

Cold storage of platelets in additive solution: the impact of residual plasma in apheresis platelet concentrates

Irene Marini,^{1*} Konstanze Aurich,^{2*} Rabie Jouni,¹ Stefanie Nowak-Harnau,¹ Oliver Hartwich,² Andreas Greinacher,² Thomas Thiele^{2,‡} and Tamam Bakchoul^{1,2,‡}

¹Centre for Clinical Transfusion Medicine, Medical Faculty of Tübingen, University of Tübingen and ²Institute of Immunology and Transfusion Medicine, University of Greifswald, Germany

**Both authors shared first authorship. †Both laboratories contributed equally to this work*

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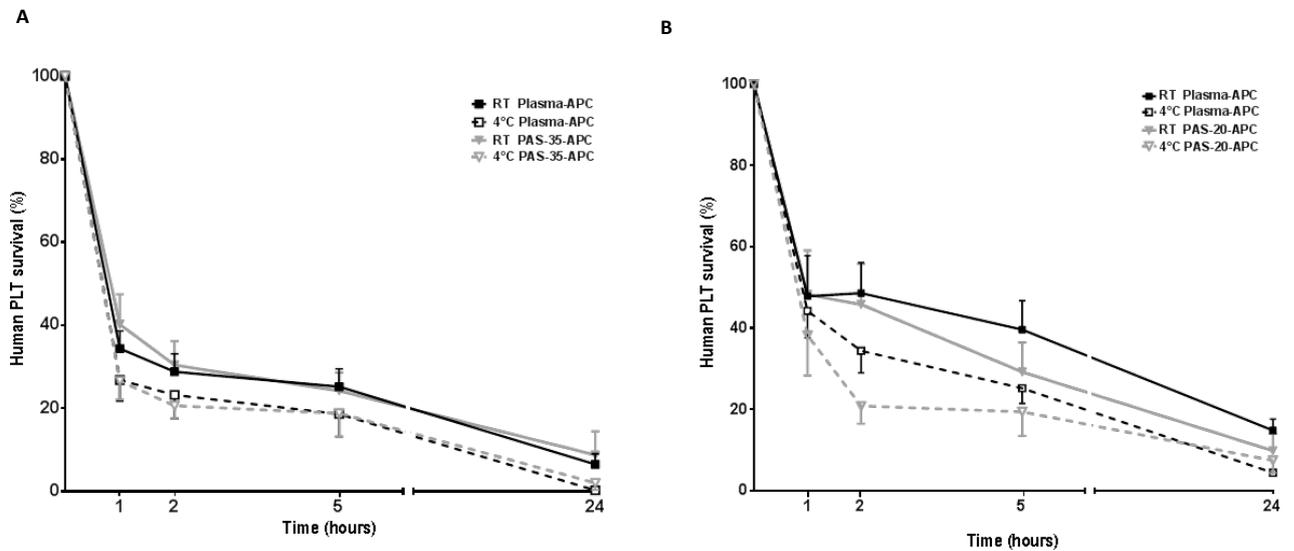
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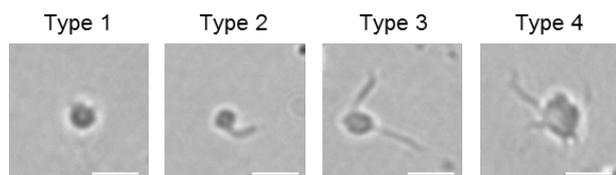
Correspondence: tamam.bakchoul@med.uni-tuebingen.de

Supplementary Figures



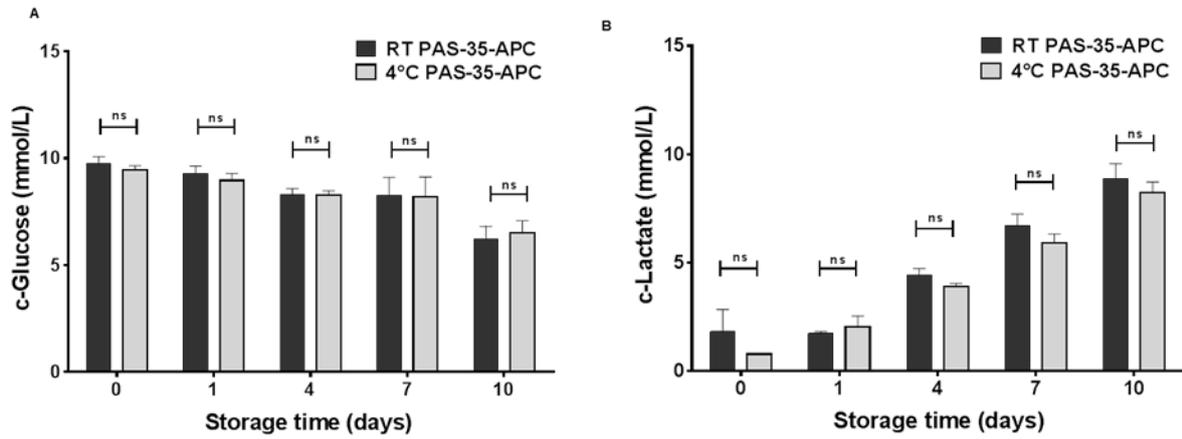
Supplementary Figure 1: Platelet survival after cold storage

After 7 days of storage at room temperature (RT, filled symbols and continues lines) or 4°C (empty symbols and dashed lines), apheresis platelet concentrates (APCs) containing (A) 35% plasma (PAS-35-APC, triangles), (B) 20% plasma (PAS-20-APC, triangles) or 100% plasma (Plasma-APC, squares) were administered into the mouse circulation via lateral tail vein. Survival of human platelets (PLTs) in mouse was analysed collecting murine blood 1, 2, 5 and 24 hours after baseline sampling. Data are shown as mean \pm SEM, n=5.



Supplementary Figure 2: Morphology of adherent platelets

Representative images of the different morphology of adherent platelets (PLTs). Type 1, without filopodia; type 2, with filopodia; type 3 lammellipodia and type 4 fully spread PLTs (scale bar 5 μ m).



Supplementary Figure 3: Apheresis platelet concentrates metabolism during cold storage

At the indicated storage time (A) c-Glucose consumption and (B) c-Lactate production for apheresis platelet concentrates (APCs) containing 35% plasma were measured. Data are shown as mean±SEM; ns, not significant, n=3.

Supplementary Table 1A and B

Platelets (PLTs) adhesion ability was analysed after 7 days of storage on fibrinogen or collagen surfaces for apheresis platelet concentrates (APCs) containing 35% plasma (PAS-35-APC; Table 1A) and 20% plasma (PAS-20-APC; Table 1B). During the post-acquisition analysis, adhered PLTs without and with filopodia (type 1 and 2, respectively), lamellipodia (type 3), or fully spread (type 4) were determined and compared to freshly isolated PLTs used as control (100%).

1A: Adhesion assay of PAS-35-APC on fibrinogen and collagen surfaces

	RT Plasma-APC	4°C Plasma-APC	RT PAS-35-APC	4°C PAS-35-APC
<u>Fibrinogen</u>	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Type 1	9±2	19±9	5±2	14±6
Type 2	40±12	63±18	33±7	56±22
Type 3	15±5	27±6	38±9	35±18
Type 4	<1	<1	<1	<1
<u>Collagen</u>	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Type 1	8±3	21±7	2±1	8±4
Type 2	26±10	55±8	11±4	25±10
Type 3	5±2	5±3	13±8	31±18
Type 4	<1	<1	<1	<1

1B: Adhesion assay of PAS-20-APC on fibrinogen and collagen surfaces

	RT Plasma-APC	4°C Plasma-APC	RT PAS-20-APC	4°C PAS-20-APC
<u>Fibrinogen</u>	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Type 1	34±15	16±2	7±4	16±7
Type 2	35±3	44±6	20±8	31±11
Type 3	11±3	27±17	15±9	15±12
Type 4	<1	8±8	<1	<1
<u>Collagen</u>	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Type 1	22±10	41±4	11±4	13±4
Type 2	25±11	31±4	20±4	25±10
Type 3	5±1	17±6	11±5	13±1
Type 4	<1	<1	<1	<1

Supplementary Methods

Preparation of apheresis platelet concentrates

To prepare PAS-35-APCs, PLTs were resuspended at the end of the separation process in 140 mL autologous plasma. The PLT suspension was then split into two bags (Fresenius Kabi, Bad Homburg, Germany); one bag received an additional 130 mL autologous plasma (Plasma-APC) and the other received 130 mL PAS. In contrast, PAS-20-APCs were produced by the addition of 80 mL autologous plasma. The PLT suspension was then split into two bags; one bag received an additional 160 mL autologous plasma (Plasma-APC) and the other received 160 mL PAS (PAS-20-APC). After preparation, all APCs (PAS-20-APC, PAS-35-APC and Plasma-APC) were allowed to rest for 1 h at RT, before subsequent storage at RT on a standard PLT agitator (Heidolph, Frankfurt, Germany) or at 4°C in a refrigerator also on a standard PLT agitator. Each unit was stored at the corresponding condition for 10 days.

In vivo studies

To assess the survival of human PLTs stored in PCs at different conditions, we used the NOD/SCID mouse model. NOD/SCID mice (NOD.CB17-Prkdc^{scid}/J, stock No. complexes, 001303) were purchased from the Jackson Laboratories (Ben Harbour, ME, USA) via Charles River, Research Models and Services (Sulzfeld, Germany). Sex- and age-matched (12-16 weeks) animals were used in this study. 200 µL containing 1×10^9 /mL PLTs were injected into the lateral tail vein. Freshly isolated human PLTs from a healthy donor were used as control. 30 min after PLT injection a blood sample was collected by tail vein punctuation to determine the baseline of circulating human PLTs (100%). The survival of human PLTs in the mouse circulation was analysed by taking murine blood after 1, 2, 5 and 24 h. Samples were prepared immediately after collection using a commercially available fixation kit (PerFix-nc Kit, Beckman Coulter, Brea, CA, USA). Briefly, 2 µL of murine blood were collected by tail vein puncture into 30 µL of acid-citrate-dextrose (ACD-A; BD Bioscience, San Diego, CA, USA). Samples were then fixed with fixation buffer (1:10) for 15 min at RT and red blood cells were lysed using 100 µL of lysis buffer. PLTs were then stained with anti-human CD41-PE-Cy5 (Beckman Coulter) and anti-mouse CD41-FITC (BD Bioscience) for 30 min in the dark at RT. Data acquisition was performed using a flow cytometer (FC) (Navios, Beckman Coulter). PLTs were distinguished from other cells by means of size (forward scatter, FSC) and granularity (side scatter, SSC). To exclude non-specific events in the PLT gate, only events that stained positive for human CD41 were included in the analysis.

Measurement of glycan changes

PLT suspension was collected from APCs and fixed using 2% paraformaldehyde (PFA, Morphisto, Frankfurt, Germany) for 20 min at RT. Thereafter, PLTs were washed twice with wash buffer (2 mL PBS, Biochrom GmbH, Berlin, Germany) and after centrifugation (650 g, 7 min, RT) the pellet was resuspended in 100 µL wash buffer. Glycan pattern was then quantified by FC using FITC-conjugated lectin *Ricinus communis* agglutinin (RCA; 0.5 µg/mL) (Vector, Burlingame, CA, USA) which binds beta-galactose. Washed PLTs from each condition were incubated with RCA for 30 min at RT. Exogenous neuraminidase (5 IU, Merck, Darmstadt, Germany) was added as a positive control.

Apoptosis

PLTs were collected from APCs and centrifuged at 700 g for 7 min. Following this, PLTs were washed once with Tyrode buffer (0.1 g glucose, 0.1 g BSA in 100 mL PBS, pH 7.4) and resuspended with 1 mM CaCl₂. Thereafter, PLTs were stained with Annexin V-FITC (Beckman Coulter) for 60 min at RT. Finally, CD41-PC5 positive cells were analysed for Annexin V-FITC binding reflecting surface exposure of phosphatidylserine. Freshly isolated washed PLTs were incubated for 60 min with 10 µM ionomycin (Abcam, Cambridge, UK) for the externalisation of phosphatidylserine and used as positive control.

Platelet aggregation

Light transmission PLT aggregation assay (LTA) was performed with a 4-channel-aggregometer (Labitec, Ahrensburg, Germany). PLT aggregation was measured by turbidimetry induced by addition of 8 µg/mL collagen (Nycomed, Konstanz, Germany), 1.5 µM ristocetin, or 80 µM ADP (both Mölab, Langenfeld, Germany).

Hypotonic shock reaction

The ability to respond to a hypotonic environment was determined as hypotonic shock reaction (HSR) by LTA. PLTs were exposed to distilled water or 0.9% sodium chloride solution as control. Due to the concentration gradient, the water diffuses into the PLTs, which leads to their swelling. As a result of the swelling, the refractive index of PLTs increases, which leads to an increase in light transmission. The percentage of HSR was calculated as $(T1-T2)/(T2-T1) \times 100$, where T1=transmission of PLT suspension in sodium chloride and T2= transmission of PLTs in distilled water.

Platelet metabolism

Glucose and lactate were analysed in the supernatant of APCs by standard methods (Flex® and Dimension Vista 500, Siemens Healthcare, Eschborn, Germany).