Effects of storage time prolongation on in vivo and in vitro characteristics of 4°C–stored platelets

Moritz Stolla,[1,2] *S. Lawrence Bailey*,[1] *Lydia Fang*,[1] *Lynda Fitzpatrick*,[1] *Irena Gettinger*,[1] *Esther Pellham*,[1] *and Todd Christoffel*[1]

BACKGROUND: Cold (4°C)-stored platelets are currently under investigation for transfusion in bleeding patients. It is currently unknown how long cold-stored platelets can be stored for clinical applications.

STUDY DESIGN AND METHODS: Twenty three subjects were recruited. Twenty-one subjects were available for in vivo assessment and received indium-111 radiolabeled, cold-stored platelets. We investigated 5- (n = 5), 10- (n = 6), 15- (n = 5), and 20-day–stored (n = 5) platelets and obtained samples for in vitro testing at baseline and after the designated storage time. Twenty three units were available for in vitro testing. Five- and 7-day (n = 5 each), room temperature (RT)-stored platelets served as the current clinical standard control.

RESULTS: In vivo, we found a continuous decline in platelet recovery from 5 to 20 days. Platelet survival reached a low nadir after 10 days of storage. Ex vivo, we observed the maximum platelet αIIbβ3 integrin response to collagen at 5 days of cold storage, and we saw a continuous decline thereafter. However, platelet integrin activation and mitochondrial membrane integrity were better preserved after 20 days at 4°C, compared to 5 days at RT. Platelet metabolic parameters suggest comparable results between 20-day cold-stored platelets and 5- or 7-day RT-stored platelets.

CONCLUSION: In summary, we performed the first studies with extended, cold-stored, apheresis platelets in plasma for up to 20 days with a fresh comparator. Storing cold-stored platelets up to 20 days yields better results in vitro, but further studies in actively bleeding patients are needed to determine the best compromise between hemostatic efficacy and storage prolongation.

Platelet transfusions can be lifesaving by facilitating hemostasis in trauma and surgical patients and can be used for bleeding prophylaxis in thrombocytopenic patients with cancer. Platelets for transfusion are currently collected from volunteer donors and stored at room temperature (RT; 22 ± 2°C). RT storage maximizes platelet recovery and survival in transfused recipients[1]; however, it also increases the opportunity for bacterial growth in the platelet bag. The US Food and Drug Administration (FDA) limits the shelf life of platelets to 5 days to reduce this bacterial risk. Some laboratories can extend the shelf life with an additional bacterial point-of-care test to 7 days.[2] The limited storage time leads to outdates, wastage, and shortages, especially in rural areas in the United States and far forward military operations. Recently released, refined guidelines by the FDA increase the requirement for bacterial testing or recommend pathogen reduction, and this highlights the magnitude of the problem for the transfusion medicine community.[3] The new guidelines will make the maintenance of an RT-stored platelet inventory more costly and labor intensive. Nevertheless, transfusion-associated sepsis from platelet transfusion remains the most common transfusion-transmitted infection and one of the principal lethal risks associated with transfusion.[2]

ABBREVIATIONS: CCCP = carbonyl cyanide m-chlorophenyl hydrazine; PS = phosphatidyl serine; RT = room temperature; BCL-2 and BCL-xl = B-cell lymphoma–2 and B-cell lymphoma–extra large.

From the [1]Platelet Transfusion Research Laboratory, Bloodworks Northwest Research Institute and [2]Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, Washington.

Address reprint requests to: Moritz Stolla, Bloodworks Northwest Research Institute 1551 Eastlake Ave, Suite 100, Seattle, WA 98102; Email: mstolla@bloodworksnw.org

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Cold storage of platelets could alleviate these issues because of very limited bacterial growth at 4°C.[4] Unfortunately, cold storage of platelets is known to reduce platelet circulatory life span to 1 to 2 days compared to an average life span of 4 to 5 days after 22°C storage.[1] Previous reports suggested superior in vitro performance of platelets stored at 4°C.[5,6] However, earlier studies about bleeding time corrections after transfusion of cold-stored platelets in patients with thrombocytopenia and healthy human subjects on antiplatelet drugs yielded contradictory results.[5,7–9] Besides, they were performed in the 1960s and 1970s and require confirmation in contemporary platelet transfusion settings, including clinical trials.[5,7–9] Other unresolved issues around 4°C storage include determination of the maximum storage time, evaluation of appropriate platelet quality markers, and acceptable storage media. Blood banks in the United States can currently apply for an FDA variance, which allows for 3-day cold storage in plasma for actively bleeding patients, based on a historic 3-day storage time limit.

To validate 20-day cold- and plasma-stored platelets as a transfusion product, we stored platelets in plasma 5, 10, 15, and 20 days at 4°C and included a fresh and 5- or 7-day RT comparator. We had previously published results on 3-day cold-stored platelets and compared them to platelets stored at 4°C for 10 and 15 days, respectively.[10] This study also suggested that there could be a benefit to plasma as storage medium.[10] Based on the 5- to 7-day storage limit for RT-stored platelets, we decided to store platelets in the 4°C test group for 5 days and increase the storage time up to 20 days to exceed our longest previous storage time. Moreover, other groups have published on in vitro performance of 21-day cold-stored platelets in the past and suggested acceptable in vitro performance.[11,12]

Our study provides essential guidance for future clinical trials. We subjected platelets to several state-of-the-art tests for platelet in vitro and in vivo quality and function. We also included tests to assess mitochondrial health and apoptosis to understand changes in platelet biology during storage at 4°C better and to characterize the cold lesion further. Twenty-day storage significantly extends the current platelet shelf life for RT-stored platelets. Our in vitro data suggest that 20-day cold-stored platelets are not inferior and could even be superior in trauma or surgery patients compared with RT-stored platelets, although this has not been specifically addressed in this study and requires further confirmation. To our knowledge, this is the first-in-human study to investigate the storage and in vivo characteristics of extended-storage cold-stored platelets in plasma up to 20 days.

MATERIALS AND METHODS

Preparation of test platelets

A standard single apheresis platelet unit (target platelet yield 3.0×10^{11}/unit) was collected from 23 healthy subjects with an automated blood collection system (Trima Accel, Terumo BCT) and stored in 100% plasma.

Only 21 of 23 subjects were available for in vivo assessment (5- [n = 5]; 10- [n = 6]; 15- [n = 5]; and 20-day–stored [n = 5] platelets) because two subjects did not return for autologous transfusion. However, in vitro results for all 23 subjects are available (5- [n = 5]; 10- [n = 7]; 15 [n = 5]; and 20-day–stored [n = 6] platelets).

Each unit achieved a final platelet concentration of $700–2100 \times 10^3$ platelets/μL, as per allowable bag parameters. We stored the units for up to 20 days at 4°C. Cold-stored units were stored without agitation. On the day of testing and transfusion, the units were allowed to equilibrate to RT for 1 hour before we took a sample for radiolabeling or in vitro testing from the unit. Units were inverted but not otherwise manipulated physically (e.g., massaged or shaken).

Preparation of control platelets

Control units were collected by apheresis in the same fashion as described above and stored in plasma (n = 5) for 7 days. We performed tests for microparticles, annexin V, glucose, lactate, and platelet yield with this control group. The original study included a dedicated 5-day RT-control group for the following tests: PAC-1 (n = 8), JC-1 (n = 7), and caspase 3,7 (n = 4). RT-stored bags were agitated as per standard requirement for clinically used units and also otherwise treated as clinically used standard-of-care platelet units.

Radiolabeling of stored platelets

We radiolabeled platelets as previously described, following the detailed Biomedical Excellence for Safer Transfusion (BEST) collaborative protocol.[13] In brief, 111-Indium Oxine (In-111, Anazao) was used to label both control and test platelets because, per our preliminary experiments, the other available isotope, chromium-51, demonstrated very poor uptake by cold-stored platelets (data not shown). Because of the short circulating times of cold-stored platelets, we considered reuse of the same isotope to measure both control and test cold-stored platelets feasible. Additionally, we collected a preinfusion radioactivity sample before the fresh (control) transfusion to account for any possible residual In-111 activity after each subject's control transfusion and adjusted our calculations accordingly.

At the end of the storage period, the subject returned to receive an In-111 radiolabeled aliquot of their 4°C stored platelets. Follow-up samples from the subject were collected approximately 2 hours after transfusion (recovery), two times on Day 1 (2-10 hours apart), and two times on Days 2 and 3 (each 2-10 hours apart), to calculate recovery and survival of the subject's 4°C–stored platelets.

The 7-day in vivo radiolabeling data were previously performed and kindly provided by Dr. Sherrill Slichter and were not previously published. It includes platelets obtained in the same fashion (apheresis platelets) and radiolabeled with the same isotope (indium-111). The 7-day RT time point is a common control group for many studies. Given the

potential risk of bacterial contamination and septic reactions for healthy human subjects, we consider it ethically irresponsible to repeat this group as long as the collection, storage, and test parameters do not change.

In vitro platelet measurements

Platelet counts of collected products were performed on the day of the collection after 2 hours of resting time to allow platelet disaggregation that might have occurred during collection and at the end of storage using an hematology analyzer (ABX Diagnostics). We performed all other in vitro tests for the "fresh" time point on the day of collection, after a 2 hour resting period. We calculated platelet yields by multiplying the platelet count by the volume of the platelet unit. At Day 0 (2 hours after collection) and after storage, in vitro measurements of glucose and lactate concentration were measured with a commercially available blood gas instrument (ABL Flex 805, Radiometer Medical). P-selectin expression, microparticle quantification, and annexin V binding were performed by flow cytometry (FACSCalibur, Beckman Coulter) as previously described.[14] We used the following antibodies: P-Selectin CD62P-FITC (BD Biosciences), GPIbα-PE (BD Biosciences), and Annexin V-PE (BD Biosciences). PAC-1 (BD Pharmingen) binding was tested by flow cytometry as previously described[15] at baseline and after stimulation with 20 μg/mL collagen (Chrono-Log) for both fresh and stored units. Caspase 3,7 activation was measured as previously described.[16] In brief, a commercially available reagent (CellEvent Caspase-3/7 Green Detection, Thermo Fisher Scientific) was incubated with platelets with or without ABT 737 and subsequently read on a flow cytometer for Fluorescence-1 (FL-1). For mitochondrial membrane potential measurements, we used the commercially available JC-1 dye (Invitrogen) as previously described.[17] In brief, JC-1 is a positively charged probe that accumulates in the negatively charged internal environment of healthy mitochondria and forms red fluorescent J-aggregates if concentrated. Upon depolarization, the J-aggregates dissipate, and green fluorescence can be detected. The FL2/FL1 ratio, therefore, helps to determine if the dye is concentrated in intact mitochondria or leaking into the cytoplasm.

Healthy human subjects research

The Western Institutional Review Board approved the research, and all human participants gave written informed consent. The clinical trial registration number for this study was NCT02754414 and the entry name Cold Apheresis Platelets in PAS (CAPP). The study was conducted following the Declaration of Helsinki.

Statistical analysis

We reported the results are reported as mean ± standard error of the mean, and statistical significance was assessed by unpaired, two-tailed Student t test unless otherwise indicated. A p value of 0.05 or less was considered significant.

RESULTS

Total platelet yield after storage

We observed a significantly lower platelet yield for platelets stored for 5 days at 4°C compared to 7-day RT storage (75 ± 2% vs. 98 ± 1%, respectively, p = 0.003) (Fig. 1). No significant differences were observed between 5 to 10 days, 10 to 15 days, and 15 to 20 days (Fig. 1). The difference between 7-day RT-stored platelets and 20-day 4°C–stored platelets remained statistically significant (75 ± 4% vs. 98 ± 1%, p = 0.0012).

In vivo platelet viability

Calculated as percentage of fresh, the recoveries of all time points were significantly lower compared to the respective previous steps (46 ± 3% vs. 31 ± 2%, p = 0.003; 31 ± 2% vs. 22 ± 2%, p = 0.009; 22 ± 2% vs. 13 ± 1%, p = 0.008), and the recovery of 7-day RT-stored platelets was significantly higher compared to 5-day cold-stored platelets (70 ± 7% vs. 46 ± 3%, p = 0.017) (Fig. 2A).

Platelet radiolabeling studies also showed a significantly lower survival of cold-stored platelets compared to RT-stored platelets. Interestingly, the maximum drop occurred early (between 0 and 10 days of storage), and after 10 days a low nadir was reached. The percentage of prestorage analysis showed significant differences between 7-day RT and 5-day cold (51 ± 8% vs. 20 ± 3%, p < 0.0001) and 5-day cold and 10-day cold (20 ± 3% vs. 8 ± 1%, p = 0.0014), but no significant decrease thereafter (Fig. 2 B,C).

Platelet metabolism markers

Glucose levels were significantly lower in RT-stored samples when compared to 5-day cold-stored samples (65 ± 2%

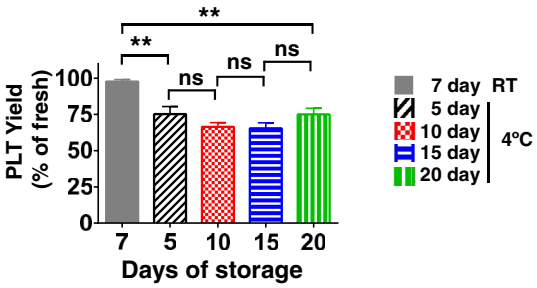

Fig. 1. In vitro platelet yields: (Total Platelet Yield in the Component [Platelet Count × Component Volume = Platelet Yield]) Platelets were stored in plasma for either 7 days at 22°C (solid gray bars), 5-day 4°C–stored (white with black diagonal stripes), 10-day 4°C–stored (red with white squares), 15-day 4°C–stored (blue with horizontal white stripes), 20-day 4°C–stored (green with vertical white stripes). Results are shown as the percentage of the respective fresh sample. ns = not significant; p < 0.01, n = 5 for RT samples, n = 5-7 for cold-stored groups. [Color figure can be viewed at wileyonlinelibrary.com]**

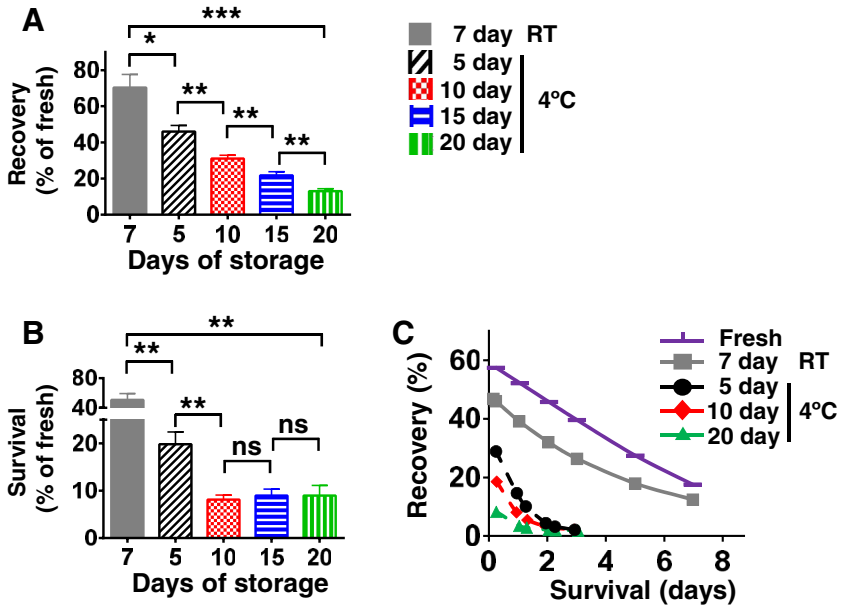

Fig. 2. In vivo platelet characteristics: Healthy human subjects received autologous radiolabeled platelets either fresh or after storage at 4°C or RT (22°C). Seven-day RT-stored (gray bars), 5-day 4°C–stored (white with black diagonal stripes), 10-day 4°C–stored (red with white squares), 15-day 4°C–stored (blue with horizontal white stripes), 20-day 4°C–stored (green with vertical white stripes). (A) Platelet recovery shown as percentage of the subject's fresh autologous radiolabeled platelets. (B) Survival shown as percentage of the subject's fresh autologous radiolabeled control platelets. (C) Representative traces taken from radiolabeling data (multiple-hit model). Fresh (purple line with horizontal intersections), 7-day RT-stored (gray line with squares), 5-day 4°C–stored (dotted, black line with circles), 10-day 4°C–stored (dotted, red line with diamonds), 20-day 4°C–stored (dotted, green line with triangles). *p < 0.05, **p < 0.01,*p < 0.001; ns = not significant, n = 5 for RT and n = 5-6 for all other groups. [Color figure can be viewed at wileyonlinelibrary.com]**

vs. 98 ± 2%, p < 0.0001) (Fig. 3A). In contrast, there was a stepwise decrease in cold-stored platelets from 5 to 20 days of storage, which was significant between 5 and 10 days, and 10 and 15 days (98 ± 2% vs. 90 ± 1%, p = 0.007; and 90 ± 1% vs. 83 ± 2%, p = 0.003 respectively) (Fig. 3A). We did not observe any significant differences between 20-day 4°C–stored platelets and 7-day RT-stored platelets. Correspondingly, the lactate concentration was significantly higher at RT than 5-day cold-stored platelets (830 ± 134% vs. 292 ± 26%, p = 0.003), and we saw a stepwise, significant increase from 5 to 10 days (292 ± 26% vs. 473 ± 41%). After 10 days, no significant changes were observed in the step-by-step analysis. Similar to the glucose analysis, we did not observe significant differences between 7-day RT-stored platelets and the last time point of the 4°C storage time period (20 days) (Fig. 3B).

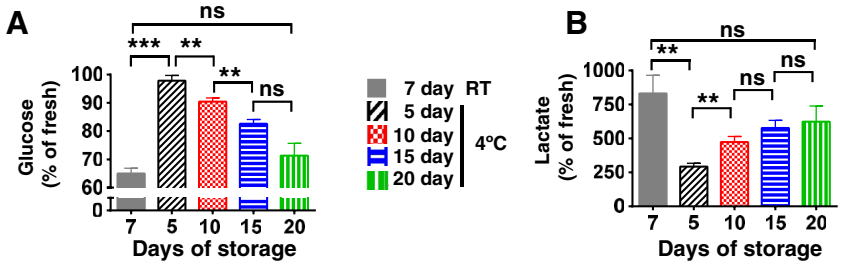

Fig. 3. In vitro platelet metabolism parameters: Glucose and lactate levels measured by blood gas analyzer. Seven-day RT-stored (gray bars), 5-day 4°C–stored (white with black diagonal stripes), 10-day 4°C–stored (red with white squares), 15-day 4°C–stored (blue with horizontal white stripes), 20-day 4°C–stored (green with vertical white stripes). (A) Glucose levels shown as percentage of corresponding fresh samples. (B) Lactate levels shown as percentage of corresponding fresh samples. **p < 0.01, ***p < 0.001; ns = not significant, n = 5 for all room temperature-stored samples, n = 5-7 for all cold-stored samples. [Color figure can be viewed at wileyonlinelibrary.com]

Platelet quality markers in vitro

Platelet microparticle release is a marker for platelet activation and increases with storage time in platelet products.[18] We found no significant differences between 7-day RT-stored and 5-day, 4°C–stored platelets (Fig. 4A). There was a stepwise increase in microparticles in cold-stored platelets from 5 to 20 days, which was significant when 20 days were compared to 7 days at RT (2326 ± 567% vs. 235 ± 32%, p = 0.0095).

Platelet α-degranulation is a hallmark of the platelet storage lesion. We tested for P-selectin expression over time in the storage bag (without agonist stimulation). We found a trend for higher P-selectin expression after 7 days compared to 5 days of cold storage (315 ± 151% vs. 1013 ± 368%, p = 0.119). When platelets were stored at 4°C, there was a continuous increase in degranulation, and by Day 20, the level was similar and remained not significantly different compared to 7-day RT-stored platelets (841 ± 215% vs. 1013 ± 368%, p = 0.685) (Fig. 4B).

Platelet αIIbβ3 integrin activation requires inside-out signaling and is critical for platelet participation in hemostasis at the site of vascular injury. Therefore, we tested platelet activation in response to collagen, an extracellular matrix protein that can cause platelet adhesion under flow and platelet aggregation by activating the immunoreceptor tyrosine-based activation motif receptor glycoprotein VI and the αIIβ1 integrin.[19] Platelet αIIbβ3 integrin activation was measured with the PAC-1 antibody, which specifically binds the activated conformation of the integrin.[20]

All samples responded appropriately to collagen before storage (fresh) (Fig. S1A, available as supporting information in the online version of this paper). We found a trend for higher PAC-1 binding levels in cold-stored platelets at all time points, but due to the high variability of this assay we found statistical significance only between 15-day cold-stored platelets compared to 5-day RT-stored platelets in the percentage of fresh analysis (26.7 ± 6.6 vs. 4.4 ± 0.3, p = 0.018) (Fig. 4C).

To see how apoptosis events compare between cold storage and RT storage we tested for mitochondrial membrane integrity, caspase 3,7 activation, and phophatidyl serine exposure. Mitochondrial membrane integrity was assessed by flow cytometry using the JC-1 dye. When the dye is highly concentrated in mitochondria, it forms J-aggregates with an emission maximum at approximately 590 nm (FL2); when more diluted in cytoplasm, the dye emits at approximately 530 nm (FL1). We used the proton gradient uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP) as positive control, to show successful disruption of the electron transport chain. All fresh samples showed a significant drop of the FL2/FL1 ratio after CCCP was added to the sample (Fig. S1B, available as supporting information in the online version of this paper). When testing stored samples, we found a markedly reduced FL2/FL1 ratio in 5-day RT-stored platelets, compared to cold-stored platelets at 5 days (2 ± 1% vs. 184 ± 62%, p = 0.009)

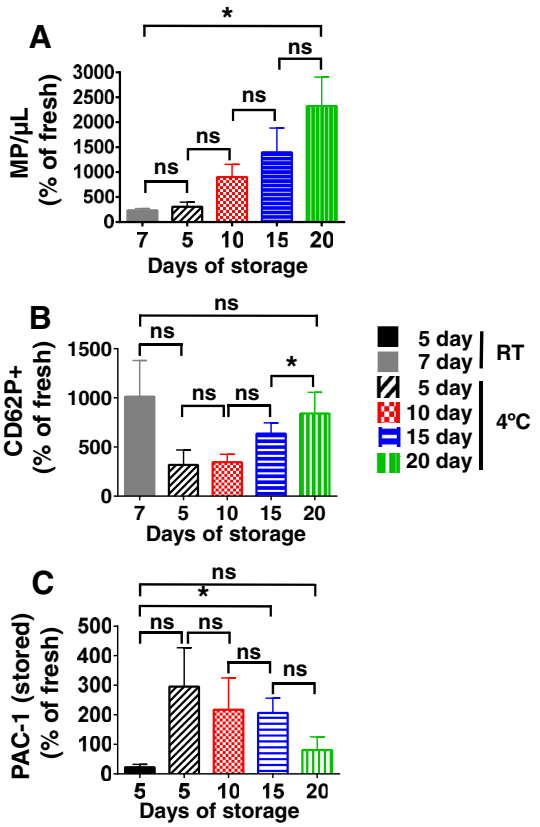

Fig. 4. In vitro platelet activation parameters: Five- or 7-day RT-stored platelets (black and gray bars respectively), 5-day, 4°C–stored (white with black diagonal stripes), 10-day 4°C–stored (red with white squares), 15-day 4°C–stored (blue with horizontal white stripes), 20-day 4°C–stored (green with vertical white stripes). (A) Microparticles (MP) measured by flow cytometry as percentage of fresh samples. Microparticles were measured without addition of an agonist. (B) Alpha-granule secretion measured by P-selectin exposure by flow cytometry (shown as percentage of corresponding fresh samples). P-selectin was measured without addition of an agonist. (C) Platelet αIIbβ3 integrin activation was measured by PAC-1 antibody binding. PAC-1 antibody binding was measured after stimulation with the agonist collagen at 20 µg/mL. *p < 0.05; ns = not significant, n = 8 for 5 day room temperature-stored samples, n = 5 for the 7 day room temperature-stored samples, n = 5-7 for all cold-stored samples. [Color figure can be viewed at wileyonlinelibrary.com]

(Fig. 5A). Surprisingly, this difference remained significant for the maximum storage time tested, suggesting a significant advantage of cold storage for mitochondrial preservation (2 ± 1% vs. 55 ± 16%, p = 0.004) (Fig. 5A). During apoptosis, mitochondrial membrane disruption is followed by caspase 3,7 activation.[21] This sequence was not found in 5-day RT or cold-stored platelets at any time point. While there was a trend for higher caspase 3,7 activation in 10 and 15 days of

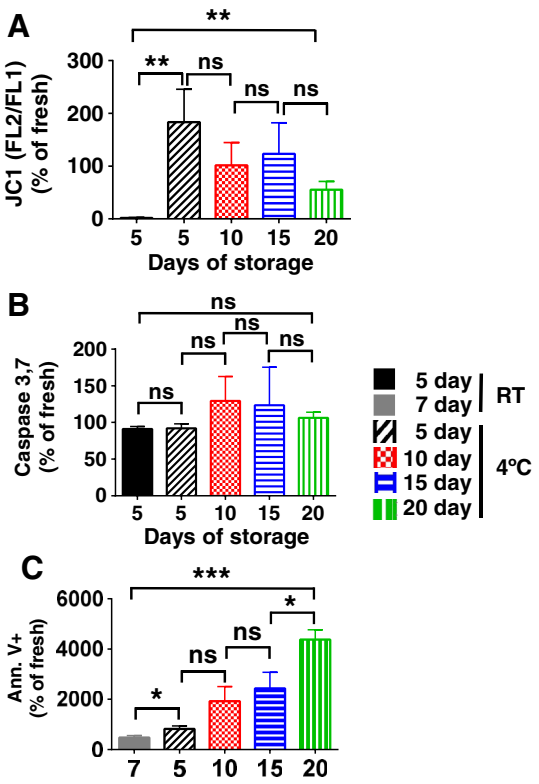

Fig. 5. In vitro platelet apoptosis parameters: Platelet mitochondrial membrane potential measured as JC-1 red (FL2) to green (FL-1) ratio. Five-day 4°C–stored (white with black diagonal stripes), 10-day 4°C–stored (red with white squares), 15-day 4°C–stored (blue with horizontal white stripes), 20-day 4°C–stored (green with vertical white stripes). (A) JC-1 (FL2/FL1) stored baseline samples as percentage of the corresponding fresh baseline samples (pre-storage) shown as mean ± standard error of the mean. (B) Caspase 3,7 activation shown as percentage of prestorage sample. (C) Annexin V binding by flow cytometry as percentage of fresh samples. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns = not significant, n = 4-7 for 5-day RT-stored samples, n = 5 for the 7-day RT-stored samples, n = 5-7 for all cold-stored samples. [Color figure can be viewed at wileyonlinelibrary.com]

cold storage in the percentage of fresh analysis, no significance was observed for any time point between the groups (Fig. 5B). All fresh samples showed an appropriate increase in caspase 3,7 activation in response to the BCL-2 and BCL-xl inhibitor ABT 737, indicating the general capacity to undergo apoptosis (positive control) (Fig. S1B, available as supporting information in the online version of this paper). Phosphatidyl serine (PS) exposure can be induced by apoptosis or activation in platelets.[22] To test for PS exposure we stained samples with annexin V without agonist for activation or apoptosis. We found significantly more annexin V binding in 5-day cold-stored platelets compared to 7-day RT-stored platelets. Cold-stored platelets showed higher PS exposure at each time point, and this was significant when 5-day 4°C storage was compared to 7-day RT storage (817 ± 118% vs. 478 ± 76%, $p = 0.043$), 15 days were compared to 20 days (2431 ± 646% vs. 4376 ± 390%, $p = 0.025$), and 20 days to 7 days at RT (4376 ± 390% vs. 478 ± 76%, $p < 0.0001$).

For this study, we did not systematically address the occurrence of micro- and macroaggregates in cold-stored units because aggregates did not impair our ability to conduct this study. Overall, over 20 units were included in this study and only one unit had to be discarded because of a large proteinaceous aggregate that had formed over storage time. This large aggregate was considered unlikely to be related to 4°C storage.

DISCUSSION

The present study was based on previous observations suggesting that cold-stored platelets stored in plasma have a higher recovery and survival compared to platelets stored in US-licensed platelet additive solutions.[10] The goal of this study was to characterize platelet in vivo and in vitro function at 4°C storage in plasma at 5, 10, 15, and 20 days and compare these data with a fresh sample and current clinical standards.

Our study has four major findings: 1) Platelet recovery continues to decline up to 20 days, and the trajectory suggests even further decline is possible after 20 days; 2) platelet survival is low after 5 days of 4°C storage and further decreases to 10 days but then reaches a low nadir; 3) platelet in vitro yield is significantly lower at 5-day 4°C storage, compared to 7-day 22°C and remains on the same level over 20 days; and 4) markers of platelet biology in vitro indicate that platelets stored for 20 days at 4°C are equivalent or better compared to 5-day RT platelets.

For licensing purposes, the FDA usually requires recovery of 66% and survival of 50% of a healthy human subject's fresh platelets for RT-stored platelet products.[23–25] Our data indicate that cold-stored platelets fail these criteria. However, these guidelines likely need to be revised for alternative platelet/hemostatic products, and this is currently under debate. The purpose of cold-stored platelets is to be transfused to trauma and surgical patients with active bleeding, and therefore platelet survival is not likely to be of high importance. However, because cold-stored platelets represent a hemostatic transfusion product, it is important to characterize its fate in vivo and thus how long we can expect a hemostatic effect. Platelet recovery is a valuable marker for this question because platelets need to circulate until hemostasis is achieved, and severe trauma cases and large surgeries with massive blood loss can take several hours. It is currently unclear how efficacious cold-stored platelets are for hemostasis in trauma and surgery patients and what the threshold levels are for recovery and survival, before this impairs hemostatic efficacy. If our in vitro

function tests indeed predict in vivo function, we project that even storage for 20 days at 4°C yields an efficacious product. Most previous studies were conducted in patients with thrombocytopenia or in healthy human subjects on aspirin.[5,7–9] A pilot trial was conducted in patients following cardiac surgery, and 14-day–stored platelets in additive solution were noninferior to RT-stored platelets.[26,27]

Our αIIbβ3 integrin activation data suggest that platelet activation after 15 days at 4°C is significantly better compared to 5-day RT-stored platelets and P-selectin and metabolism parameters are comparable between these time points and storage temperatures. These findings alleviate potential concerns about platelet quality and the cold-storage lesion. In fact, the maximum αIIbβ3 preactivation during cold storage was achieved at the earliest time point (5 days, data not shown). If there is a cold-priming effect of platelets that renders them more effective for in vivo hemostasis, it occurs early and there appears to be little change over time. The response to collagen decreased over storage time, suggesting that there are limits for platelet storage at 4°C. Other groups have shown better platelet in vitro function in cold-stored platelets including after extended storage times with various methods and assays.[5,6,28–32] In the current study, our goal was to display the in vivo results in parallel with the in vitro function results. Unfortunately, we did not have enough subjects in the study to perform meaningful correlation studies, but the decline in in vitro integrin activation, mitochondrial membrane potential, and glucose levels appears to correlate with the stepwise decrease in in vivo recovery, and negatively correlate with lactate values.

Our studies that used a mitochondrial marker and caspase activation show a peculiar pattern: While the mitochondrial membrane potential is clearly affected over time in cold-stored platelets and severely reduced in RT-stored platelets, this did not result in significant caspase activation. This suggests that early stages but not later stages of apoptosis are reached during RT or 4°C storage. An alternative explanation is that mitochondrial damage occurs independently of a canonical apoptosis pathway activation in platelets during storage. Mitochondrial membrane integrity correlated with αIIbβ3 activation response to collagen, suggesting a causal relationship between mitochondrial health and integrin activation. Our findings corroborate results by Bynum et al.,[29] who showed better mitochondrial preservation and a good correlation between platelet function and mitochondrial function in cold-stored platelets.[29]

However, our results contradict a study by Li et al.[33] that showed caspase 3 activation in RT-stored platelets after 7 days.[33] While Li et al. see activation already at 3 and 5 days, a different group reports that caspase activation during platelet storage is a relatively late event.[34] Therefore, it is possible that we did not store platelets long enough at both RT and 4°C to detect caspase activation. We confirmed successful caspase activation by ABT 737, and this indicates that the lack of response was not a problem with the assay itself or an intrinsic biologic inability to activate caspase after storage. Van der Wal et al.[35] showed caspase 9 activation after cold exposure for 4 hours and rewarming to 37°C for 1 hour, which are very different conditions compared to the conditions in our protocol.[35] In a recent study, Marini et al.[36] showed more PS exposure when platelets were stored at 4°C compared to 22°C after 7 days, and this was independent of the percentage of plasma in the storage unit.[36] It is difficult to come to conclusions about apoptosis in platelets by PS exposure alone, since there are activation- and apoptosis-specific pathways for PS exposure, and whether there is cross-talk between these pathways is unclear.[21,22] While the mitochondrial membrane integrity is better preserved at 4°C storage compared to RT storage, we observed a continuous decline in membrane integrity over 20 days. Mitochondria are critical not only for apoptosis but also for calcium-dependent platelet activation.[22,37] Taken together, our data suggest that the cold lesion in platelets triggers platelet activation similar to the response to agonists but does not lead to the activation of classical apoptosis effectors like caspase 3,7 activation.

We found a continuous decrease in glucose and increase in lactate over storage time, indicating that metabolism continues at 4°C even though it is markedly slowed when compared to RT storage. Interestingly, 20-day 4°C storage showed comparable glucose levels to 7-day 22°C storage. Our study corroborates data published by Braathen et al.[12] who showed much slower glucose metabolism and lower lactate levels in cold platelets. However, they stored platelets in additive solution and not in 100% plasma.[12] As described previously in the literature, the yield in cold-stored platelets in plasma is lower likely because of aggregate formation between plasma fibrinogen and preactivated αIIbβ3 integrins on platelets.[28] Our data suggest that the maximum aggregation happens early during storage and the platelet yield remained stable from 5 to 20 days.

Our study has limitations; most importantly, it is not a clinical trial and we cannot make definitive recommendations about hemostatic efficacy. While it is intuitive to suggest that platelet integrin activation correlates with hemostatic efficacy in vivo, this has yet to be proven. To assess hemostatic efficacy in healthy human subjects is notoriously difficult. The bleeding time has often been suggested by some[8,38,39] but is criticized by others due to a poor predictive value for surgical bleeding.[40] Fecal occult blood is often present only in patients with thrombocytopenia, and antiplatelet reagents do not reliably lead to detectable fecal blood loss.[41] Furthermore, the short-term effect of a hemostatic product like cold-stored platelets is likely hard to measure in a fecal occult blood test. We found a continuous decrease in recovery to approximately 15% of the recovery of RT-stored platelets. This could suggests that storing platelets at 20 days at 4°C reduces recovery to an extent that hemostatic efficacy is compromised. Ultimately, to find middle ground between storage time prolongation, in vitro and in vivo function, and in vivo viability represents a balancing act and will have to be addressed in future studies.

In summary, our study is the first to compare cold-stored platelets up to 20 days to fresh and RT-stored platelets in plasma in healthy human subjects. We found that 20-day cold-storage share many similarities with 5- and 7-day RT storage but also have specific advantages and disadvantages. The best compromise between recovery, hemostatic efficacy, and storage time prolongation will need to be determined in future clinical trials in actively bleeding patients.

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SUPPORTING INFORMATION

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

Fig. S1. Supplementary figure.