In vitro and *in vivo* effects of short-term cold storage of platelets in PAS-C

Cold (4°C)-stored platelets (CSP) were the standard of care in the 1960s and 1970s but fell out of favor when their short *in vivo* survival was discovered. Since then, room temperature-stored platelets (RSP) have been the standard of care. Septic transfusion reactions from bacterially contaminated RSP remain the most common transfusion-transmitted infection. In addition, accumulating data questioning the efficacy and safety of RSP, together with a short shelf life, highlight an unmet medical need for an alternative product. Currently, CSP are being re-evaluated for bleeding patients, for whom immediate function matters more than long circulation time. An added benefit of CSP is that bacterial growth is markedly reduced at 4°C. However, how to store CSP best is poorly understood. Some groups reported increased wastage due to aggregates in CSP stored in plasma. Platelet additive solutions (PAS) were developed to reduce transfusion reactions and limit metabolic damage, but one group showed that PAS prevented aggregates in CSP.[1] Other groups reported decreasing platelet counts despite the use of PAS during cold storage, suggesting persistent microaggregates.[2-4] While numerous *in vitro* studies on CSP in PAS exist,[4-8] only one study looked at the effect of PAS on *in vivo* kinetics of transfused CSP, but lacked fresh comparators.[9]

In the current study, we investigated CSP in PAS (PAS-CSP) and compared them to CSP in plasma (P-CSP) and room temperature-stored platelets in plasma (P-RSP). All platelets were stored for 5 days. We collected a standard single apheresis platelet unit from six healthy subjects stored in either 100% plasma at 22°C or 4°C, or 65% PAS-C (Intersol) and 35% plasma at 4°C. In this study, we included historical controls for P-CSP and P-RSP,[10,11] but all units were collected by apheresis and stored in the same fashion as described below. Concerns regarding risks for healthy human volunteers and their safety, in addition to the high costs of *in vivo* radiolabeling studies, made repeating control groups that have already been studied and published both redundant and ethically burdensome for this small study.

We included PAS-C because it is currently licensed in the USA and we previously obtained *in vivo* data that favored PAS-C over PAS-F (Isoplate) for CSP.[9] All three groups

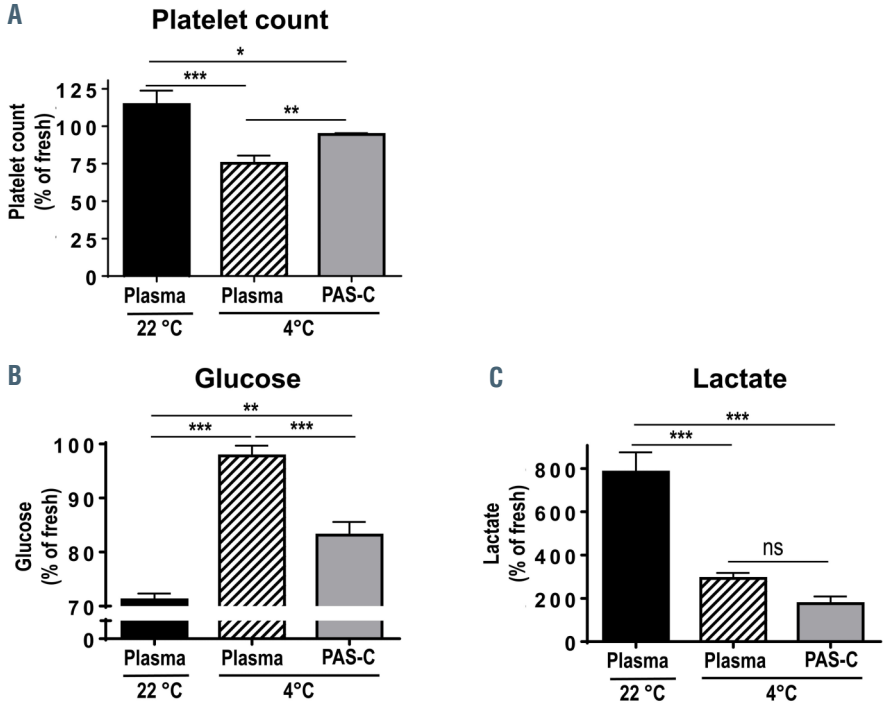

Figure 1. *In vitro* platelet characteristics. Platelets stored at room temperature in plasma (P-RSP) (Plasma, 22°C, solid black bars), platelets stored cold in plasma (P-CSP) (Plasma, 4°C, striped bars), and platelets stored cold in platelet additive solution (PAS-CSP) (PAS-C, 4°C, solid gray bars) were tested fresh and on day 5 after storage. (A) Platelet count measured by an ABX Hemanalyzer, P-RSP (n=18), P-CSP (n=5), PAS-CSP (n=6). (B) Glucose measured by a blood gas analyzer, P-RSP (n=6), P-CSP (n=5), PAS-CSP (n=6). (C) Lactate measured by a blood gas analyzer, P-RSP (n=6), P-CSP (n=5), PAS-CSP (n=6). Data are shown as percentage of fresh and as mean + standard error of mean. *P<0.05, **P<0.01, ***P<0.001. ns = not significant

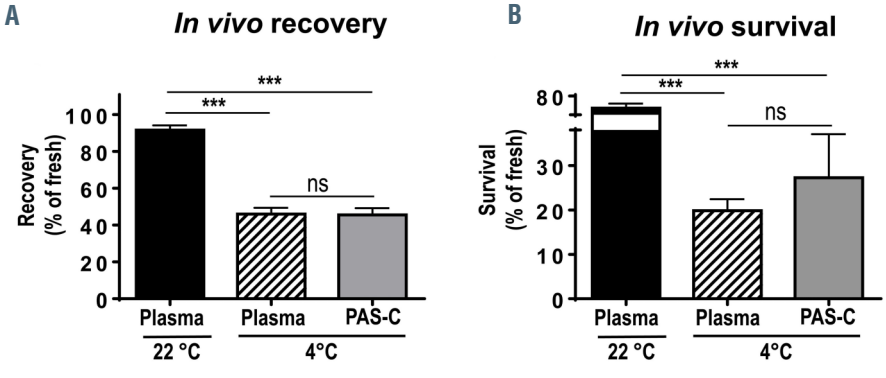

Figure 2. *In vivo* platelet characteristics. Healthy human subjects received autologous radiolabeled fresh platelets or platelets stored for 5 days in plasma at room temperature (P-RSP) (Plasma, 22°C, solid black bars), in plasma at 4°C (P-CSP) (Plasma, 4°C, striped bars), or in plasma additive solution at 4°C (PAS-CSP) (PAS-C, 4°C, solid gray bars). (A) Recovery of transfused platelets after 2 hours, P-RSP (n=21), P-CSP (n=5), PAS-CSP (n=5). (B) Survival of transfused platelets, P-RSP (n=21), P-CSP (n=5), PAS-CSP (n=5). Data shown as percentage of fresh, mean + standard error of mean. ***P<0.001, ns = not significant

(PAS-CSP, P-CSP, and P-RSP) comprised different cohorts (no matching between groups). The annexin V and P-selectin data were obtained from a separate group of four volunteers whose platelets were collected by apheresis and stored in mini-storage bags to clarify a role of these platelet activation parameters. All apheresis units were collected and bags stored at room temperature were agitated as per standard blood banking protocols. Cold-stored units were stored without agitation. We radiolabeled platelets as previously described with minor modifications.[12]

The Western Institutional Review Board approved the research, and all human participants gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki and registered with ClinicalTrials.gov identifier NCT02754414. We assessed statistical significance by one way analysis of variance (ANOVA) with the Tukey correction for multiple comparisons. To minimize biological variability, and following recommendations by Murphy *et al.*, we present the normalized stored data (percentage of fresh values). The absolute data are shown in *Online Supplementary Figures S1-S3*.

As previously described, CSP counts were significantly lower than P-RSP counts. PAS-CSP counts were significantly higher than those of P-CSP, corroborating findings from others and our group (Figure 1A).[1,11] Consistent with high metabolic activity at room temperature, glucose levels at day 5 were lowest in P-RSP. Post-storage levels of glucose in PAS-CSP were significantly lower than in P-CSP (Figure 1B). While P-RSP showed the highest lactate levels, cold storage reduced lactate production, with a trend for lower levels in PAS-CSP than in P-CSP (Figure 1C). These findings hint at persistent, detectable metabolic activity up to 5 days, even though the metabolism is markedly slowed at 4°C. As expected, platelet *in vivo* markers decreased significantly, but the recovery of PAS-CSP and P-CSP did not differ significantly (Figure 2A). We observed a trend for longer survival in PAS-CSP than in P-CSP (Figure 2B). To obtain more insights into the biological health of stored platelets, we studied mitochondrial membrane potential as an early marker of apoptosis. We observed significantly better-preserved membrane potential in P-CSP than in P-RSP. PAS-CSP and P-CSP values did not differ significantly as percentage of fresh values, but the absolute data showed significantly better preservation in PAS-CSP (Figure 3A, and *Online Supplementary Figure S2D*). All cells responded appropriately to the uncoupler CCCP (2-[2-(3-chlorophenyl)hydrazinylyidene]propanedinitrile) (*Online Supplementary Figure S2C, D*). There was a trend for more caspase activation in P-CSP, but overall, we did not see significant differences in this marker of late apoptosis (Figure 3B). Adding ABT 737 induced caspase activation before and after storage, indicating that platelets had the capacity to undergo apoptosis (*Online Supplementary Figure S2E, F*). We did not find significant differences in procoagulant activity, but there was a trend to higher levels at room temperature (Figure 3C). Similarly, we observed higher P-selectin levels at room temperature, but although this was significant when compared to PAS-CSP, it was not when compared to P-CSP (Figure 3D).

Integrin activation was greatest in P-CSP, significantly more than in P-RSP. The PAS-CSP integrin response was lower than the P-CSP one, but the difference was not statistically significant (Figure 3E-G). The largest difference

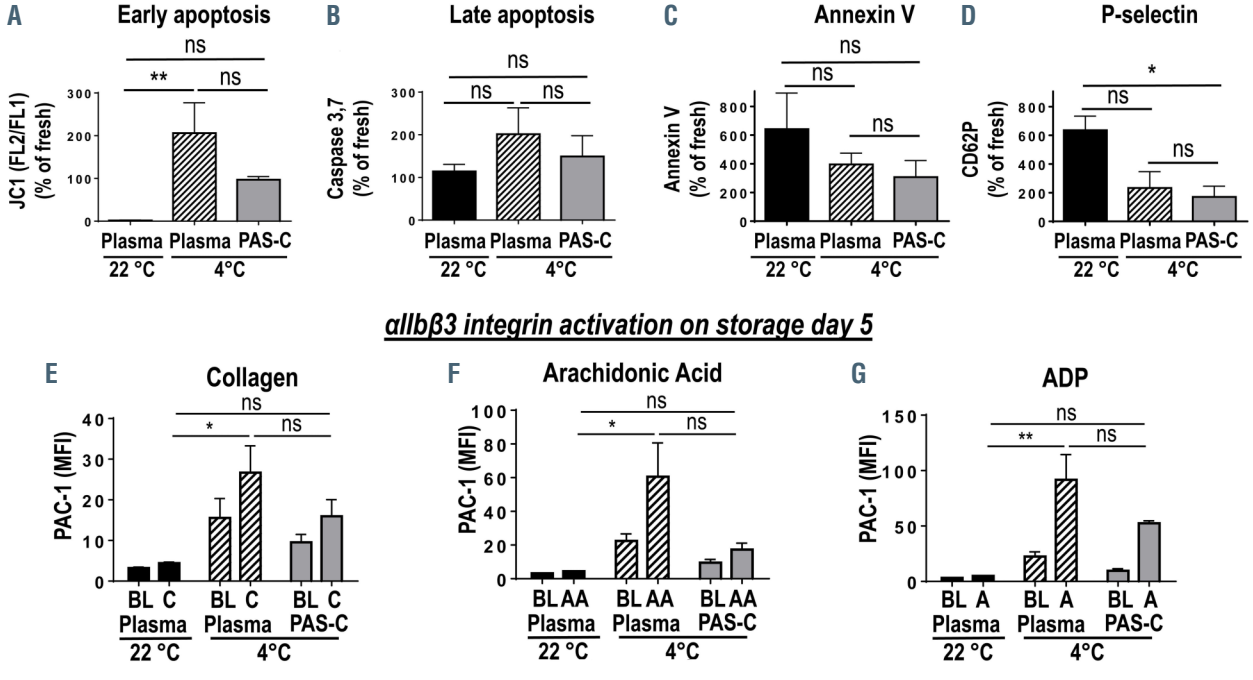

Figure 3. *In vitro* apoptosis and activation parameters. Apoptosis and parameters of platelet activation were measured in fresh platelets and platelets stored for 5 days in plasma at room temperature (P-RSP) (Plasma, 22 °C, solid black bars), in plasma at 4°C (P-CSP) (Plasma, 4°C, striped bars), or in plasma additive solution at 4°C (PAS-CSP) (PAS-C, 4 °C, solid gray bars). (A) Platelet mitochondrial membrane potential measured by JC-1 dye red (FL2) to green (FL-1) ratio, P-RSP (n=7), P-CSP (n=5), PAS-CSP (n=5). (B) Caspase 3,7 activation measured by flow cytometry, P-RSP (n=5), P-CSP (n=5), PAS-CSP (n=5). (C) Procoagulant activity measured by annexin V binding by flow cytometry. P-RSP (n=4), P-CSP (n=4), PAS-CSP (n=4). (D) α-granule degranulation was measured by CD62P binding by flow cytometry. P-RSP (n=4), P-CSP (n=4), PAS-CSP (n=4). Platelet αIIbβ3 integrin activation was measured by PAC-1 antibody binding by flow cytometry at baseline (*BL*) and after stimulation (E) with collagen (*C*), P-RSP (n=5), P-CSP (n=7), PAS-CSP (n=5). (F) with arachidonic acid (*AA*), P-RSP (n=5), P-CSP (n=5), PAS-CSP (n=5). or (G) with ADP (*A*) P-RSP (n=5), P-CSP (n=5), PAS-CSP (n=5). (A-C) Data are shown as percentage of values for fresh platelets, mean + standard error of mean. (E-G) Absolute data, mean + standard error of mean. **P*<0.05, ***P*<0.01, ****P*≤0.001.

between P-CSP and PAS-CSP was after stimulation with arachidonic acid (Figure 3F).

After only 5 days, we found that PAS prevented a cold-induced decrease in platelet count, likely by preventing microaggregates.[1,9] One report suggests that aggregate formation is prevented by continuous agitation during cold storage.[8] We observed a platelet count decrease independently of agitation in preliminary studies (*Online Supplementary Figure S3C*). In a previous study including over 20 units, we saw one large proteinaceous aggregate in one single unit, while a study with frequent manipulation and rewarming led to much more frequent detection of aggregates (*Online Supplementary Figure S3D*). Other investigators attempted to store cold platelets with repeated rewarming episodes (temperature cycling).[13] To prevent aggregates, rewarming had to be accompanied by agitation, an approach we have thus far not incorporated in our studies. Metabolically active platelets under normal storage conditions convert most of the supernatant glucose into lactate in P-RSP. Replacing plasma with PAS-C removes sugars but adds acetate, which modifies glucose utilization and can suppress lactate generation in RSP. Accordingly, the level of lactate in PAS-CSP stored for 5 days was lower than that in P-CSP or P-RSP, indicating that replacing plasma with PAS had a beneficial effect.[10,11] We did not observe activation differences between P-CSP and PAS-CSP after stimulation with various agonists similar to what has been described before for PAS/plasma CSP in aggregometry experiments[1] and 100% plasma CSP.[11] However, whether *in vitro* responses of CSP to agonists predict *in vivo* hemostasis is not well understood. Nevertheless, the fact that CSP in PAS and plasma have similar responses suggests that both media provide the right environment for platelet function testing *in vitro*. In our study, the mitochondrial membrane potential was best preserved in P-CSP and best predicted integrin activation, highlighting the need for an intact energy supply for platelet activation.[10,11] It is likely that our 5-day storage time was not enough to induce later stages of apoptosis: a recent study of CSP in PAS-F did not find significant differences up to day 15, compared to baseline, in some markers of apoptosis.[6] Our study provides a limited analysis of the complex apoptotic process and further studies are needed to explore this in further detail.

Small sample sizes and donor-to-donor variability may also have affected the outcomes of this study. An additional limitation is the lack of an earlier testing time point before the 5-day maximum. PAS may have additional benefits by reducing allergic and febrile adverse reactions, although this has not been systematically studied with CSP. During storage, the levels of inflammatory mediators are lower in CSP than in RSP. Whether PAS further reduces inflammatory levels in CSP remains to be investigated, but data from RSP support this idea. Most European countries utilize pathogen reduction technology (PRT). Two previous studies looked into the effects of combining PAS/PRT with 4°C storage.[14,15] The authors found mostly comparable results with the notable exception of reduced clot retraction and reduced GPIbα-levels with PRT. There may also be a benefit in promoting coagulation with PRT. [14,15]

In summary, we found that PAS had positive effects on *in vitro* parameters while not negatively affecting *in vivo* kinetics. Our data along with the results from other groups suggest that CSP in PAS are a safe and efficacious product and could have a practice-changing impact on the blood banking industry in the coming years.

S. Lawrence Bailey,[1] Lydia Y. Fang,[1] Lynda Fitzpatrick,[1] Daire Byrne,[1] Esther Pellham[1] and Moritz Stolla[1,2]

[1]Bloodworks Northwest Research Institute, Seattle, WA and [2]University of Washington Medical Center, Department of Medicine, Division of Hematology, Seattle, WA, USA

Correspondence:

MORITZ STOLLA - mstolla@bloodworksnw.org

doi:10.3324/haematol.2021.279865

Received: August 25, 2021.

Accepted: December 16, 2021.

Pre-published: December 23, 2021.

Disclosures: MS received research funding from Cerus Corp. and Terumo BCT

Contributions: SLB analyzed the data, established assays, and performed experiments, EP, DB and LYF performed experiments. LF performed apheresis collection procedures. MS designed the study, analyzed the data and wrote the manuscript.

Acknowledgments: the authors would like to thank Dr. Sherrill Slichter and the members of the cold-stored platelet interest group organized by the Department of Defense for helpful discussions. We thank Renetta Stevens and Tena Petersen for administrative support.

Funding: this project received funding support from the Department of Defense, award n.. W81XWH-12-1-0441

References

1. Getz TM, Montgomery RK, Bynum JA, Aden JK, Pidcoke HF, Cap AP. Storage of platelets at 4 degrees C in platelet additive solutions prevents aggregate formation and preserves platelet functional responses. Transfusion. 2016;56(6):1320-1328.
2. Johnson L, Tan S, Wood B, Davis A, Marks DC. Refrigeration and cryopreservation of platelets differentially affect platelet metabolism and function: a comparison with conventional platelet storage conditions. Transfusion. 2016;56(7):1807-1818.
3. Johnson L, Vekariya S, Wood B, Tan S, Roan C, Marks DC. Refrigeration of apheresis platelets in platelet additive solution (PAS-E) supports in vitro platelet quality to maximize the shelf-life. Transfusion. 2021;61(Suppl 1):S58-S67.
4. Reddoch-Cardenas KM, Montgomery RK, Lafleur CB, Peltier GC, Bynum JA, Cap AP. Cold storage of platelets in platelet additive solution: an in vitro comparison of two Food and Drug Administration-approved collection and storage systems. Transfusion. 2018;58(7):1682-1688.
5. Marini I, Aurich K, Jouni R, et al. Cold storage of platelets in additive solution: the impact of residual plasma in apheresis platelet concentrates. Haematologica. 2019;104(1):207-214.
6. Reddoch-Cardenas KM, Peltier GC, Chance TC, et al. Cold storage of platelets in platelet additive solution maintains mitochondrial integrity by limiting initiation of apoptosis-mediated pathways. Transfusion. 2021;61(1):178-190.
7. Braathen H, Sivertsen J, Lunde THF, et al. In vitro quality and platelet function of cold and delayed cold storage of apheresis platelet concentrates in platelet additive solution for 21 days. Transfusion. 2019;59(8):2652-2661.
8. Hegde S, Wellendorf AM, Zheng Y, Cancelas JA. Antioxidant prevents clearance of hemostatically competent platelets after long-term cold storage. Transfusion. 2021;61(2):557-567.
9. Stolla M, Fitzpatrick L, Gettinger I, et al. In vivo viability of extended 4 degrees C-stored autologous apheresis platelets. Transfusion. 2018;58(10):2407-2413.
10. Zimring JC, Slichter S, Odem-Davis K, et al. Metabolites in stored platelets associated with platelet recoveries and survivals. Transfusion. 2016;56(8):1974-1983.
11. Stolla M, Bailey SL, Fang L, et al. Effects of storage time prolongation on in vivo and in vitro characteristics of 4 degrees C-stored platelets. Transfusion. 2020;60(3):613-621.
12. The Biomedical Excellence for Safer Transfusion (BEST) Collaborative. Platelet radiolabeling procedure. Transfusion. 2006;46(Suppl 3):59S-66S.
13. Vostal JG, Gelderman MP, Skripchenko A, et al. Temperature cycling during platelet cold storage improves in vivo recovery and survival in healthy volunteers. Transfusion. 2018;58(1):25-33.
14. Agey A, Reddoch-Cardenas K, McIntosh C, et al. Effects of Intercept pathogen reduction treatment on extended cold storage of apheresis platelets. Transfusion. 2020;61(1):167-177.
15. Six KR, Devloo R, Compernolle V, Feys HB. Impact of cold storage on platelets treated with Intercept pathogen inactivation. Transfusion. 2019;59(8):2662-2671.