Tagen wurden Proben untersucht in Bezug auf die Freisetzung von endogenem Annexin V, das transmembrane mitochondriale Potential ($\Delta\psi$) sowie die klassischen Marker der Plättchenqualität (pH, LDH-Freisetzung, hypotone Schockreaktion (HSR)). **Ergebnisse:** In allen Einheiten blieben Plättchenzahl und LDH-Freisetzung im initialen Bereich. Alle Einheiten zeigten einen Abfall des pH, der HSR und des $\Delta\psi$ und einen Anstieg der Freisetzung von endogenem Annexin V. Während der gesamten Lagerungszeit wiesen M-Einheiten diesbezüglich die geringsten Werte auf. Sieben Tage hindurch blieben M- und C-Einheiten vergleichbar ($p > 0,05$), während geringere Werte in I-Einheiten beobachtet wurden. Die Unterschiede zu C-Einheiten erreichten hier statistische Signifikanz ab Tag 1 (pH: $p < 0,0001$), Tag 5 (Annexin-V-Freisetzung: $p < 0,014$) und Tag 7 (HSR, $\Delta\psi$: $p \leq 0,003$). Nach der PRT-Behandlung korrelierten $\Delta\psi$ und die Freisetzung von endogenem Annexin V signifikant ($p < 0,001$) mit pH und HSR. **Zusammenfassung:** Während der Lagerung aller Einheiten sanken die HSR- und $\Delta\psi$ -Werte, während die Azidität und die Annexin-V-Freisetzung zunahmen. Während die M- und C-Einheiten vergleichbar blieben, zeigten die I-Einheiten im letzten Lagerungsdrittel signifikant niedrigere Werte. Ursächlich hierfür können Unterschiede der PRT-Behandlung oder einfach der Lagerungsmedien sein. Jedoch sollte die klinische Relevanz in künftigen Untersuchungen ermittelt werden.

Introduction

The psoralen-based INTERCEPT BLOOD SYSTEM (Cerus Corp, Concord, CA, USA) [1–3] and the riboflavin-based MIRASOL-PRT (CaridianBCT Biotechnologies, Lakewood, CO, USA) [4–6] are designed for pathogen reduction (inactivation) of platelet (PLT) concentrates (PCs), which bear the risk of bacterial contamination and growth. Pathogen reduction technologies (PRTs) could affect structural and functional integrity of PLTs during storage. For example, mitochondrial function may be disturbed by targeting mitochondrial nucleic acids. Provided that this would lead to an impaired mitochondria-based oxidative phosphorylation delivering much more biologic energy in form of adenosine triphosphate (ATP) than the anoxidative pathway of the cytosol, direct consequences for energy maintenance and in vitro quality during storage [7] could be expected.

A variety of markers including activation as well as morphologic and metabolic changes are routinely used to monitor PC quality. Rather unusual markers in this field are the detection of disruption of the transmembrane mitochondrial potential ($\Delta\psi$) to indicate a disturbed mitochondrial function or the release of endogenous annexin V. The latter was shown to be correlated with PLT shape change [8] and may therefore provide a sensitive marker for cellular alteration. Due to a putative binding pocket for the phosphatidylserine (PS) head group [9], studies on endogenous annexin V suggest that PS recognition forms the basis of its physiological function [10]. Blood PLTs were the first cells for which it was demonstrated that changes in the environment, e.g. a rise in receptor activator concentration or a rise in intracellular Ca^{2+} , could enhance PS exposure at the outer membrane leaflet [11, 12], which is also promoted by a disturbed mitochondrial function [13]. Apart from induction of phagocytosis [14], PS exposure exhibits procoagulant and proinflammatory activities [15]. This may depict a novel significance for the biological function of annexin V by shielding PS exposure of the dying cell until being released into the plasma pool. Another clinical significance of annexin V is the fact that due to its high affinity to negatively charged phospholipid membranes, annexin V acts as a strong anticoagulant by competing with the binding sites of factors VIIIa and Va in the tenase and prothrombinase complex assembly [16], probably interfering with the clinical efficacy of PCs.

Material and Methods

Preparation of PCs

Nine triple-dose PLT collections were performed using the Trima Accel apheresis collection device, version 5.1 according to the manufacturer's instructions (CaridianBCT Biotechnologies). All donors gave written informed consent and passed eligibility criteria based on German [17] and European [18] requirements. Collection targets per procedure were 10.0×10^{11} PLTs in 330 ml of autologous plasma. Collection units were kept un-

disturbed for 2 h at ambient temperature prior to splitting and subsequent processing to allow dissociation of any PLT aggregates. All units were leukodepleted by the process-controlled leukoreduction system. Immediately after splitting, single units were designated to PRT treatment with psoralen-UVA (I) or riboflavin-UVB (M) or remained untreated (C) to serve as controls.

PRT Treatment

Prior to PRT treatment with the INTERCEPT BLOOD SYSTEM as described previously [19–21], 180 ml of InterSol (Fenwal, Deerfield, IL, USA; composition: Na_3 citrate $2 \text{ H}_2\text{O}$ 318 mg, Na acetate $3 \text{ H}_2\text{O}$ 442 mg, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 105 mg, NaH_2PO_4 305 mg, NaCl 452 mg) was added to I units. M units received 150 ml of SSP+ (MacoPharma, Langen, Germany; composition: Na_3 citrate $2 \text{ H}_2\text{O}$ 3.18 g, Na acetate $3 \text{ H}_2\text{O}$ 4.42 g, $\text{NaH}_2\text{PO}_4 \cdot 2 \text{ H}_2\text{O}$ 1.05 g, NaH_2PO_4 3.05 g, NaCl 4.05 g, KCl 0.37 g, $\text{MgCl}_2 \cdot 6 \text{ H}_2\text{O}$ 0.30 g) after PRT treatment with MIRASOL-PRT according to the manufacturers' instructions [22–24]. Untreated C units were resuspended in 150 ml of InterSol plus 30 ml saline to compensate for the addition of photosensitizers. The overall plasma ratio was 35–40%.

Sampling and Storage of PCs

Prior to splitting and PRT treatment, a sample volume of 4 ml was taken aseptically from the whole collection unit and diluted with 6 ml of saline to compensate for the addition of PLT additive solution (PAS). Thereafter, the collection unit was split equally into 3 single units (I, M, C). All 27 single units were stored for 8 days in PL2410 plastic bags at $22 \pm 2^\circ\text{C}$ on an horizontal flatbed agitator (Helmer Laboratories, Noblesville, IN, USA) running at $50\text{--}60 \times \text{min}^{-1}$. Additional samples were taken aseptically on storage days 1, 5, 7, and 8.

Analysis of Cell Count, LDH Release, pH, Hypotonic Shock Response (HSR) and Bacterial Contamination

Evaluation of PLT count, contamination with red and white blood cells via flow cytometry, LDH release, HSR, and bacterial contamination with the Bactec[®] culturing system (Becton Dickinson, San Jose, CA, USA) were performed as described previously [25, 26]. The pH value was analyzed at 37°C on an automated blood gas analyzer (Rapidlab 1260, Siemens Medical Solutions Diagnostics mbH, Fernwald, Germany) and corrected to 22°C .

Analysis of Released Annexin V

Annexin V release was analyzed by ELISA technology (ELISA Annexin V, Haemochrom Diagnostica GmbH, Essen, Germany) from PLT-poor plasma (PPP) obtained immediately after sampling by centrifugation at $2,000 \times g$ for 30 min and frozen at -70°C until being analyzed. In brief, immediately after thawing, PPP was incubated in a microwell precoated with rabbit polyclonal antibodies (F(ab')₂ fragments) specific for human annexin V. Peroxidase-labelled polyclonal anti-annexin V antibody and tetramethylbenzidine/ H_2O_2 were used as conjugate and substrate, respectively. The reaction was stopped after 5 min with 0.45 mol/l sulphuric acid, and the absorbance was read at 450 nm.

Assessment of the $\Delta\psi$

Upon accumulation in mitochondria with polarized membranes, the dye JC-1 (J-aggregate-forming lipophilic cationic fluorochrome 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) shows red fluorescence emission (aggregate state). When staying in the cytosol (because of depolarized mitochondrial membranes), JC-1 changes the fluorescent emission spectrum from red to green (monomer state). To evaluate the $\Delta\psi$, a commercially available JC-1 assay (Mitoscreen BD[™], Becton Dickinson) was used and analyzed on a FACScan (Becton Dickinson) according to the manufacturer's instructions. Data were analyzed with the software CELLQuest Pro (Becton Dickinson) and expressed as percentage of cells with polarized/depolarized mitochondrial potential.

Fig. 1. Boxplot curves of cell quality parameters during 8 days of storage.

*Significant compared to C (untreated) units.

ⁱSignificant compared to I (psoralen-UVA-treated) units.

^mSignificant compared to M (riboflavin-UVB-treated) units.

Table 1. Correlation coefficients (Spearman test) for bivariate correlation between annexin V release/JC-1 signal and pH, LDH release and HSR

	Annexin V			JC-1 signal (% depolarized PLTs)		
	C	I	M	C	I	M
pH value (22 °C)	-0.82	-0.61	-0.48	-0.54	-0.85	-0.55
LDH release, U/10 ⁹ PLTs	0.08	0.50	0.51	0.47	0.53	0.10
HSR, %	-0.13	-0.69	-0.74	-0.14	-0.83	-0.71

C = untreated, I = psoralen-UVA-treated, M = riboflavin-UVB-treated units.
Italic letters indicate significance ($p < 0.05$).

Fig. 2. Correlation (Spearman test) between annexin V release and JC-1 signal in PRT treated PLTs; c = correlation coefficient.

Statistical Analysis

Results obtained are expressed as median $\pm$ 95% confidence interval and analyzed by computer software (SPSS 15.0 for Windows, SPSS Software GmbH, Munich, Germany). Statistical analysis within a study group was conducted using the Friedman test, while the Kruskal-Wallis test was applied for the comparison of all three types of units. In case of significant differences ($p < 0.05$), post-hoc paired comparisons were made with the Mann-Whitney U test. Here, according to Bonferroni, a p value of less than 0.017 was related to a significance level of 5%. Correlation analysis was performed using the Spearman test for nonparametric correlations.

Results

The whole collection units yielded a median volume of 338 ml (range 326–344 ml). Median PLT dose was 9.4×10^{11} (range 8.8 – 10.7×10^{11}), corresponding to a median PLT concentration of $2,796 \times 10^9/l$ (range, $2,620$ – $3,138 \times 10^9/l$). No serious adverse effects were observed in any of the apheresis procedures. All units were tested under sterile conditions after 16 days of incubation under aerobic and anaerobic conditions. Residual leukocytes and erythrocytes were well within the range of international guidelines [18]. Boxplot curves of cell quality parameters, including PLT count, LDH release, pH at 22 °C, HSR, annexin V release, and JC-1 signal from day 1 to day 8 of storage, are illustrated in figure 1. Day 0 values obtained prior to splitting and PRT treatment were $1,075 \pm 57 \times$

$10^9/l$ for PLT count, 55.6 ± 6.8 U/10⁹ PLTs for LDH in supernatant, 7.17 ± 0.06 for pH at 22 °C, $76.2 \pm 5.0\%$ for HSR, 1.78 ± 0.33 ng/ml for annexin V in PC supernatant, and $4.5 \pm 5.7\%$ for the percentage of depolarized cells. The correlation between released annexin V, $\Delta\psi$ and conventional cell quality parameters examined is shown in table 1. The correlation between annexin V release and $\Delta\psi$ is depicted in figure 2.

PLT counts and LDH release of all units changed only slightly during storage and revealed no significant differences among the study groups. During storage, annexin V release of all units increased steadily and was accompanied by a concomitant disruption of $\Delta\psi$. M units exhibited the lowest values for annexin V release, whereas I units had the highest, being significantly higher than those of C and M units by day 5 of storage. Regarding $\Delta\psi$, no significant differences were noted between M and C units during 7 days of storage, while I units demonstrated significantly higher $\Delta\psi$ disruption from the beginning of day 7. This was irrespective of the mode of $\Delta\psi$ expression (percentage of depolarized cells or mean fluorescence intensity (MFI) ratio (FL2/FL1) to also include partially depolarized cells) (fig. 3). On day 8, however, there were significant differences in $\Delta\psi$ between untreated controls and both types of PRT units, including M units. The analysis of more classical markers of the PLT storage lesion revealed no significant differences with respect to acidity between C and

Fig. 3. MFI ratio FL2/FL1

The MFI ratio of I units as compared to untreated (C) and riboflavin-UVB-treated (M) units was significantly lower by day 7 of storage (* $p < 0.001$).

M units, whereas pH of I units was significantly lower already by day 1 of storage. Corresponding to the low annexin V release, HSR of M units exceeded that of the other units until day 5, but fell below C units by day 7 of storage. During the entire storage period, I units had the lowest values for HSR proving statistical significance versus C units by day 7 of storage. After PRT treatment, annexin V release and $\Delta\psi$ were significantly correlated with HSR and pH value (table 1).

Discussion

PRT procedures may increase blood safety but also bear the potential to impair functionality and viability of the treated blood cells. In this study, we evaluated the effects of two different PRTs focusing on annexin V release and $\Delta\psi$ using apheresis-derived triple-dose PLTs stored in PAS for up to 8 days.

Because leukoreduced PCs with less than 1×10^6 white blood cells per unit were used, supernatant annexin V can be assumed to be entirely related to the release from PLTs. Given the steady increase of annexin V release and $\Delta\psi$ disruption in all study units, cellular alterations occurred during storage even in untreated PLTs that are reinforced by decreasing values of HSR and pH. Interestingly, the correlation between annexin V release and $\Delta\psi$ with HSR was significantly more pronounced in PRT-treated compared to untreated units, suggesting a higher degree of cellular injury after PRT treatment.

$\Delta\psi$ disruption is associated with the apoptotic pathway [10]. Thus, apart from increase in cellular injury, PRT treatment may enhance the apoptotic suicide machinery in stored PLT products. Comparing both PRTs, annexin V release and $\Delta\psi$ disruption were more pronounced after the psoralen-based PRT. With riboflavin as photosensitizer, annexin V release was even lower and $\Delta\psi$ remained comparable to untreated

units during 7 days of storage which is in accordance with previous reports on $\Delta\psi$ [27]. The results obtained for $\Delta\psi$ in a former investigation on riboflavin-UVB treated PLTs [28] were 2 to 3 times higher than those obtained in the present study. Here, untreated and PRT-treated PLTs were resuspended in PAS instead of 100% of autologous plasma as performed in the previous investigation. Thus, a protective role for mitochondrial integrity could be supposed for PLT storage in PAS.

Our results of annexin V release and $\Delta\psi$ were underlined by the analysis of more conventional markers of the PLT storage lesion like pH and HSR. For pH, lower values were observed with I units. However, pH of all units complied with international requirements (AABB standards: pH ≥ 6.2 [29], Council of Europe recommendations: pH ≥ 6.4 [18]) throughout the entire storage period. As compared to untreated C units, the lower annexin V release of M units combined with higher HSRs, at least until storage day 5, could have resulted from differences in glucose metabolism, leading to better energy maintenance after PRT treatment with riboflavin-UVB. As reported previously, compared to untreated PLTs, riboflavin-UVB-treated PLTs exhibited an obviously up-regulated mitochondria-based respiration [25], which delivers profoundly more ATP than the anaerobic glycolytic pathway. On the other hand, PRT treatment with the psoralen-based technology appeared to impair oxidative phosphorylation [26]. As a consequence, I units mainly performed anaerobic glycolysis, resulting in less ATP generation and, as observed here, in lower HSRs and higher annexin V release.

We conclude that, due to the high correlation with HSR and pH, the analysis of annexin V release and of the JC-1 signal (solely or combined) may indicate the degree of cellular injury and provides a sensitive and practical approach for quality monitoring of PCs, especially after PRT treatment. Results obtained demonstrated comparability between riboflavin-UVB-treated and untreated PLTs, but significantly

lower values in psoralen-UVA-treated PLTs. It cannot completely be excluded that, apart from differences in PRT technology, differences in storage media (in contrast to InterSol, SSP+ contains additional amounts of K⁺ and Mg²⁺) could have contributed to differences in cell quality observed. Since differences to untreated controls became evident only later during storage, a storage period of maximally 7 days could be recommended for both kinds of PRT. The impact for PLT behavior in vivo, however, should be answered in the near future.

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Disclosure

The authors declared no conflict of interest.

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