Comparison between manufacturing sites shows differential adhesion, activation, and GPIbα expression of cryopreserved platelets

Katrijn R. Six,[1,2] Willem Delabie,[1] Katrien M.J. Devreese,[2,3] Lacey Johnson,[4] Denese C. Marks,[4,5] Larry J. Dumont,[6,7] Veerle Compernolle,[1,2,8] and Hendrik B. Feys[1,2]

BACKGROUND: Transfusion of cryopreserved platelets (cryoplatelets) is not common but may replace standard liquid-preserved platelets (PLTs) in specific circumstances. To better understand cryoplatelet function, frozen concentrates from different manufacturing sites were compared.

STUDY DESIGN AND METHODS: Cryoplatelets from Denver, Colorado (DEN); Sydney, Australia (SYD); and Ghent, Belgium (GHE) were compared (n = 6). A paired noncryopreserved control was included in Ghent. Microfluidic-flow chambers were used to study PLT adhesion and fibrin deposition in reconstituted blood. Receptor expression was measured by flow cytometry. Coagulation in static conditions was evaluated by rotational thromboelastometry (ROTEM).

RESULTS: Regardless of the manufacturing site, adhesion of cryoplatelets under shear flow (1000/sec) was significantly ($p < 0.05$) reduced compared to control. Expression of GPIbα was decreased in a subpopulation of cryoplatelets comprising 45% ± 11% (DEN), 63% ± 9% (GHE), and 94% ± 6% (SYD). That subpopulation displayed increased annexin V binding and decreased integrin activation. PLT adhesion, agglutination, and aggregation were moreover decreased in proportion to that subpopulation. Fibrin deposition under shear flow was normal but initiated faster (546 ± 163 sec GHE) than control PLTs (631 ± 120 sec, $p < 0.01$), only in the absence of tissue factor. In static conditions, clotting time was faster, but clot firmness decreased compared to control. Coagulation was not different between manufacturing sites.

CONCLUSION: Cryopreservation results in a subset of PLTs with enhanced GPIbα shedding, increased phosphatidylserine expression, reduced integrin response, and reduced adhesion to collagen in microfluidic models of hemostasis. The proportion of this phenotype is different between manufacturing sites. The clinical effects, if any, will need to be verified.

ABBREVIATIONS: CRP-XL = cross-linked collagen-related peptides; cryoplatelet(s) = cryopreserved platelet(s); DEN = cryoplatelets from Denver, Colorado; GHE = cryoplatelets from Ghent, Belgium; PC(s) = platelet concentrate(s); ROTEM = rotational thromboelastometry; RT = room temperature; TF = tissue factor; SYD = cryoplatelets from Sydney, Australia.

From the [1]Transfusion Research Center, Belgian Red Cross-Flanders, Ghent, Belgium; the [2]Faculty of Medicine and Health Sciences, Department of Diagnostic Sciences, Ghent University, Ghent, Belgium; and the [3]Coagulation Laboratory, Department of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium; [4]Research & Development, Australian Red Cross Blood Service, Sydney, Australia; and the [5]Sydney Medical School, University of Sydney, Sydney, Australia; [6]Blood Systems Research Institute, Denver, Colorado; the [7]Geisel School of Medicine at Dartmouth, Lebanon, New Hampshire, USA; and the [8]Blood Service of the Belgian Red Cross-Flanders, Mechelen, Belgium.

Address reprint requests to: Hendrik B. Feys, Ottergemsesteenweg 413, Ghent 9000, Belgium; e-mail: hendrik.feys@rodekruis.be.

This research was supported by the Foundation for Scientific Research of the Belgian Red Cross Flanders. KS is a fellow of the Special Research Fund of Ghent University. The Australian government funds the Australian Red Cross Blood Service to provide blood and blood products to the Australian community.

The views, opinions, and/or findings contained in this report are those of the author (s) and should not be construed as an official Department of Defense position, policy, or decision unless so designated by other documentation.

Received for publication December 22, 2017; revision received March 22, 2018; and accepted March 22, 2018.

doi:10.1111/trf.14828

TRANSFUSION 2018;58;2645–2656

The demand for platelet concentrates (PCs) has increased progressively over the years[1] due to an aging population and an increased number of patients suffering from chronic thrombocytopenia. Platelets (PLTs) are transfused for prophylaxis in patients with hematologic malignancies or to treat active bleeding.[2] Room temperature (RT) storage of PCs results in better transfusion yields than cold-stored PLTs.[3] However, it also limits the shelf life to between 4 and 7 days, resulting in a logistic challenge that all blood banks continuously face.[4] In addition, these conditions give rise to a substantial risk of bacterial transmission. Consequently, cold storage and cryopreservation have remained of interest as an alternative to standard PC banking[5–7] for nonprophylactic use, particularly for remote areas with low-quality transportation infrastructure or distant rural communities or during military operations when transport of liquid-stored PCs is hampered.[8] In selected cases, cryopreserved platelets (cryoplatelets) could serve as backup product in emergencies for an acutely bleeding patient when supply of regular liquid stored PC is exhausted.

Several cryopreservation methods have been published aiming to minimize PLT damage. Dimethyl sulfoxide (DMSO) is the most commonly used cryoprotectant, although several others have been investigated.[5,9–11] Most protocols include hyperconcentration of PLTs before freezing to reduce the amount of DMSO transfused. This requires reconstitution after thawing, most commonly in plasma or saline.[12]

Cryoplatelets have been transfused in healthy volunteers, generally yielding lower count increments compared to liquid-stored PLTs.[5,13,14] This is caused by biochemical changes during cryopreservation that can be detected in vitro including decreased PLT aggregation, deficient signal transduction, altered morphology, differential expression of certain membrane markers, and microparticle release.[14–18] Recent studies suggest that cryoplatelets are procoagulant,[19,20] but these experiments were performed in static conditions when hemostasis was not restricted by convective hydrodynamic forces. Studies of PLT adhesive and procoagulant function of cryoplatelets under shear flow are lacking. In addition, comparison between cryopreservation procedures and cryopreserved products manufactured in different facilities is not available. This study combines both questions in a comparison of cryoplatelets from three unrelated international blood institutions, using microfluidic-flow chamber perfusion and real-time video microscopy.

MATERIALS AND METHODS

Study design

Platelet concentrates were prepared and cryopreserved in three different manufacturing sites: Denver, CO, (DEN); Sydney, Australia (SYD); and Ghent, Belgium (GHE). Primary products from DEN and SYD were from apheresis and stored in plasma with ACD-A. Those from GHE were from pooling of six buffy coats in CPD-plasma with 65% (vol/vol) additive solution (SSP+, Macopharma). Cryopreservation protocols were similar for all three manufacturing sites and were described previously.[14,21] In brief, a 27% (vol/vol) DMSO with 0.9% NaCl (wt/vol) in water was added to the PC to achieve a final concentration of 5% to 6% (vol/vol) DMSO. These PCs were hyperconcentrated by centrifugation, resuspended in the remaining supernatant, and then stored at −80°C. Cryopreserved DEN and SYD products were shipped (World Courier) on dry ice to GHE where these were immediately transferred to a −80°C freezer until use.

Concentrates from DEN were thawed for 8 minutes at 37°C and were then held undisturbed for 30 minutes before resuspension in 25 mL of 0.9% (wt/vol) NaCl in water at RT. Those from SYD and GHE were resuspended in 250 mL of ABO/D-matched plasma (30°C) immediately after thawing for 5 minutes at 37°C. After resuspension, SYD and GHE PLTs were held undisturbed for 1 hour at RT before experiments. Paired liquid-preserved control PCs from DEN and SYD could not be transported to GHE because of the logistic challenge. Therefore, paired control PCs were only included in Ghent (GHE CTR). These were "sham" treated with all preparation and handling methods but without freezing, therefore controlling for confounding effects of DMSO, hyperconcentration, resuspension, and incubation. Preliminary experiments using microfluidic-flow chambers found no difference between sham-treated and untreated control PLTs (data not shown). GHE CTR PLTs were resuspended in 250 mL of ABO/D plasma and incubated for 1 hour at RT, as for the paired GHE cryoplatelets. From every manufacturing site, six unrelated cryoplatelet units were included (n = 6).

Blood reconstitution for microfluidic-flow chambers and thromboelastometry

Fresh blood samples were taken from healthy consenting nonmedicated volunteers in hirudin vacutainers (REF 08128812001, Diapharma Group Inc.) or in sodium citrate vacutainers (REF 366575, BD Diagnostics). Hirudin vacutainers were used for microfluidic-flow chambers studying PLT adhesion alone, in the absence of coagulation.[22] Citrate was used for microfluidic-flow chambers with recalcification to study PLT adhesion in combination with fibrin deposition (i.e., coagulation).[23]

Blood cells were separated by centrifugation (13 min at 250 × *g* without brake) generating PLT-rich plasma and concentrated red blood cells (RBCs). PLT-poor plasma was obtained by centrifugation of that PLT-rich plasma for 10 minutes at 4500 × *g*. Blood reconstitution was performed by mixing the RBCs with the corresponding PLT-poor

plasma and with PLTs from the various cryo- or control groups, aiming for a mean of 40% hematocrit and 250×10^9 PLTs/L. Complete blood and PLT counts after reconstitution were determined with an automated hematology analyzer (PocH-1000i, Sysmex, Kobe, Japan). The PLT count before cryopreservation was with the hematology analyzers from the respective manufacturing sites: PocH-1000i, Sysmex (GHE); XE 2100D, Sysmex (DEN); and CellDyn Ruby, Abbott Diagnostics (SYD).

Perfusion-flow chamber experiments

Adhesion of PLTs to immobilized collagen was examined by measuring surface coverage as a function of time during perfusion of reconstituted, hirudin anticoagulated blood in microfluidic channels coated with collagen as described.[22] Briefly, microfluidic-flow chambers were coated with 50 μg/mL of collagen (horm collagen Type III, Takeda Pharma) in an isotonic glucose solution (pH 2.7) overnight at 4°C. Reconstituted blood was labeled for 10 minutes at 37°C with 3,3′-dihexyloxacarbo-cyanine iodide in a final concentration of 1 μmol/L (Sigma-Aldrich, St Louis, MO). Labeling efficiency was tested using flow cytometry and was not different between PCs. A microfluidic pump (Mirus evo nanopump, Cellix Ltd.) was used to generate a wall shear stress of 50 dyne/cm^2 corresponding to a wall shear rate of 1000/sec. PLT deposition was recorded as a function of perfusion time using real-time video microscopy and accompanying acquisition software (Carl Zeiss, Oberkochen, Germany). PLT surface coverage (%) was retrieved by image data analysis (Zen 2 [blue edition], Carl Zeiss). A linear regression of PLT surface coverage with time was used to calculate the PLT adhesion rate (Prism Version 6.07, GraphPad Software Inc.).

Adhesion of PLTs in combination with fibrin deposition was examined by measuring median fluorescence increase as a function of time during perfusion of reconstituted and recalcified blood under perfusion flow, as described.[23] Instead of hirudin, citrate was used to anticoagulate fresh blood for reconstitution. The reconstituted blood was mixed during perfusion with one tenth volume of recalcification buffer (HBS; 10 mmol/L HEPES, 155 mmol/L NaCl, pH 7.4) containing 100 mmol/L $CaCl_2$ and 37.5 mmol/L $MgCl_2$. Separately operated syringe pumps (Exigo syringe pump, Cellix) were used. PLT and fibrin deposition under flow was examined in channels coated with only collagen for contact activation or with collagen plus purified recombinant human lipidated tissue factor (TF, Dade Innovin) at approximately 100 pmol/L in HBS (Siemens Healthcare GmbH) for TF-based activation. Before perfusion, the reconstituted blood was spiked with 70 μg/mL of Alexa Fluor 405-labeled fibrinogen (Sigma-Aldrich) in addition to 3,3′-dihexyloxacarbocyanine iodide to label PLTs as above. For investigation of TF-mediated coagulation, reconstituted blood samples were supplemented with 4 μmol/L corn trypsin inhibitor (Enzyme Research Laboratories) to inhibit FXIIa-based contact activation. Image acquisition and analysis were as described.[23] In brief, PLT adhesion rate (/sec) was measured by linear regression of the green fluorescent signal increase as a function of perfusion time. The variables retrieved for fibrin deposition included coagulation rate (/sec), which is the linear portion of fibrin deposition kinetics and clotting time (sec), which is the lag time indicating the moment of coagulation onset. This analysis takes into account thrombus growth in the z-plane. The outcome variables were extracted from the raw fluorescence data using a software plugin developed in MatLab (MathWorks). All flow chamber studies were performed in duplicate and the mean of two technical repeats was used.

Flow cytometry

Expression of P-selectin (phycoerythrin-anti-CD62P, Life Technologies), phosphatidylserine (peridinin-chlorophyll-Cy5.5 Annexin V, BD Biosciences), activated integrin $\alpha_{IIb}\beta_3$ (fluorescein-labeled PAC1, BD Biosciences) and GPIbα (fluorescein-labeled anti-CD42b, Life Technologies) was analyzed with an acoustic focusing flow cytometer (Attune, Life Technologies). PLTs were incubated with the labeled antibodies for 10 minutes in buffer 10 mmol/L HBS/1 mmol/L $MgSO_4$ at RT. Samples were diluted 20-fold immediately before readout. Phosphatidylserine measurement was in buffer supplemented with 2 mmol/L $CaCl_2$. Integrin $\alpha_{IIb}\beta_3$ activation was measured with PAC1 for resting PLTs or after activation using cross-linked collagen-related peptides (CRP-XL; 0.25 μg/mL, University of Cambridge). Threshold gates were set including 0.5% of 10,000 events incubated with corresponding isotype antibody controls. For phosphatidylserine controls, a sample containing labeled annexin V was prepared without $CaCl_2$. The percentage of positive events or median fluorescent intensities were determined for 10,000 events staining positive for CD61 (allophycocyanin-labeled anti-CD61, Life Technologies). The number of microparticles was determined using calibration beads (Biocytex) and the accompanying gating strategy provided by the manufacturer. Microparticles were defined as GPIbα positive events with sizes smaller than 0.9 μm. The result is expressed as the fraction of microparticles per 10,000 all-size events positive for GPIbα.

Western blotting to determine GPIbα ectodomain shedding

Samples (1 mL) were taken from the PLT bag immediately before freezing at −80°C and immediately after thawing. These experiments were performed on GHE PLTs only for logistic reasons. Metalloproteinase activity was quenched immediately with 50 mmol/L EDTA. Centrifugation was performed at 4500 × *g* for 1 minute at RT. The PLT pellet was resuspended in reducing sample buffer (60 mmol/L Tris, 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 0.01% [wt/vol]

bromophenol blue, and 40 mmol/L dithiothreitol). The supernatant was 0.2 μm filtered first and then prepared for electrophoresis by addition of reducing sample buffer. Equal volumes of sample were loaded onto 4% to 15% polyacrylamide Tris-Glycine TGX gels (Bio-Rad). The GPIbα protein or its ectodomain were detected with the mouse monoclonal anti-GPIbα (Clone 8H211) antibody (Antibodies Online) and a secondary peroxidase conjugated polyclonal antibody (Cell Signaling Technologies). Antibodies were prepared in Tris-buffered saline (pH 7.4, 25 mmol/L Tris with 150 mmol/L NaCl, and 2 mmol/ KCl) with 5% (wt/vol) skimmed milk. Membranes were developed in a imaging system (ChemiDoc MP, Bio-Rad). Densitometry was performed using (ImageLab v4.0.1, Bio-Rad).

PLT aggregation

Platelet aggregation and agglutination were examined with light transmission at 37°C (Chrono-Log, Helena Laboratories). Ristocetin-induced PLT agglutination was with 1.5 mg/mL ristocetin (American Biochemical and Pharmaceuticals). Aggregation was with a combination of 10 μmol/L protease activated receptor-1 activating peptide (PAR1AP, Sigma-Aldrich), 20 μmol/L 2-(methylthio)adenosine 5-diphosphate trisodium salt hydrate (MeSADP, Santa Cruz Biotechnology) and 5 μmol/L epinephrine (Sigma-Aldrich). Aggregation cuvettes contained 250×10^9 PLTs/L in a final volume of 500 μL, diluted in their respective supernatant (obtained by centrifugation at $4500 \times g$ for 10 min). The signal was calibrated using the respective PLT-free supernatants. Maximal amplitude (%) is reported.

Thrombin generation assay

Generation of thrombin in vitro was analyzed with the thrombin generation assay kit from Technoclone GmbH according to the manufacturer's instructions with minor modifications. Samples were prepared in 96-well microplates. PCs were diluted to 10×10^9, 50×10^9, or 250×10^9/L in a fixed 40% (vol/vol) volume of heterologous human pooled plasma and 5% (vol/vol) of saline (0.9% [wt/vol] NaCl in water) and supplemented with 1 pmol/L TF, 4.0 μmol/L corn trypsin inhibitor, 0.5 μmol/L fluorogenic substrate (Z-G-G-RAMC) and 7.5 mmol/L $CaCl_2$ (final concentrations). Samples were immediately analyzed in a microplate reader (Infinite F200PRO, Tecan Group Ltd.) with filter settings for excitation at 360 nm and emission at 460 nm. The fluorescent signal was recorded as a function of time for a total of 120 minutes at 37°C. The raw signal was converted to thrombin concentrations based on a calibration kit and a script in Excel (Microsoft) provided by the manufacturer.[24]

Rotational thromboelastometry

Rotational thromboelastometry (ROTEM) was performed on reconstituted and citrate anticoagulated blood using a ROTEM delta analyzer (Tem Innovations) according to the manufacturer's recommendations. ROTEM provides dynamic information on the speed of coagulation initiation, kinetics of clot growth, clot strength, and breakdown of the clot. Contact activation is with ellagic acid and phospholipids. Activation of the TF pathway with lipidated TF was performed as described previously.[25] The following variables were analyzed: clotting time (sec) to examine speed of fibrin formation, clot formation time (sec) to examine clot formation kinetics, and maximum clot firmness (mm) to examine firmness of the clot. Time between in vitro reconstitution of blood and analysis was 120 minutes at maximum.

Statistical analysis

Results are reported as mean with standard deviation (SD). Comparison between sites was by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons correction. Comparison of paired GHE and GHR-CTR concentrates was with a two-tailed paired t-test. Comparison of thrombin generation output data by varying PLT concentration and production site was with two-way ANOVA with Tukey's multiple comparison's correction. Results from statistical analysis are depicted on top of the panels. All statistical analyses were performed using computer software (Prism, Version 6, GraphPad Software Inc.).

RESULTS

PLT recovery and storage time

Table 1 shows the mean PLT content of the products in the study before and after cryopreservation. Mean recovery was more than 90% for all manufacturing sites. It should be noted that the PLT count after thawing was determined using the GHE hematology analyzer while for the PLT count before freezing cell counters from the respective manufacturing sites were used. Storage duration was longest for SYD and shortest for GHE cryoplatelets.

PLT adhesion and fibrin deposition under perfusion flow

In reconstituted blood, the adhesion rate of cryoplatelets measured by surface coverage was decreased at least threefold compared to control ($p < 0.05$; green signal in Video S1 [available as supporting information in the online version of this paper] and Fig. 1A). SYD PLTs were more affected ($p < 0.05$) than GHE or DEN. Similar observations were made in the presence of restored calcium levels both in conditions with TF (green signal in Video S2 [available as supporting information in the online version of this paper] and Fig. 1B) and without TF (green signal in Video S3 [available as supporting information in the online version of this paper] and Fig. 1C). Cryoplatelets also bound to control surfaces without collagen, leading to localized

TABLE 1. PLT recovery and storage duration*

Center	PLT count ($\times 10^9$) Before cryo	After cryo	Recovery (%)	Storage (days)
DEN	374.6 ± 40.7	354.9 ± 71.4	94.1 ± 10.8	558 ± 138
SYD	524.2 ± 17.3	504.9 ± 67.5	98.6 ± 7.7	758 ± 12
GHE	345.8 ± 29.1	316.7 ± 17.2	92.1 ± 8.6	21 ± 12

* Data are given as mean ± SD (n = 6).

fibrin deposition (Fig. 1D). This was not observed with control PLTs.

The rate of fibrin deposition following adhesion of cryoplatelets was variable, but not different from control PLTs in the presence of TF (violet signal in Video S2 and Fig. 2A). In the absence of TF, fibrin deposition rates were slightly decreased for cryoplatelets (violet signal in Video S3, Fig. 2B). The clotting time was not different when TF was present (Fig. 2C). In the absence of TF, however, clotting time decreased for cryoplatelets (GHE, 546 ± 163 sec) compared to control PLTs (GHE-CTR, 631 ± 120 seconds; Fig. 2D). This indicates that cryoplatelets enhance contact activation of coagulation under flow compared to control. There were no significant differences between SYD, DEN, and GHE PLTs for fibrin deposition under hydrodynamic flow, despite the differences in PLT binding (Fig. 1).

GPIbα ectodomain shedding in a subset of cryoplatelets

Almost all events in the cryopreserved PCs were positive for GPIbα (Fig. 3A), similar to control PLTs. Although control PLTs had uniformly bright GPIbα expression, the cryoplatelets also contained a dim GPIbα subpopulation, comprising 45% ± 11% (DEN), 63% ± 9% (GHE), and 94% ± 6% (SYD) of all GPIbα positive events (Figs. 3B and 3C). The dim and bright subpopulations were distinguishable but not always entirely resolved in different cryoplatelet preparations (Fig. S1, available as supporting information in the online

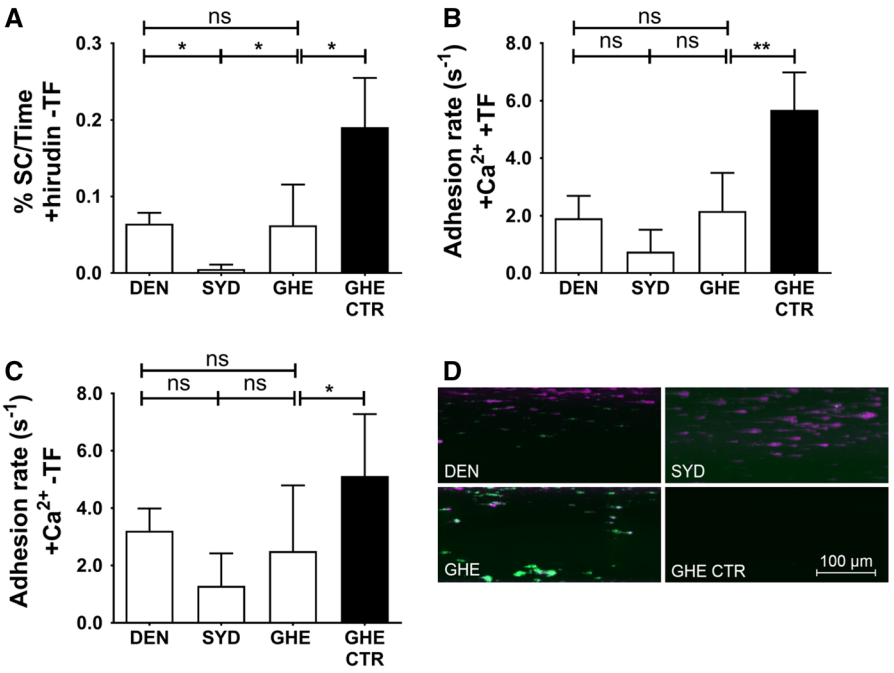

Fig. 1. Platelet adhesion under shear flow. Perfusion of blood reconstituted with cryopreserved and control PLTs was at 1000/sec. (A) The PLT surface coverage as a function of perfusion time (% SC/Time) in channels coated with only collagen (Video S1). This condition was with hirudin-anticoagulated blood without TF. (B, C) The rate of PLT adhesion in citrated blood with restored Ca^{2+} levels. These channels were coated with (B) collagen and TF (Video S2) or with (C) collagen only (Video S3). (D) PLT (green) and fibrin (violet) accumulation in a part of the channel not coated with collagen. Images were acquired at endpoint (magnification, 100×). Comparison was between DEN, SYD, and GHE (open bars). A separate paired comparison between GHE and control noncryoplatelets was also performed (GHE CTR, closed bars). Data are shown as mean ± SD (n = 6). Statistical analysis results are shown as *p < 0.05, **p < 0.01, or not significant (ns). [Color figure can be viewed at wileyonlinelibrary.com]

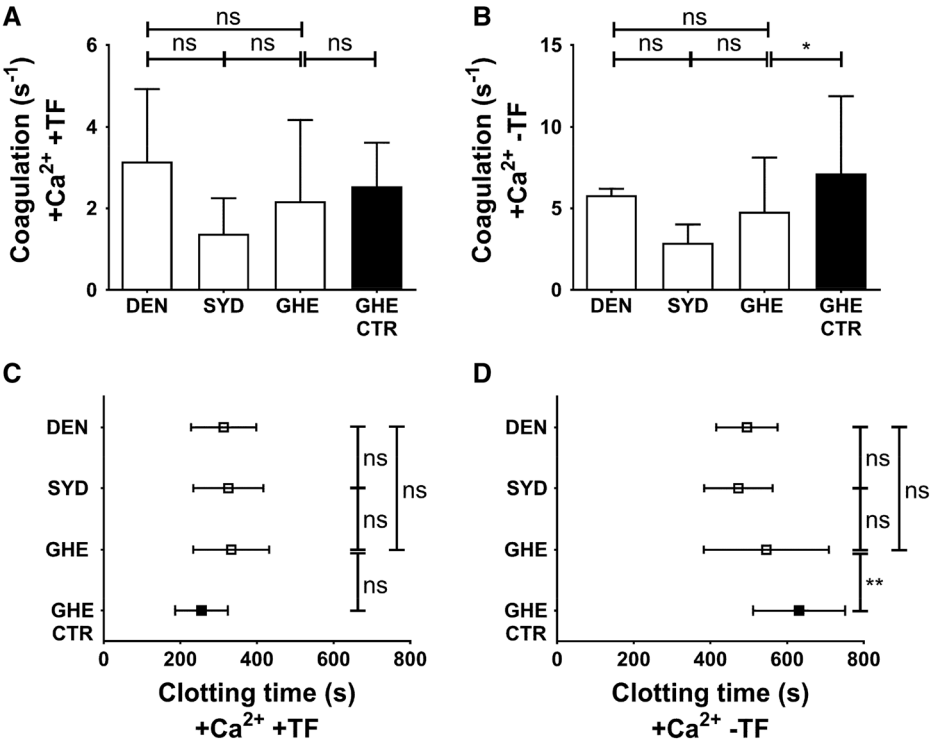

Fig. 2. Coagulation under shear flow. Perfusion of blood reconstituted with cryopreserved and control PLTs was at 1000/sec with real-time restoration of Ca^{2+} levels. Fibrin accumulation was followed over time. Accumulation rate (/sec) of fibrin in channels coated with (A) collagen and TF or with (B) only collagen. The clotting time (sec) indicates the moment of coagulation onset in channels coated with (C) collagen and TF or with (D) only collagen. Comparison was between DEN, SYD, and GHE (all open symbols). A separate paired comparison between GHE and control noncryoplatelets was also performed (GHE CTR, closed symbols). Data are shown as mean ± SD (n = 6). Statistical analysis results are shown as *$p < 0.05$, **$p < 0.01$, or not significant (ns).

version of this paper). Ristocetin induced PLT agglutination was significantly decreased in cryoplatelets (Fig. 3D) and SYD PLTs were more affected ($P < 0.001$) than GHE or DEN. The ristocetin response was associated with the fraction of GPIbα-bright PLTs (Fig. 3C).

Western blotting of PLT lysate and of the PLT-free supernatant indicated significant GPIbα ectodomain shedding after cryopreservation (Fig. 4). The signal of full-length GPIbα in PLT cell lysates significantly decreased (Fig. 4A). The signal of GPIbα ectodomain in PLT-free supernatant correspondingly increased three- to sevenfold in cryoplatelets compared to before cryopreservation (Figs. 4A and 4B). This significant variation in the level of ectodomain shedding confirms the variation of GPIbα expression found in flow cytometry (Fig. S1).

Platelet aggregation was significantly reduced for cryoplatelets (Fig. 5A) compared to control, despite a strong agonist blend of PAR1AP, MeSADP, and epinephrine. Integrin $\alpha_{IIb}\beta_3$ activation by CRP-XL was also significantly reduced compared to control (Fig. 5B). Again, SYD PLTs were more affected ($p < 0.01$) than GHE or DEN. The aggregation and integrin activation response furthermore followed the fraction of GPIbα-bright PLTs (Fig. 3C). In addition, the bright GPIbα subpopulation had normal integrin activation, while the GPIbα-dim subpopulation did not (representative data in Fig. 5C). Similarly, the bright subpopulation had low levels of annexin V binding, while the dim one had high levels of annexin V binding (representative data in Fig. 5D). This resulted overall in higher number of annexin V–positive events (Fig. S2A). In line with this, the percentage of microparticles was increased after cryopreservation (Fig. S2B). The highest number was found in SYD cryoplatelet preparations.

The refractive properties of the GPIbα-dim subpopulation were altered. In forward mode, less light (median intensity, $1.9 \times 10^5 \pm 0.2 \times 10^5$) was scattered compared to the GPIbα-bright subpopulation either in GHE cryoplatelets (median intensity, $3.3 \times 10^5 \pm 0.5 \times 10^5$) or GHE-CTR control PLTs (median intensity, $3.3 \times 10^5 \pm 0.4 \times 10^5$; Fig. S3). Collectively, these data suggest that the GPIbα-dim subpopulation is indicative of the partial damage caused by cryopreservation.

Function of cryoplatelets in reconstituted whole blood under static conditions

Coagulation under static conditions in vitro was examined with thromboelastometry. Reconstituted whole blood containing

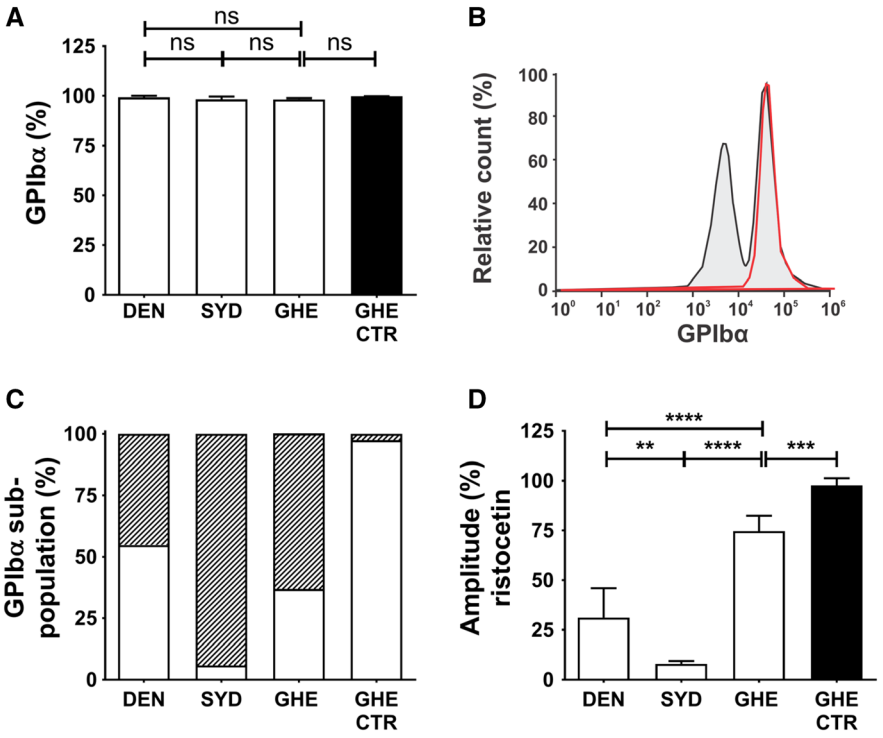

Fig. 3. GPIbα expression and PLT activation. (A) The percentage of GPIbα-positive events determined in flow cytometry. (B) Representative histogram of GPIbα signals in flow cytometry in paired control GHE CTR (red) and cryopreserved GHE (black) PCs denoting dim and bright GPIbα subpopulations. (C) Within the GPIbα-positive population, events were gated for dim or bright GPIbα expression based on the respective histograms as per B. The population of dim GPIbα (hatched bars) is overlaid with the population of bright GPIbα (open bars) expressing events. (D) Ristocetin-induced PLT agglutination was measured and data are shown as maximal amplitude (%). Comparison was between DEN, SYD, and GHE (all open symbols). A separate paired comparison between GHE and control noncryoplatelets was also performed (GHE CTR, closed symbols). Data are shown as mean ± SD (n = 6). Statistical analysis results are shown as $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, or not significant (ns). [Color figure can be viewed at wileyonlinelibrary.com]

GHE cryoplatelets showed decreased clotting times compared to GHE-CTR indicating that initiation of coagulation was faster irrespective of TF (47 ± 6 sec vs. 58 ± 5 sec; Fig. 6A) or contact activation (158 ± 30 sec vs. 203 ± 11 sec; Fig. 6B). In line with this, the lag time between initiating thrombin generation and initial thrombin formation was short for all cryoplatelet preparations (Fig. 6C). Peak thrombin concentrations were significantly higher (Fig. 6D). Both lag time and peak thrombin were dependent on the dose of PLTs as well, irrespective of cryopreservation. Data between SYD, DEN, and GHE were not different for these parameters, in line with the coagulation experiments under shear flow. Despite shortening lag times with cryoplatelets, however, the subsequent clot formation rate and clot firmness were reduced compared to control (Fig. 7). This was independent of TF.

DISCUSSION

Freezing cells damages their integrity, often irreversibly decreasing the cell's ability to function optimally.[26] Excessive release of microparticles and decreased responses to agonists in vitro are known indicators of PLT cryopreservation-induced damage.[4,18] Despite these changes, no serious adverse events were documented in a recent Phase I dose-escalation trial in hematology-oncology patients.[27] Older studies even suggested clinical efficacy of cryoplatelets, although count increments were consistently lower in comparison to liquid-preserved PLTs.[28,29] Cryoplatelets are promising as they can be shipped over great distances, but this will require significant standardization of the production process. In addition, a deep understanding of cryoplatelet biochemical quality and its markers is required. This study has examined cryoplatelets produced in different countries and shipped to one investigation site, with a view to understanding cryoplatelet variability and function under conditions of shear flow.

A key finding in this study was the presentation of a GPIbα-dim subpopulation representing 45% to 94% in cryoplatelets, but less than 5% in controls. This observation was reported first by Barnard et al.[17] in a seminal paper on receptor density of cryoplatelets. Cryoplatelet preparations with an abundant GPIbα-dim subpopulation displayed

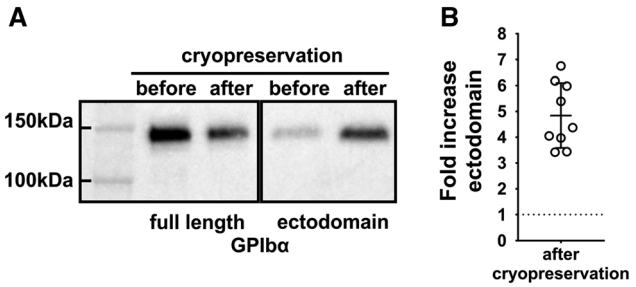

Fig. 4. Ectodomain shedding of GPIbα. (A) A representative Western blot of the GPIbα receptor in the PLT cell lysate (full length) or the PLT supernatant (ectodomain) is shown. All samples were loaded on the same gel, but not adjacent to each other as indicated by the panel borders. Molecular weight markers are in kilodalton (kDa). Paired samples before and after cryopreservation are shown. (B) Densitometry of the GPIbα ectodomain signal was performed for samples before and after cryopreservation. The signal after cryopreservation is expressed relative to that before. Data from individual biologic repeats (n = 9) are shown. Mean is shown as a horizontal line and error bars represent the SD.

reduced or absent aggregation, poor integrin activation, and reduced PLT adhesion under shear. In addition, the GPIbα-dim subpopulation itself was unresponsive to agonists and had increased annexin V binding and altered refractive properties.

Platelets can actively decrease the GPIbα receptor number on the plasma membrane by internalization and by ectodomain shedding. For instance, PLT activation by PAR1AP decreases GPIbα levels to below 60% of resting control PLTs. This does not affect their subsequent adhesion to collagen in flow chambers because internalized GPIbα receptors are quickly restored upon contact with the immobilized collagen surface.[30] These observations imply that GPIbα internalization is not sufficient to sustainably decrease PLT adhesion to collagen. The GPIbα decrease during cryopreservation therefore is not governed by receptor internalization but by ectodomain shedding,[15] in a subset of PLTs. Contrary to GPIbα internalization by PLT activation, ectodomain shedding is irreversible. Moreover, the GPIbα-dim PLTs have GPIbα levels well below 50% of normal, which is a critical value for accomplishing adhesion

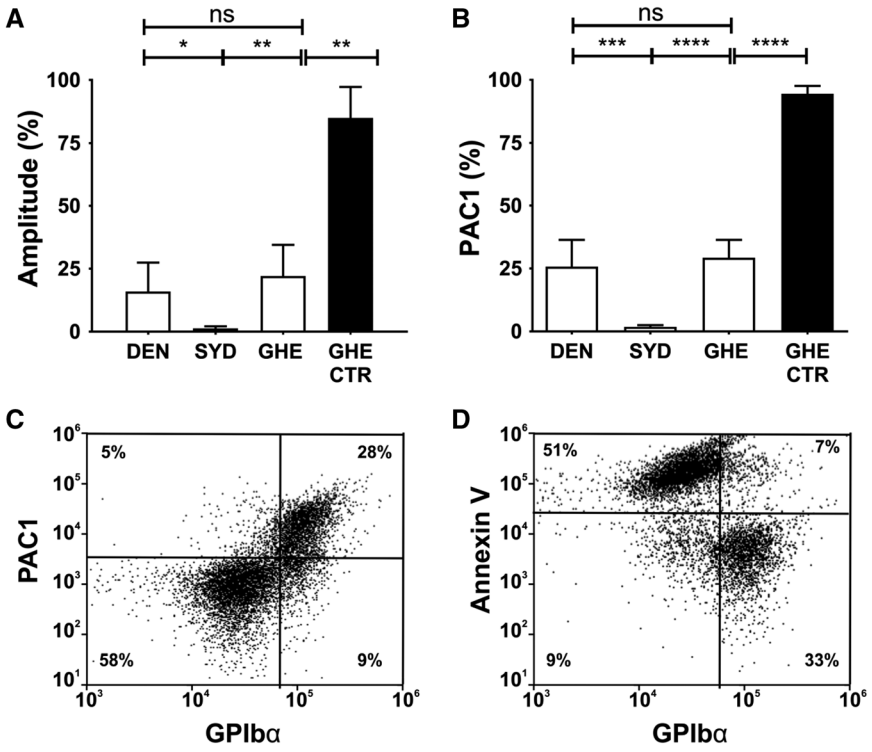

Fig. 5. Platelet activation and GPIbα expression levels. (A) PLT aggregation in response to a mix of PAR1AP, MeSADP, and epinephrine. Maximal amplitude (%) is shown. (B) The percentage of PLTs expressing activated integrin $\alpha_{IIb}\beta_3$ in response to stimulation with CRP-XL measured by binding of PAC1. (C) A representative dot plot of PAC1 binding versus GPIbα expression. The plot is divided by a quadrant indicating subpopulations expressing high or low signals of PAC1, GPIbα, or both. (D) A representative dot plot of annexin V binding versus GPIbα expression. The plot is divided as in C. Comparison was between DEN, SYD, and GHE (all open symbols). A separate paired comparison between GHE and control noncryoplatelets was also performed (GHE CTR, closed symbols). Data are shown as mean ± SD (n = 6). Statistical analysis results are shown as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, or not significant (ns).

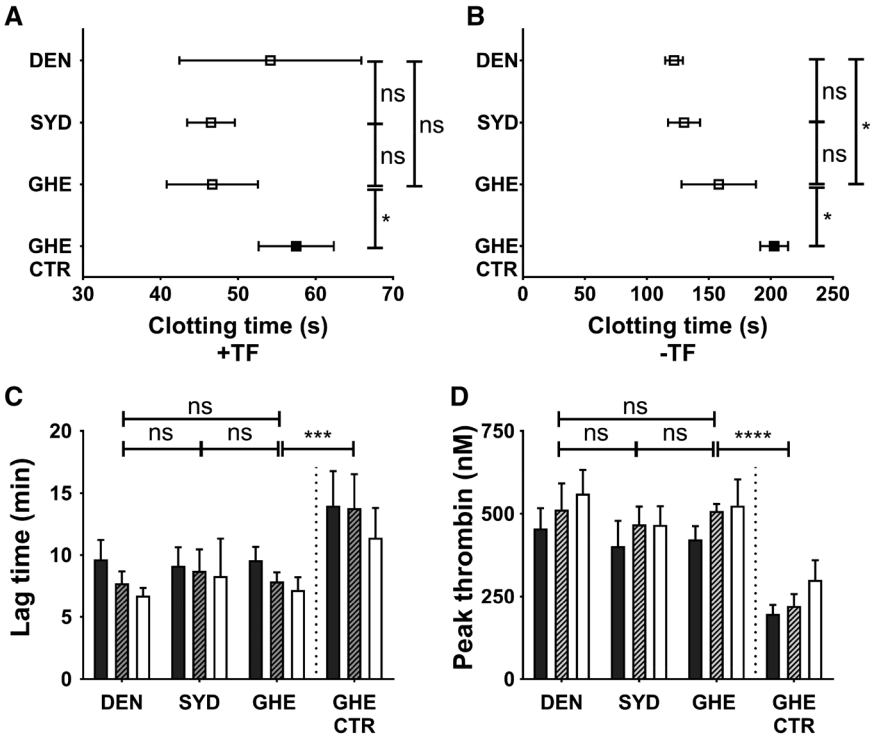

Fig. 6. Coagulation initiation in static conditions. Blood was reconstituted with cryopreserved (open symbols) or control PLTs (closed symbols) and studied in the presence of Ca^{2+} by ROTEM using (A) TF or (B) ellagic acid to initiate coagulation. The clotting time (sec) is the lag time from adding the activating agent to the blood until the elastogram tracing reached 2 mm. (C, D) Thrombin generation was measured in the presence of TF and increasing PLT concentrations of 10×10^9/L (filled bars), 50×10^9/L (hatched bars), and 250×10^9/L (open bars). (C) The lag time (min) measures the time until threshold thrombin concentrations are reached. Statistics are indicated for the conditions with 250×10^9 PLTs/L. (D) Peak thrombin (nmol/L) measures the peak amount of thrombin formed. Statistical significance is indicated for the conditions with 250×10^9 PLTs/L. Cryoplatelets from DEN, SYD, and GHE were used and the latter was paired to control noncryoplatelets GHE CTR (dashed line). Data are shown as mean ± SD (n = 6). Statistical analysis results are shown as *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, or not significant (ns).

to collagen under flow.[30–32] Consequently, these PLTs contribute most if not all to the decrease in adhesion and by extrapolation to the decrease in overall cryoplatelet function.

Furthermore, that fraction was remarkably variable between production sites as well as between PLT preparations within a given site (Fig. S1). This suggests that cryoplatelet quality may be improved if the number of dysfunctional PLTs can be consistently controlled at low levels. It is currently not clear, however, what makes certain PLTs more susceptible to cryopreservation-induced damage. Maybe differences in primary PLT concentration and age, cryoprocess steps, (preliminary) exposure to temperature cycles[33], and/or (bio)chemicals contribute. Whether shedding inhibitors could prevent cryopreservation-induced damage depends on what comes first. If GPIbα shedding is secondary to a damaging event, then shedding inhibitors will not help but in the case that shedding sparks downstream lesions, inhibitors may be able to mitigate damage. For age-related storage lesion of liquid PLTs, specific inhibition of GPIbα shedding rescued the rapid clearance of aged PLTs to some extent.[34] Future research can always exploit the GPIbα-dim subpopulation as a biomarker for the extent of damage. The impact of directed modifications in the cryoprocess can be easily monitored using this particular marker in flow cytometry.

Cryopreservation-induced damage was particularly obvious in assays that specifically study PLTs, like integrin activation, microparticle release, and aggregation. This is in line with previous publications[14–18], which were mostly under static conditions (or extremely low shear) that do not always mimic the biophysical environment of blood vessel injury. Our data now confirm that in shear flow, cryoplatelet adhesion rates to immobilized collagen were also significantly decreased compared to control. This was both with and without anticoagulation, indicating that cryoplatelets are less adhesive irrespective of simultaneous thrombin and fibrin formation. Unlike controls, cryoplatelets also bound to uncoated parts of the channel during perfusion. This suggests that cryoplatelets are less selective than control PLTs for binding to nonbiologic substrates. This requires further investigation because cryoplatelets may be lost by nonspecific adhesion to transfusion bags, lines, or needles.

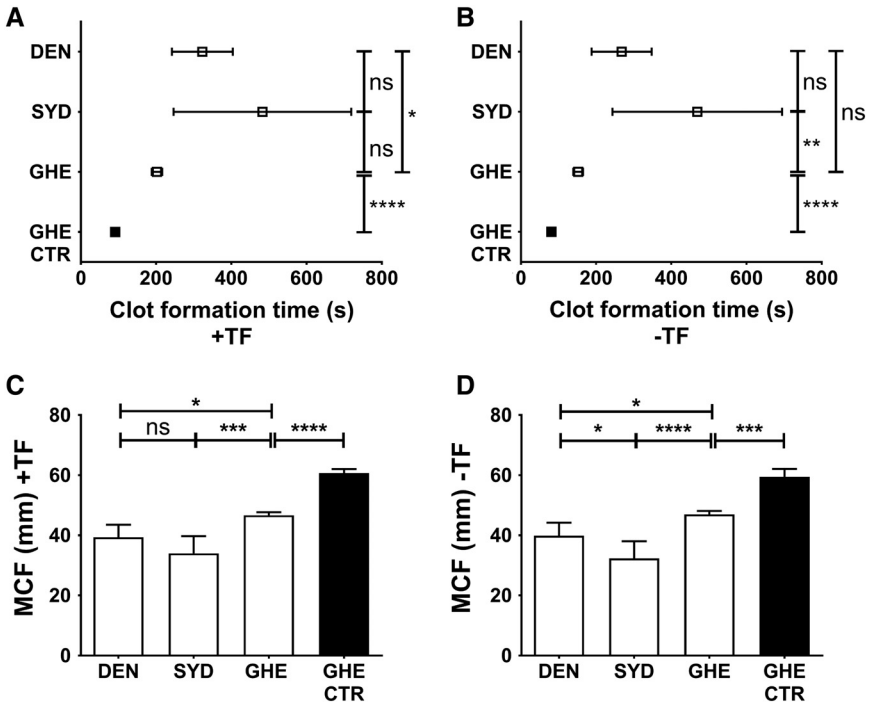

Fig. 7. Coagulation propagation in static conditions. Blood was reconstituted with cryopreserved (open symbols) or control PLTs (closed symbols) and studied in the presence of Ca^{2+} by ROTEM using (A, C) TF or (B, D) ellagic acid to initiate coagulation. (A, B) The clot formation time (sec) was measured between the clotting time (Figs. 5A and 5B) and the moment a clot firmness of 20 mm has been reached. (C, D) Maximum clot firmness (MCF) reflects the absolute strength of the clot. Comparison was between DEN, SYD, and GHE (all open symbols). A separate paired comparison between GHE and control noncryoplatelets was also performed (GHE CTR, closed symbols). Data are shown as mean ± SD (n = 6). Statistical analysis results are shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, or not significant (ns).

Cryopreservation-induced damage was less prominent in assays that focus on coagulation. In ROTEM, cryoplatelets had more rapid clotting times compared to controls even though clot formation times and maximum clot firmness were decreased. Further, in terms of thrombin generation, cryoplatelets demonstrated faster clotting times than controls and higher peak thrombin, all in a dose-dependent manner. Johnson and colleagues[19,35] had previously shown that the presence of cryoplatelet supernatant shortens time to coagulation presumably by the activity of microparticles, but these experiments were conducted in static conditions, allowing coagulation factors to diffuse freely in bulk. In this case, the cascade can quickly assemble tenase and prothrombinase complexes on the abundant negatively charged phospholipid surface provided by damaged cryoplatelets and microparticles. Under shear flow, factor availability depends on convective hydrodynamic forces rather than diffusion.[36] Our data confirm, however, that also under shear flow, coagulation is not affected by cryopreservation of PLTs. In contrast, contact-induced coagulation initiation in the absence of TF was even shorter than control. This is remarkable because with or without TF, significantly fewer PLTs were adhering to the surface during perfusion. Clinical trials are required to investigate whether the procoagulant role of cryoplatelets is sufficient to stop bleeding in patients.

The effect of cryopreservation for SYD cryoplatelets was greater than that for the other manufacturing sites particularly in PLT assays. The GPIbα-dim subpopulation was more abundant than in the other sites, the aggregation response was lower, integrin activation was less, and PLT adhesion under shear was the lowest with SYD cryoplatelets. Despite this, the differences between production sites were less obvious in PLT-based coagulation assays. It is not clear why SYD PLTs were particularly more damaged than those from the other sites. SYD and GHE PLTs were resuspended the same way with similar plasma so differences in reconstitution procedure are not likely. Irregularities in deep-freeze conditions could be one of the reasons, but differences in primary product preparation are also possible. The SYD PLTs were collected by apheresis in plasma, while GHE PLTs were prepared by pooling of buffy coats in plasma with SSP+. The SYD PLTs were frozen for 2 years, which is the maximum reported shelf-life.[37] Although preliminary data suggest that cryoplatelets stored for 4 years are equivalent to those stored for 2 years,[38] the effect of extended storage on the GPIbα subpopulation and PLT function have not been reported. The SYD PLTs also had the highest initial PLT content in comparison to the other sites, which effectively reduces the amount of DMSO per PLT unit. These variables might have contributed to the observations.

We conclude that cryoplatelets are less adhesive in shear flow conditions, in proportion to a subset of damaged GPIbα-dim PLTs, which decrease the effective PLT dose. The size of the affected subpopulation may be indicative of the extent of damage. Different production sites may have different cryoplatelet qualities especially in PLT function assays. Finally, even though fewer cryoplatelets bind collagen under flow, these nonetheless enhance contact activation, provide sufficient support for TF activation and normal coagulation under shear flow.

ACKNOWLEDGMENTS

The authors thank Michael Luypaert and Esha Wauters for their technical support in performing ROTEM analyses.

AUTHOR CONTRIBUTIONS

KS, VC, LJ, LJD, DCM, and HBF designed research, provided cryopreserved platelet products, and performed quality control experiments; KS and WD performed experiments; KD was responsible for the thromboelastometry assays; HBF and KS wrote the manuscript; VC, LJ, LJD, DCM, and HBF supervised the study; and all authors critically reviewed and amended the manuscript.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

REFERENCES

1. Desborough MJ, Smethurst PA, Estcourt LJ, et al. Alternatives to allogeneic platelet transfusion. Br J Haematol 2016;175: 381-92.
2. Liumbruno G, Bennardello F, Lattanzio A, et al. Recommendations for the transfusion of plasma and platelets. Blood Transfus 2009;7:132-50.
3. Murphy S, Gardner FH. Effect of storage temperature on maintenance of platelet viability—deleterious effect of refrigerated storage. N Engl J Med 1969;280:1094-8.
4. Johnson L, Tan S, Wood B, et al. Refrigeration and cryopreservation of platelets differentially affect platelet metabolism and function: a comparison with conventional platelet storage conditions. Transfusion 2016;56:1807-18.
5. Valeri CR, Feingold H, Marchionni LD. A simple method for freezing human platelets using 6% dimethylsulfoxide and storage at −80°C. Blood 1974;43:131-6.
6. Cap AP. Platelet storage: a license to chill! Transfusion 2016;56: 13-6.
7. Reddoch KM, Pidcoke HF, Montgomery RK, et al. Hemostatic function of apheresis platelets stored at 4°C and 22°C. Shock 2014;41(Suppl 1):54-61.
8. Hess JR, Lelkens CC, Holcomb JB, et al. Advances in military, field, and austere transfusion medicine in the last decade. Transfus Apher Sci 2013;49:380-6.
9. Balduini CL, Mazzucco M, Sinigaglia F, et al. Cryopreservation of human platelets using dimethyl sulfoxide and glycerol-glucose: effects on "in vitro" platelet function. Haematologica 1993;78:101-4.
10. Nie Y, de Pablo JJ, Palecek SP. Platelet cryopreservation using a trehalose and phosphate formulation. Biotechnol Bioeng 2005; 92:79-90.
11. Taylor MA. Cryopreservation of platelets: an in-vitro comparison of four methods. J Clin Pathol 1981;34:71-5.
12. Valeri CR, Ragno G, Khuri S. Freezing human platelets with 6 percent dimethyl sulfoxide with removal of the supernatant solution before freezing and storage at −80 degrees C without postthaw processing. Transfusion 2005;45:1890-8.
13. Spector JI, Yarmala JA, Marchionni LD, et al. Viability and function of platelets frozen at 2 to 3 C per minute with 4 or 5 per cent DMSO and stored at −80 C for 8 months. Transfusion 1977;17:8-15.
14. Dumont LJ, Cancelas JA, Dumont DF, et al. A randomized controlled trial evaluating recovery and survival of 6% dimethyl sulfoxide-frozen autologous platelets in healthy volunteers. Transfusion 2013;53:128-37.
15. Johnson LN, Winter KM, Reid S, et al. Cryopreservation of buffy-coat-derived platelet concentrates in dimethyl sulfoxide and platelet additive solution. Cryobiology 2011;62:100-6.
16. Valeri CR, Macgregor H, Ragno G. Correlation between in vitro aggregation and thromboxane A2 production in fresh, liquid-preserved, and cryopreserved human platelets: effect of agonists, pH, and plasma and saline resuspension. Transfusion 2005;45:596-603.
17. Barnard MR, MacGregor H, Ragno G, et al. Fresh, liquid-preserved, and cryopreserved platelets: adhesive surface receptors and membrane procoagulant activity. Transfusion 1999;39:880-8.
18. Waters L, Padula MP, Marks DC, et al. Cryopreserved platelets demonstrate reduced activation responses and impaired signaling after agonist stimulation. Transfusion 2017;57:2845-57.
19. Johnson L, Coorey CP, Marks DC. The hemostatic activity of cryopreserved platelets is mediated by phosphatidylserine-expressing platelets and platelet microparticles. Transfusion 2014;54:1917-26.
20. Cid J, Escolar G, Galan A, et al. In vitro evaluation of the hemostatic effectiveness of cryopreserved platelets. Transfusion 2016;56:580-6.
21. Johnson L, Reade MC, Hyland RA, et al. In vitro comparison of cryopreserved and liquid platelets: potential clinical implications. Transfusion 2015;55:838-47.
22. Van Aelst B, Feys HB, Devloo R, et al. Microfluidic flow chambers using reconstituted blood to model hemostasis and platelet transfusion in vitro. J Vis Exp 2016;109:e53823.
23. Six KR, Devloo R, Van Aelst B, et al. A microfluidic flow chamber model for platelet transfusion and hemostasis measures platelet deposition and fibrin formation in real-time. J Vis Exp 2017;120:e55351.

24. Hemker HC, Kremers R. Data management in thrombin generation. Thromb Res 2013;131:3-11.
25. Chitlur M, Sorensen B, Rivard GE, et al. Standardization of thromboelastography: a report from the TEG-ROTEM working group. Haemophilia 2011;17:532-7.
26. Iwatani M, Ikegami K, Kremenska Y, et al. Dimethyl sulfoxide has an impact on epigenetic profile in mouse embryoid body. Stem Cells 2006;24:2549-56.
27. Slichter SJ, Dumont LJ, Cancelas JA, et al. Treatment of bleeding in severely thrombocytopenic patients with transfusion of dimethyl sulfoxide (DMSO) cryopreserved platelets (CPP) is safe - report of a phase 1 dose escalation safety trial. Blood 2016;128:1030.
28. Daly PA, Schiffer CA, Aisner J, et al. Successful transfusion of platelets cryopreserved for more than 3 years. Blood 1979;54: 1023-7.
29. Towell BL, Levine SP, Knight WA 3rd, et al. A comparison of frozen and fresh platelet concentrates in the support of thrombocytopenic patients. Transfusion 1986;26:525-30.
30. van Zanten GH, Heijnen HF, Wu Y, et al. A fifty percent reduction of platelet surface glycoprotein Ib does not affect platelet adhesion under flow conditions. Blood 1998;91: 2353-9.
31. Cauwenberghs N, Ajzenberg N, Vauterin S, et al. Characterization of murine anti-glycoprotein Ib monoclonal antibodies that differentiate between shear-induced and ristocetin/botrocetin-induced glycoprotein Ib-von Willebrand factor interaction. Haemostasis 2000;30:139-48.
32. Kageyama S, Matsushita J, Yamamoto H. Effect of a humanized monoclonal antibody to von Willebrand factor in a canine model of coronary arterial thrombosis. Eur J Pharmacol 2002; 443:143-9.
33. Hoffmeister KM, Felbinger TW, Falet H, et al. The clearance mechanism of chilled blood platelets. Cell 2003;112: 87-97.
34. Chen W, Liang X, Syed AK, et al. Inhibiting GPIbalpha shedding preserves post-transfusion recovery and hemostatic function of platelets after prolonged storage. Arterioscler Thromb Vasc Biol 2016;36:1821-8.
35. Raynel S, Padula MP, Marks DC, et al. Cryopreservation alters the membrane and cytoskeletal protein profile of platelet microparticles. Transfusion 2015;55:2422-32.
36. Zhu S, Lu Y, Sinno T, et al. Dynamics of thrombin generation and flux from clots during whole human blood flow over collagen/tissue factor surfaces. J Biol Chem 2016;291: 23027-35.
37. Valeri CR, Srey R, Lane JP, et al. Effect of WBC reduction and storage temperature on PLTs frozen with 6 percent DMSO for as long as 3 years. Transfusion 2003;43:1162-7.
38. Noorman F, Strelitski R, Badloe J. Frozen platelets can be stored for 4 years at −80°C without affecting in vitro recovery, morphology, receptor expression, or coagulation profile. Transfusion 2014;54:74A.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Fig. S1 Platelet subpopulations by GPIbα expression. GPIbα expression was determined by flow cytometry on 10,000 platelets. Isotype negative control (blue) was compared to (A) non cryopreserved control platelets of GHE-CTR (red) and to all GHE cryopreserved platelets (black). Three different GHE products are shown in panels B to D. The dotted line represents the arbitrary gate determined for dim (left) and bright (right) GPIbα subpopulations. The histograms were normalized to display relative count.

Fig. S2 Annexin V binding and microparticles. (A) The percentage of GPIbα positive events binding annexin V. (B) Microparticles were defined as events smaller than 0.9 μm. The relative number of microparticles to the total number of GPIbα positive events is depicted. Comparison was between cryopreserved platelets from Denver, CO (DEN), Sydney (Australia, SYD) and Ghent (Belgium, GHE) (open bars). A separate paired comparison was between cryopreserved platelets (GHE) and control non-cryopreserved platelets (GHE CTR, filled bars). Data are shown as mean ± SD (n = 6). Statistical analysis results are shown as ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ or not significant (ns).

Fig. S3 Scatter properties of cryopreserved platelets. The median intensity (MI) of the forward scattered light (FSC) in flow cytometry for subpopulations of cryoplatelets (open bars) or control platelets (closed bars) based on the GPIbα signal as shown in Fig. S1. All samples were from the Belgian (GHE) site. Data are shown as mean ± SD (n = 6). Statistical analysis results are shown as ** $P < 0.01$ or not significant (ns)

VIDEO. S1 ADHESION_MOVIE.

VIDEO. S2 COAGULATION_CG+TF_MOVIE.

VIDEO. S3 COAGULATION_CG_MOVIE.