

AWARD NUMBER: W81XWH-12-1-0441

TITLE: Pathogen-Reduced, Extended Platelet Storage in Platelet Additive Solution (PAS)

PRINCIPAL INVESTIGATOR: Moritz Stolla, MD

CONTRACTING ORGANIZATION: Bloodworks Northwest, Seattle, WA

REPORT DATE: December 2021

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

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13. SUPPLEMENTARY NOTES					
14. ABSTRACT This grant pertains to finding novel approaches for storage of platelets for transfusion. Our project proposes to determine the efficacy of using a pathogen inactivation technique (Mirasol) coupled with a platelet additive solution (PAS) to extend the life of stored platelets. Our project also aims to determine how long acceptable platelet viability can be maintained in platelets stored at 4°C.					
15. SUBJECT TERMS bleeding, extended storage, hemorrhage, hemostasis, InterSol, Mirasol, pathogen inactivation, pathogen reduction technology, platelet additive solution, platelet recovery and survival, platelet storage, platelet storage solution, platelets, thrombocytopenia, transfusion, whole blood					
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Pathogen-Reduced, Platelet Additive Solution, Extended Stored Platelets (PREPS)

(Previously Pathogen-Reduced, Plasmalyte-Extended Stored Platelets)

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Final Report

15-SEP-2012 to 14-SEP-2021

INTRODUCTION: The purpose of this project is to find better ways to store platelets for patients that need platelet transfusions. A deeper mechanistic understanding of the effects of collection and storage on platelet function could greatly aid in improving the availability and efficacy of platelets both on the battlefield and in the civilian transfusion setting. In this research study, we are interested in evaluating the novel combinations of collection, storage and pathogen reduction approaches on the structural and functional properties of platelets and on platelet viability and function following transfusion.

KEY WORDS: 4°C storage, bleeding, cold storage, extended storage, hemorrhage, hemostasis, Isoplate, Intersol, pathogen inactivation, pathogen reduction, pathogen reduction technology, PRT, platelet additive solution, PAS, platelet recovery and survival, platelet storage, platelet storage solution, platelets, refrigerated storage, thrombocytopenia, transfusion, whole blood.

ACCOMPLISHMENTS: The following major goals are described in the July 2, 2014 revised Statement of Work (Appendix A), Novel Approaches to Storage of Platelets for Transfusion.

1. Evaluation of structural and functional changes to platelets during enhanced collection, storage and pathogen reduction (enhanced platelets).
2. Evaluation of enhanced platelets in animal models of trauma and hemorrhage.
3. Evaluate enhanced autologous platelets in normal subjects.
4. Evaluation of enhanced platelet recovery and survival, bleeding time and hemostatic activity in thrombocytopenic patients with and without acute hemorrhage.

In 2015 following discussions with the Grants Officer's Representative, a focus to this research award was expanded and added additional exploration of cold-stored platelets.

5. Exploring a variety of storage conditions, in vitro tests and mechanisms and in vivo recovery and survival as related to cold storage of platelets.

During the course of this award, our research focused on the exploration of cold-storage of platelets as related to SOW #1 and #5 above. The summary below addresses the key research accomplishments through project publications and presentations as related to the SOW through development of research protocol, study implementation, data and analysis of the following four studies enrolling healthy human research subjects.

Research Project #1 - Assessment of Whole Blood Cold Stored Platelets - Brrr Study

An evaluation of changes in the structural and functional properties of platelets stored as whole blood under refrigeration has been completed. Enrollment for this study began in October 2013 and ended in February 2015. Data analysis was completed in April 2015.

Discussion and results of this study can be found in the attached Final Report, ASH Abstract and AABB publication (Appendix B).

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#1a. Final Report - Award Number W81XWH-12-1-0441, EDMS 5570 Log Number 11105004
'Storage of Platelets in Whole Blood at 4°C' - April 15, 2015.

#1b. American Society of Hematology Annual Meeting; Orlando, FL December 2015.
In Vivo Viability of Platelets Stored in Whole Blood at 4°C. Slichter SJ, Fitzpatrick L, Pellham E, Jones MK, Bailey SL, Gettinger I. *Blood* 2015; 126(23):2338.

#1c. S. Slichter^{1,2}, L. Fitzpatrick¹, B. Osborne¹, T. Christoffel¹, I. Gettinger¹, E. Pellham¹, S. Bailey¹, MK. Jones¹, M. Herzig³, A. Cap³. Platelets Stored in Whole Blood at 4°C: in vivo Post-transfusion Platelet Recoveries and Survivals and in vitro Hemostatic Function. *Transfusion* JUN2019; 59:2084-2092.

¹ Bloodworks Northwest, Platelet Transfusion Research Laboratory, Seattle, WA

² Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, WA

³U.S. Army Institute of Surgical Research, Fort Sam Houston, TX

Research Projects - #2 Cold Apheresis Platelets in Isoplate or Intersol (CAPI) and #3 Cold Apheresis Platelets in Plasma (CAPP).

From January 2016 to April 2017, our research evaluated apheresis platelets stored at 4°C either in a platelet additive solution (PAS), such as InterSol or Isoplate, or stored in plasma. The protocol, entitled Cold Apheresis Platelets in Isoplate (CAPI), called for an apheresis platelet unit to be collected from a healthy subject and divided into two units. One half of the split unit was stored in plasma at 4°C for 3 days (control), the other half was stored at 4°C in a PAS/plasma mixture or in plasma alone (test) for 10 or 15 days. Subjects received radiolabeled platelet infusions on Day 3 (control) and Day 10 or 15 (test) to evaluate platelet recovery and survival. In addition to the in vivo platelet recovery and survival assays a number in vitro metabolic and functional platelet assays were performed on Day 3 and at the end of storage.

In April 2017 we revised the protocol to use plasma, not PAS, as the storage solution. The study was re-titled Cold Apheresis Platelets in Plasma (CAPP). We soon discovered that the stored/indium signal was swamped by the fresh/chromium signal when calculations were performed in accordance with the 2005 Biomedical Excellence for Safer Transfusion (BEST) method. This approach yielded unusable data outputs when comparing products of very different signal strengths.

In May 2017 we modified the protocol to replace the chromium label with a second indium label of fresh platelets administered a week after the stored radiolabeled platelets. We evaluated in vivo platelet recovery and survival assays of apheresis platelets stored for 5, 10 and 15 days at 4°C in comparison to fresh platelets. Additionally, we continue to compare in vitro metabolic and functional platelet assays on the day of collection to those at the end of storage.

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We also evaluated 5 day InterSol (PAS) 4°C stored platelets in comparison to fresh platelets and continue to compare in vitro metabolic and functional platelet assays on the day of collection to those at the end of storage. Enrollment for all of these studies ended in October 2018.

Discussion and results of these studies can be found in the attached MHSRS abstracts/posters, AABB abstracts/posters and AABB and *Haematologica* publications (Appendix C).

#2a. Military Health System Research Symposium (MHSRS); Kissimmee, FL August 2017

In Vivo Viability of Extended 10-Day 4°C Stored Autologous Platelets

M. Stolla^{1,2}, L. Fitzpatrick¹, I. Gettinger¹, S. Bailey¹, E. Pellham¹, T. Christoffel¹, S. Slichter^{1,2}

¹ Bloodworks Northwest, Platelet Transfusion Research Laboratory, Seattle, WA

² Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, WA

#2b. Military Health System Research Symposium (MHSRS); Kissimmee, FL August 2018

In Vivo Viability of Extended 20-Day 4°C Stored Autologous Platelets

M. Stolla^{1,2}, L. Fitzpatrick¹, S. Bailey¹, L. Fang¹, I. Gettinger¹, J. Corson¹, B. Osborne¹, E. Pellham¹, T. Christoffel¹

¹ Bloodworks Northwest, Platelet Transfusion Research Laboratory, Seattle, WA

² Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, WA

#2c. American Association of Blood Banks Annual Meeting; Boston, MA October 2018

Effects of Extended Cold-Storage on Platelet Function, Apoptosis and In Vivo Characteristics in Healthy Human Subjects

M. Stolla^{1,2}, L. Fitzpatrick¹, S. Bailey¹, L. Fang¹, I. Gettinger¹, J. Corson¹, B. Osborne¹, E. Pellham¹, T. Christoffel¹

¹ Bloodworks Northwest, Platelet Transfusion Research Laboratory, Seattle, WA

² Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, WA

#2d. M. Stolla^{1,2}, L. Fitzpatrick¹, I. Gettinger¹, S. Bailey¹, E. Pellham¹, T. Christoffel¹, S. Slichter^{1,2}

In Vivo Viability of Extended 4°C Stored Autologous Apheresis Platelets. *Transfusion* 2018; 58(10):2407-2413. PMID: 30203458

¹ Bloodworks Northwest, Platelet Transfusion Research Laboratory, Seattle, WA

² Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, WA

#2e. American Association of Blood Banks Annual Meeting; San Antonio, TX October 2019

Effects of Platelet Additive Solution on In Vivo and In Vitro Characteristics in 4°C Stored

Platelets. M. Stolla^{1,2}, L. Fitzpatrick¹, S. Bailey¹, L. Fang¹, I. Gettinger¹, E. Pellham¹, T. Christoffel¹ *Transfusion* 2019; 59(10): (S3):21A.

¹ Bloodworks Northwest, Platelet Transfusion Research Laboratory, Seattle, WA

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² Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, WA

#2f. M. Stolla^{1,2}, S. Bailey¹, L. Fang¹, L. Fitzpatrick¹, I. Gettinger¹, E. Pellham¹, T. Christoffel¹
Effects of Storage Time Prolongation on In Vivo and In Vitro Characteristics of 4°C Stored Platelets. *Transfusion* 2020; 60(3):613-621. PMID: 32017135

¹Bloodworks Northwest, Platelet Transfusion Research Laboratory, Seattle, WA

²Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, WA

#2g. S. Bailey, L. Fang, L. Fitzpatrick, D. Byrne, E. Pellham, M. Stolla. Effect of PAS-C on Short-Term Cold-Stored Platelets in Vitro and in Vivo. *Haematologica*. Accepted for publication Dec.2021.

Research Project #4 - Reversal of Aspirin Antiplatelet Therapy with Cold Stored Platelets (RASP).

The purpose of this study is to compare the reversal responses of 4°C cold stored platelets (CSP) versus 7 day, 22°C room temperature stored platelets (RTP) when given to healthy adult subjects who have received a loading dose of aspirin antiplatelet therapy. The primary endpoint is $\alpha\text{IIb}\beta\text{3}$ (GPIIb-IIIa) integrin activation as determined by ARU (Verify Now Aspirin [ARU Test]) at 1h post autologous transfusion, with additional testing at 4h and 24h post autologous transfusion as secondary endpoints.

STATUS:

Study active recruitment began DEC2020 and enrollment ended in SEP2021.

Number of subjects recruited/original planned target: 13/40

Number of subjects screened/original planned target: 12/40

Number of subjects enrolled/original planned target: 12/18

Number of subjects completed/original planned target: 10*/18

*data was collected from 10 subjects, however two subjects did not complete both study periods. One subject's room temperature infusion was cancelled due to a QC failure of the sample before the infusion and the final subject's cold storage study period was cancelled due to available study funds to complete the required testing.

Discussion and analysis of the current data can be found in the attached ASH abstract and slide presentation (Appendix D) .

We plan on continuing to analyze RASP Study samples using Bloodworks Northwest Investigator funding. Planned RASP activities include testing on frozen donor samples to further clarify mechanism to explain novel results. Testing may include but is not limited to the following; characterization of platelet derived microparticles and thrombin generation potential of frozen plasma samples (donor samples from before transfusion, after transfusion, and the apheresis unit after storage), metabolomics to clarify the effect of ASA on endogenous platelet population,

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effect of Transfusion (RSP vs CSP) on ASA effect, predictors in the Storage Bag for ASA reversal in RSP and predictors in the Storage Bag for pro-coagulant function in CSP.

#3a. American Society of Hematology Annual Meeting; Atlanta, GA December 2021.

Safety and Efficacy of 14-Day Cold Stored Platelets in Reversing Effects of Aspirin.

V. J. Li, MD^{1,2}, S. Bailey, BS², M. Bawcom-Randall², D. Byrne², J. Miles², J. Corson, RN² and M. Stolla, MD^{2,3}

Blood 2021 138(Suppl 1): 353 NOV2021. (Abstract and Oral Presentation – Slides attached)

¹Division of Hematology, University of Washington/Fred Hutchinson Cancer Research Center, Seattle, WA

²Bloodworks Northwest Research Institute, Seattle, WA;

³Department of Medicine, University of Washington, Seattle, WA

IMPACT: A deeper understanding of the effects of cold storage on platelet function could greatly aid in improving the availability of platelets on the battlefield and in the civilian transfusion setting. We have nothing to report related to impact on other disciplines, impact on technology transfer or impact on society beyond science and technology.

CHANGES/PROBLEMS:

CHANGES: During 2015, following communication with our Grants Officer's Representative work on pathogen-reduced, platelet additive solution, extended stored platelets was suspended and efforts were redirected towards exploration of cold stored apheresis platelets.

PROBLEMS: In November 2016, we received Notice of Minor Non-Compliance after an audit by the Compliance Administrator, Regulatory Affairs of the University of Washington. We submitted and received approval for study modifications in accordance with the audit recommendations. Details of this audit were submitted in the Annual Report dated 09OCT2017.

PRODUCTS:

PUBLICATIONS:

#1c. S. Slichter^{1,2}, L. Fitzpatrick¹, B. Osborne¹, T. Christoffel¹, I. Gettinger¹, E. Pellham¹, S. Bailey¹, MK. Jones¹, M. Herzig³, A. Cap³. Platelets Stored in Whole Blood at 4°C: in vivo Post-transfusion Platelet Recoveries and Survivals and in vitro Hemostatic Function. *Transfusion* JUN2019; 59:2084-2092.

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³U.S. Army Institute of Surgical Research, Fort Sam Houston, TX

Acknowledgement of federal support - Yes

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#2d. M. Stolla^{1,2}, L. Fitzpatrick¹, I. Gettinger¹, S. Bailey¹, E. Pellham¹, T. Christoffel¹, S. Slichter^{1,2}
In Vivo Viability of Extended 4°C Stored Autologous Apheresis Platelets. *Transfusion* 2018;
58(10):2407-2413. PMID: 30203458

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#2f. M. Stolla^{1,2}, S. Bailey¹, L. Fang¹, L. Fitzpatrick¹, I. Gettinger¹, E. Pellham¹, T. Christoffel¹
Effects of Storage Time Prolongation on In Vivo and In Vitro Characteristics of 4°C Stored
Platelets. *Transfusion* 2020; 60(3):613-621. PMID: 32017135

¹ Bloodworks Northwest, Platelet Transfusion Research Laboratory, Seattle, WA

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Acknowledgement of federal support – Yes

#2g. S. Bailey, L. Fang, L. Fitzpatrick, D. Byrne, E. Pellham, M. Stolla. Effect of PAS-C on Short-Term Cold-Stored Platelets in Vitro and in Vivo. *Haematologica*. Accepted for publication Dec.2021.

Acknowledgement of federal support - Yes

ABSTRACTS:

#1b. American Society of Hematology Annual Meeting; Orlando, FL December 2015.
In Vivo Viability of Platelets Stored in Whole Blood at 4°C. Slichter SJ, Fitzpatrick L, Pellham E, Jones MK, Bailey SL, Gettinger I. *Blood* 2015; 126(23):2338.

Acknowledgement of federal support - No

#2e. American Association of Blood Banks Annual Meeting; San Antonio, TX October 2019
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Transfusion 2019; 59(10): (S3):21A.

¹ Bloodworks Northwest, Platelet Transfusion Research Laboratory, Seattle, WA

² Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, WA

Acknowledgement of federal support - No

ABSTRACTS with POSTER PRESENTATIONS:

#2a. Military Health System Research Symposium (MHSRS); Kissimmee, FL August 2017
In Vivo Viability of Extended 10-Day 4°C Stored Autologous Platelets
M. Stolla^{1,2}, L. Fitzpatrick¹, I. Gettinger¹, S. Bailey¹, E. Pellham¹, T. Christoffel¹, S. Slichter^{1,2}

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² Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, WA

Acknowledgement of federal support - No

#2b. Military Health System Research Symposium (MHSRS); Kissimmee, FL August 2018

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¹ Bloodworks Northwest, Platelet Transfusion Research Laboratory, Seattle, WA

² Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, WA

Acknowledgement of federal support - No

#2c. American Association of Blood Banks Annual Meeting; Boston, MA October 2018

Effects of Extended Cold-Storage on Platelet Function, Apoptosis and In Vivo Characteristics in Healthy Human Subjects

M. Stolla^{1,2}, L. Fitzpatrick¹, S. Bailey¹, L. Fang¹, I. Gettinger¹, J. Corson¹, B. Osborne¹, E. Pellham¹, T. Christoffel¹

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² Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, WA

Acknowledgement of federal support – No

ABSTRACT WITH ORAL PRESENTATION

#3a. American Society of Hematology Annual Meeting; Atlanta, GA December 2021.

Safety and Efficacy of 14-Day Cold Stored Platelets in Reversing Effects of Aspirin.

V. J. Li, MD^{1,2}, S. Bailey, BS², M. Bawcom-Randall², D. Byrne², J. Miles², J. Corson, RN² and M. Stolla, MD^{2,3}

Blood 2021 138(Suppl 1): 353 NOV2021. (Abstract and Oral Presentation – Slides attached)

¹ Division of Hematology, University of Washington/Fred Hutchinson Cancer Research Center, Seattle, WA

² Bloodworks Northwest Research Institute, Seattle, WA;

³ Department of Medicine, University of Washington, Seattle, WA

Acknowledgement of federal support – yes

PRESENTATIONS:

Training and professional development has included presentations on Cold Stored Platelets by Dr. Stolla at the following academic institutions, National Blood Foundation and FDA:

SEP-2018 to SEP-2019

- University of Cincinnati, Hoxworth Blood Center – Grand Rounds
- Yale University – Department of Laboratory Medicine – Grand Rounds
- University of Washington – Department of Laboratory Medicine – Grand Rounds

SEP-2019 to SEP-2020

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- November 2019 – Presentation to the FDA-BPAC meeting on Cold Stored Platelet Studies data and analysis. In Vitro, Preclinical and In Vivo Recovery and Survival Studies of Cold-Stored Platelets.

SEP-2020 to SEP-2021

- October 2020 – National Blood Foundation – Scientific Symposium at AABB 2020.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

List of Bloodworks Northwest Personnel receiving pay from the research effort below:

<i>Name:</i>	<i>Sherrill J. Slichter, MD</i>
<i>Project Role:</i>	<i>Principal Investigator</i>
<i>Name:</i>	<i>Moritz Stolla, MD</i>
<i>Project Role:</i>	<i>Principal Investigator</i>
<i>Name:</i>	<i>Lynda Fitzpatrick, RN</i>
<i>Project Role:</i>	<i>Study Coordinator/Research Nurse</i>
<i>Name:</i>	<i>Jill S Corson, RN</i>
<i>Project Role:</i>	<i>Study Coordinator/Research Nurse</i>
<i>Name:</i>	<i>Barbara Osborne, RN</i>
<i>Project Role:</i>	<i>Study Coordinator/Research Nurse</i>
<i>Name:</i>	<i>Jeffrey A Miles, BS, MS</i>
<i>Project Role:</i>	<i>Clinical Research Coordinator</i>
<i>Name:</i>	<i>Morgan Randall, BA</i>
<i>Project Role:</i>	<i>Clinical Research Coordinator</i>
<i>Name:</i>	<i>Irena Gettinger, BA</i>
<i>Project Role:</i>	<i>Lab Technologist</i>
<i>Name:</i>	<i>Daire Byrne, BS,</i>
<i>Project Role:</i>	<i>Lab Technologist</i>
<i>Name:</i>	<i>Esther Pellham, BS</i>
<i>Project Role:</i>	<i>Lab Technologist</i>
<i>Name:</i>	<i>Shawn Bailey, BS</i>
<i>Project Role:</i>	<i>Lab Technologist</i>
<i>Name:</i>	<i>Lydia Fang, BS, MS</i>
<i>Project Role:</i>	<i>Lab Technologist</i>
<i>Name:</i>	<i>Chomie Usaneerungrueng, BS</i>
<i>Project Role:</i>	<i>Lab Technologist</i>
<i>Name:</i>	<i>MaryKay Jones, MT</i>
<i>Project Role:</i>	<i>Lab Technologist</i>
<i>Name:</i>	<i>Todd Christoffel, MT</i>
<i>Project Role:</i>	<i>Lab Technologist</i>

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List of Bloodworks Northwest and University of Washington Personnel performing study specialized laboratory testing and **NOT** receiving pay from this award:

<i>Name:</i>	<i>Katie Houck</i>
<i>Project Role:</i>	<i>Research Tech, Dong Lab, BWNW</i>
<i>Name:</i>	<i>Ava M. Obenaus, MS</i>
<i>Project Role:</i>	<i>Graduate Student, Sniadecki Lab, University of Washington</i>
<i>Name:</i>	<i>Molly Y. Mollica, MS</i>
<i>Project Role:</i>	<i>Graduate Student, Sniadecki Lab, University of Washington</i>

Terumo Blood and Cell Technologies is a subcontractor on this award (Award Amendment P0006 14SEP2015) and has been responsible for submitting separate reports for review as required by the GOR. This process was established at the onset of Terumo's contract and receipt of funds. In 2019, Terumo returned a portion of the unspent funds to BWNW as technical actions related to the Terumo sub-award were completed.

SPECIAL REPORTING REQUIREMENTS:

QUAD CHART: Dated 14DEC2021 FINAL (Appendix E).

APPENDICES:

- Appendix A - Statement of Work - Novel approaches to storage of platelets for transfusion. Rev. 14 07-02.
- Appendix B – Research Project #1 - Assessment of Whole Blood Cold Stored Platelets - Brrr Study - Final Report, Abstracts and Publication.
- Appendix C - Research Projects - #2 Cold Apheresis Platelets in Isoplate or Intersol (CAPI) and #3 Cold Apheresis Platelets in Plasma (CAPP) – Abstracts with Poster Presentation and Publications.
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Appendix A - Statement of Work - Novel approaches to storage of platelets for transfusion.
Rev. 14 07-02.

APPENDIX A

REVISED STATEMENT OF WORK

Title: Novel approaches to storage of platelets for transfusion.

Background: Platelets are transfused to prevent bleeding and induce hemostasis, and can thus be critical in saving lives following trauma. Currently, platelets isolated from volunteers are stored at room temperature with gentle agitation for up to 5 days before transfusion. This short shelf-life severely compromises platelet inventories and creates chronic shortages for two important reasons: (1) platelets age during this period, and are functionally not as desirable as fresh platelets; and (2) storage at room temperature increases the risk of bacterial contamination. There is an urgent need to develop novel methods of storing platelets to minimize or even eliminate these issues. This need is particularly acute in the deployed military setting where platelet products are in especially short supply and are essentially unavailable far-forward, near the point of injury where they might be of greatest utility.

It is possible that manipulation of several collection and storage parameters, such as choice of apheresis systems, storage medium and temperature, and implementation of pathogen reduction technologies may improve platelet shelf life, safety and effectiveness. It is well known that platelets are sensitive to physical stimuli such as shear and contact with artificial surfaces, which may be activating and cause premature release of hemostatic and inflammatory mediators prior to or during storage. Selection of collection systems that minimize physical damage to platelets, in conjunction with storage optimization, could significantly enhance platelet product quality. Similarly, the current common practice of storing platelets in citrated plasma at room temperature may lead to significant product degradation due to the activity of endogenous proteases or other mechanisms. Use of platelet additive solutions (PAS), might reduce platelet stress. Alternatively, storage of platelets within whole blood under refrigeration may provide other factors that maintain important aspects of platelet function that need to be evaluated, as a potentially preferred product for battlefield polytrauma. This was once standard-of-care in transfusion medicine, but was abandoned once it was shown that refrigeration led to accelerated *in vivo* platelet clearance over about 48 hours rather than over one week. While not conducive to maintaining circulating platelet counts in thrombocytopenic cancer patients, this strategy might provide adequate platelet hemostatic capacity to bleeding trauma patients and improve platelet availability for such patients. This possibility has been inadequately evaluated, particularly in clinical studies. Finally, addition of a pathogen reduction technology to some combination of the preceding approaches may yield further benefit by impeding bacterial growth, which is the principal lethal transfusion risk associated with platelet transfusion. Pathogen reduction would have the greatest impact in resource-constrained settings such as the deployed military environment or the

developing world, where full transfusion transmitted disease testing is unavailable.

A deeper mechanistic understanding of the effects of collection and storage on platelet function could greatly aid in improving the availability and efficacy of platelets both on the battlefield and in the civilian transfusion setting. In this research proposal we are interested in evaluating the effect of novel combinations of collection, storage and pathogen reduction approaches on the structural and functional properties of platelets and on the functional consequences during transfusion.

We hypothesize that the *“collection of platelets in a manner that minimizes physical damage combined with an alternative storage medium and/or temperature and pathogen reduction technology will improve platelet shelf life, safety and function.”* We propose the following Specific Aims to test this hypothesis.

Aim 1. Evaluation of structural and functional changes to platelets during enhanced collection, storage and pathogen reduction (enhanced platelets)

We will evaluate changes in the structural and functional properties of platelets including metabolism, protein and microRNA expression, shape changes, cytoskeletal rearrangement, membrane fluidity, receptor expression and distribution, microparticle formation, aggregation in response to agonists, whole blood clotting function, adhesion and aggregation under high shear conditions.

Aim 2. Evaluation of enhanced platelets in animal models of trauma and hemorrhage

We will evaluate the hemostatic efficacy and inflammatory characteristics of enhanced platelets and these observations will be correlated with *in vitro* findings from Aim 1. Enhanced platelets will be optimized for collection methods, storage mediums (plasma, PAS etc.), Temperature (refrigerated, room temperature, temperature cycling etc.) and exposure to pathogen reduction technology.

Aim 3. Evaluate enhanced autologous platelets in normal subjects

As the first step in the *in vivo* evaluation of human platelets, autologous platelets will be obtained from whole blood or apheresis procedures. The whole blood or apheresis platelets will have been subjected to various storage conditions with or without pathogen reduction. The efficacy of the platelets obtained from these products will be assessed by determining the recovery and survival of the subjects' autologous radiolabeled platelets following re-infusion. The results of these studies will be correlated with the studies performed in Aims 1 and 2.

Aim 4. Evaluation of enhanced platelet recovery and survival, bleeding time and hemostatic activity in thrombocytopenic patients with and without acute hemorrhage.

We will evaluate the shelf life, safety, and efficacy of enhanced platelets in patients and correlate these findings with observations from Aims 1, 2, and 3 in order to optimize platelet product collection and storage conditions.

We expect our results to generate important information on how changes in platelet collection, storage medium and temperature, and exposure to pathogen reduction technologies affect stored platelet structure and function, as well as shelf life and *in vivo* efficacy.

Our collaborators at the Puget Sound Blood Center, led by Dr. Sherrill J. Slichter, have extensive experience in studying platelet biology and transfusion medicine. Dr. Slichter's laboratory and clinical study group has made a number of the seminal observations on the effectiveness of platelet transfusion strategies.

A CRADA will cover collaborative research between the Puget Sound Blood Center and the USAISR Coagulation and Blood Research Task Area. This collaborative effort is envisioned to lead to development of new platelet storage techniques. Joint authorship in publications and inventors rights will be shared by both parties.

Collaboration:

USAISR agrees to:

1. evaluate changes in the structural and functional properties of platelets following collection, storage and pathogen reduction approaches that incorporate a number of combinations of currently available technologies, or technologies in advanced development (enhanced platelets).
2. evaluate the hemostatic efficacy and inflammatory characteristics of enhanced platelets in animal models of trauma and hemorrhage, and these observations will be correlated with *in vitro* findings.
3. evaluate the shelf life, safety, and efficacy of enhanced platelets in thrombocytopenic patients with acute hemorrhage and correlate these findings with observations from Aims 1 and 2 in order to optimize platelet product collection and storage conditions.
4. engage in analysis of data and validation of findings related to changes in platelet structure, function, and viability following enhanced collection, storage and pathogen reduction.

5. write manuscripts and scientific reports, submit invention disclosures and patents.

Puget Sound Blood Center agrees to:

1. identify candidate platelet collection, storage and pathogen reduction approaches to test in model *in vitro* and *in vivo* systems with the goal of improving platelet storage life, safety and efficacy. As needed, transfer candidate technologies to USAISR for *in vitro* and *in vivo* testing as described above.

2. conduct *in vitro* and *in vivo* platelet product testing as described above.

3. conduct clinical studies of enhanced platelets such as recovery and survival experiments in normal volunteers and thrombocytopenic patients. In addition, PSBC will perform bleeding time assays and trials of hemostatic efficacy studies in thrombocytopenic patients with and without acute hemorrhage. The first collection/storage conditions to be evaluated will be platelets stored within whole blood units under refrigeration.

4. engage in analysis of data and validation of findings related to changes in platelet structure, function, and viability following enhanced collection, storage and pathogen reduction procedures.

5. write manuscripts and scientific reports, submit invention disclosures and patents.

From time to time, USAISR personnel may work in the Puget Sound Blood Center's laboratories and Puget Sound Blood Center's personnel may work in USAISR's laboratory as necessary to accomplish the goals of this collaboration.

Pathogen-Reduced, Platelet Additive Solution, Extended Stored Platelets (PREPS)

(Previously Pathogen-Reduced, Plasmalyte-Extended Stored Platelets)

Award Number W81XWH-12-1-0441 Log Number 11105004987987431 `

Final Report

15-SEP-2012 to 14-SEP-2021

Appendix B – Research Project #1 - Assessment of Whole Blood Cold Stored Platelets - Brrr Study - Final Report, Abstracts and Publication.

#1a. Final Report - Award Number W81XWH-12-1-0441, EDMS 5570 Log Number 11105004
'Storage of Platelets in Whole Blood at 4°C' - April 15, 2015.

#1b. American Society of Hematology Annual Meeting; Orlando, FL December 2015.
In Vivo Viability of Platelets Stored in Whole Blood at 4°C. Slichter SJ, Fitzpatrick L, Pellham E, Jones MK, Bailey SL, Gettinger I. *Blood* 2015; 126(23):2338.

#1c. S. Slichter^{1,2}, L. Fitzpatrick¹, B. Osborne¹, T. Christoffel¹, I. Gettinger¹, E. Pellham¹, S. Bailey¹, MK. Jones¹, M. Herzig³, A. Cap³. Platelets Stored in Whole Blood at 4°C: in vivo Post-transfusion Platelet Recoveries and Survivals and in vitro Hemostatic Function. *Transfusion* JUN2019; 59:2084-2092.

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Final Report

Award Number W81XWH-12-1-0441, EDMS 5570

Log Number 11105004

“Storage of Platelets In Whole Blood at 4°C”

April 15, 2015

Introduction:

Since the 1970's, it has been known that platelet survivals are much better maintained at 22°C compared to 4°C while platelet recoveries are not significantly different based on storage temperature (Table 1).

Number of Observations	STORAGE CONDITIONS		PLATELET	
	Time (Days)	Temperature (°C)	Recovery (%)	Survival (Days)
15	1	22	51 ± 3	8.2 ± 0.2
8	1	4	61 ± 7	1.3 ± 0.1
11	3	22	40 ± 3	7.9 ± 0.2
8	3	4	40 ± 5	1.0 ± 0.1

Legend: Platelet concentrates were prepared from normal subjects donated whole blood. After storage of the platelet concentrate, aliquots of the stored autologous platelets were labeled with ⁵¹Cr, and the labeled platelets were injected into the platelet donor. Samples were obtained post-infusion to determine radiolabeled platelet recoveries and survivals. Data are reported as the average ± 1 S.E.⁽¹⁾

Approximately 80% of the platelets given in the U.S. are transfused into hematology/oncology patients where prolonged post-transfusion survivals of the stored platelets are important to decrease the need for frequent transfusions. Thus, the standard practice has been to store all platelets at 22°C regardless of the patient's clinical condition. However, for surgical/trauma patients, a short platelet life-span may be acceptable as these patients may only require immediate hemostasis until the vascular system can be repaired. The limited shelf-life of 5 days with 22°C storage severely limits platelet availability particularly at far-forward combat facilities, suggesting that we need to consider other options to support the platelet needs of these patients.

Increasingly, it has been recognized that trauma patients may be best supported with a ratio of 1 red cell, 1 plasma, and 1 platelet.⁽²⁻³⁾ Therefore, the question becomes whether component therapy may be the best strategy to provide blood products for these patients or whether the transfusion of whole blood (WB) stored at 4°C would meet their needs. The major concern is the viability and function of platelets stored within WB at 4°C as platelets have never been stored in the cold for longer than 3 days and then only as platelet concentrates.

Purpose:

The purpose of this study was to determine the recovery and survival of autologous platelets that have been stored within WB for up to 22 days.

Primary Endpoint:

To determine how long autologous platelets can be stored in WB at 4°C with average post-storage platelet recoveries of $\geq 50\%$ of the same donor's fresh platelet recoveries and platelet survivals of ≥ 1 day.

Experimental Design:

- Normal subjects donated a unit of WB.
- WB was stored at 4°C for 12 days (non-rotated) or for 10, 15, and 22 days (rotated end-over-end).
- At end of storage:
 - A platelet concentrate was prepared from the stored WB, and the platelets were labeled with ^{111}In .
 - A 50 ml blood sample was drawn from the subject, a fresh platelet sample was prepared from this blood, and the platelets were labeled with ^{51}Cr .
 - The subject was injected simultaneously with their autologous radiolabeled stored and fresh platelets.
 - Serial samples were drawn post-injection from the subject to determine the recovery and survival of the stored compared to the fresh platelets.

Results:

The first experiment was to store the WB obtained from 7 normal subjects for 12 days at 4°C without agitation until the end of storage. After storage, the WB was thoroughly mixed before a platelet concentrate was prepared (Table 2).

Table 2						
12 DAY 4°C WHOLE BLOOD PLATELET STORAGE						
Subject (#)	PLATELET RECOVERY (%)			PLATELET SURVIVAL (Days)		
	Fresh	Stored	% of Fresh	Fresh	Stored	% of Fresh
1	28	27	96*	8.1	2.4	30
2	50	15	30	4.9	1.9	38
4	63	19	30	9.8	1.9	19
5	73	27	37	8.7	1.3	15
6	41	35	85	8.5	2.5	30
7	53	15	28	8.9	1.5	16
8	44	14	32	8.9	1.1	12
Ave $\pm$ S.D.	50 $\pm$ 15	22 $\pm$ 8	48 $\pm$ 29	8.3 $\pm$ 1.6	1.8 $\pm$ 0.5	23 $\pm$ 10
*Without this result, average = 40 $\pm$ 22%.						

There was a very wide standard deviation for the donors' platelet recoveries as a percentage of fresh. The first subject had an unexpectedly reduced fresh platelet recovery with a stored recovery that was 96% of the same donor's fresh recovery. Without the data from subject 1, platelet recoveries averaged only 40 $\pm$ 22% of the same donor's fresh recoveries. However, platelet survivals were all ≥ 1 day. In addition, 52 $\pm$ 12% of the donor's initial WB platelets were lost during storage (Table 3). These post-storage platelet results – both because of the poor platelet recoveries and platelet losses – were considered unacceptable.

Table 3

BASELINE SUBJECT DATA AND WHOLE BLOOD PLATELET AND HEMATOCRIT DATA

Subject (#)	Subject's HCT	Subject's Platelet Count ($10^3/\mu\text{L}$)	PLATELET COUNTS*			TOTAL PLATELET COUNTS	HEMATOCRITS	
			Whole Blood ($\times 10^3/\mu\text{L}$)		Loss (%)**	Whole Blood ($\times 10^{10}$) Post-Storage	Whole Blood Pre-Storage	Whole Blood Post-Storage
			Pre-Storage	Post-Storage				
1	39	248	180	110	39	4.7	31	31
2	43	227	152	66	57	3.2	37	36
4	44	377	297	183	38	8.8	39	38
5	44	239	194	86	56	4.1	38	38
6	38	191	177	49	72	2.3	33	31
7	44	188	174	97	44	4.7	38	38
8	44	215	202	88	56	4.7	38	38
Ave $\pm$ 1 S.D.	42 $\pm$ 3	244 $\pm$ 65	197 $\pm$ 47	97 $\pm$ 43	52 $\pm$ 12	4.6 $\pm$ 2.0	36 $\pm$ 3	36 $\pm$ 3

* Bag rotated end-over-end before platelet count obtained.

** Platelet loss during storage as a percent of the baseline platelet count.

Flow cytometry experiments demonstrated that the platelet loss during storage was not due to formation of either platelet aggregates or an excessive number of microparticles. Our hypothesis was that the platelets must be adhering to the walls of the bag to account for most of the platelet loss during storage. We then determined that, if the WB was rotated end-over-end during storage rather than only mixing the bag at the end of storage, $76 \pm 4\%$ of the initial platelets were maintained within the WB during 21 days of storage (Table 4).

Table 4

EVALUATION OF PLATELET LOSS DURING 4°C WB PLATELET STORAGE*

	STORAGE DAY (% of Baseline Platelet Count)				
	4	8	13	17	21
Rotation of WB End-Over-End During Storage	78	69	78	74	81
Bench Top With Bag Rotated End-Over-End At End Of Storage	58	56	64	48	48

*Two units of whole blood of the same blood type were pooled at baseline, mixed, and split into two bags for storage.

This constant end-over-end rotation of the WB during storage was then used for all future experiments. Platelet counts pre- and post-storage for up to 22 days confirmed the reproducibility of our original experiment (Table 5).

Table 5				
WHOLE BLOOD ROTATED END-OVER-END DURING 4°C PLATELET STORAGE				
Storage Time (Days)	N	WB PLATELET COUNTS $\times 10^{10}$		Post-Storage Yield (% of Baseline)
		Baseline	Post-Storage	
10	10	9.5 $\pm$ 2.1	7.0 $\pm$ 1.4	74%
15	10	10.0 $\pm$ 1.0	7.6 $\pm$ 1.4	76%
22	3	12.9 $\pm$ 1.9	9.2 $\pm$ 0.7	71%

In vivo platelet recoveries and survivals were measured for platelets separated from rotated WB stored at 4°C for 10, 15, and 22 days (Table 6).

Table 6							
WHOLE BLOOD ROTATED END-OVER-END DURING 4°C PLATELET STORAGE							
Storage Time (Days)	N	PLATELET RECOVERY (%)			PLATELET SURVIVAL (Days)		
		Fresh	Stored	% of Fresh	Fresh	Stored	% of Fresh
10	10	50 $\pm$ 10	26 $\pm$ 7	51%	8.0 $\pm$ 1.0	1.3 $\pm$ 0.3	16%
15	10	55 $\pm$ 11	27 $\pm$ 11	49%	8.0 $\pm$ 0.1	1.2 $\pm$ 0.4	16%
22	3	62 $\pm$ 16	15 $\pm$ 1	25%	8.4 $\pm$ 0.6	1.1 $\pm$ 0.7	12%

Both platelet recoveries and survivals met our acceptance criteria for up to 15 days of storage but not for 22 days. Data on *in vitro* platelet assays are given in Table 7. These data demonstrate that cold stored platelets are highly activated, suggesting that these platelets may provide immediate hemostasis for actively bleeding patients.

Table 7									
IN VITRO ASSAYS OF ROTATED WHOLE BLOOD 4°C PLATELET STORAGE									
Storage Time (Days)	N	Microparticles (%)		Annexin V Binding (% Positive)		P-Selectin (% Positive)		TGT Peak (nM Thrombin)	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
10	10	1.9 $\pm$ 0.9	21.7 $\pm$ 19.0	6 $\pm$ 6	32 $\pm$ 13	11 $\pm$ 11	68 $\pm$ 17	63 $\pm$ 25	177 $\pm$ 74
15	10	1.2 $\pm$ 0.9	20.0 $\pm$ 10.4	5 $\pm$ 6	51 $\pm$ 21	10 $\pm$ 11	76 $\pm$ 10	83 $\pm$ 16	162 $\pm$ 37
22	3	1.0 $\pm$ 0.8	36.3 $\pm$ 6.3	10 $\pm$ 8	56 $\pm$ 7	7 $\pm$ 2	92 $\pm$ 1	109 $\pm$ 62	171 $\pm$ 54

Conclusions:

- End-over-end rotation of WB during 4°C storage is required to reduce platelet adherence to the walls of the bag.
- Platelet yields in the WB post-storage average 7.0 to 9.2 $\times 10^{10}$. Thus, the FDA requirement of 5.5 $\times 10^{10}$ platelets/concentrate are easily met.

- At storage times between 10 to 15 days:
 - Stored recoveries average 50% of fresh recoveries.
 - Stored survivals average >1 day.
 - Proposed post-storage criteria for WB 4°C stored platelets are met.
- Based on *in vitro* measurements, the platelets are highly activated.

Future Studies:

It will be necessary to document the hemostatic efficacy of platelets stored within WB at 4°C. This will likely require large transfusion trials monitoring bleeding outcomes in surgical or trauma patients.

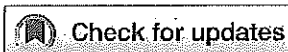
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401. BASIC SCIENCE AND CLINICAL PRACTICE IN BLOOD TRANSFUSION: POSTER II | DECEMBER 3, 2015

***In Vivo* Viability of Platelets Stored in Whole Blood at 4°C**

Sherrill J. Slichter, MD, Lynda Fitzpatrick, R.N., Esther Pellham, B.S., MBA, Mary Kay Jones, MT(ASCP), S. Lawrence Bailey, B.S., Irena Gettinger, B.A.



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Split-Screen



Abstract

Background: Trauma surgeons have suggested that the optimal transfusion support for actively bleeding patients may be a 1:1:1 ratio of red cells, plasma and platelets (plts). Therefore, the only reason to give component therapy - rather than whole blood (WB) - for these patients may be because of the poor post-transfusion survivals of plts stored at 4°C. However, for trauma/surgical patients, even short plt survivals may be sufficient to allow the surgeon enough time to repair the injury. We report the "first ever" data on the post-storage viability of plts stored at 4°C beyond 3 days. In the studies to be reported here, plts were stored within WB for up to 22 days at 4°C.

Study Design/Methods: Normal subjects donated a unit of WB that was stored at 4°C either for 12 days with mixing only at the end of storage or for 10, 15, or 22 days with end-over-end rotation of the WB throughout storage. After storage, a plt concentrate was prepared from the WB using standard centrifugation procedures. At the end of WB storage, the donor donated a 40 ml blood sample from which fresh plts were prepared. The autologous stored and fresh plts were radiolabeled with ¹¹¹In or ⁵¹Cr, respectively, before simultaneous transfusion into their donor. Serial post-transfusion blood samples were collected to determine stored *versus* fresh plt recoveries and survivals. Our pre-

defined acceptance criteria were: 1) stored plt recoveries should average $\geq 50\%$ of the same donor's fresh plt recoveries; and 2) plt survivals should average ≥ 1 day.

Results/Findings: When the WB was mixed only at the end of storage, plt yields in the WB were just 48% of baseline values with average plt counts of $4.6 \pm 2.0 \times 10^{10}$. This amount of plt loss was considered unacceptable (Table). In contrast, with continuous end-over-end rotation of the WB during storage, plt yields after 15 days were 76% of baseline values and plt counts averaged $7.6 \pm 1.4 \times 10^{10}$ (well within FDA guidelines that require 5.5×10^{10} plts/concentrate prepared from WB). Radiolabeled autologous plt recoveries averaged $27 \pm 11\%$ ($49 \pm 16\%$ of fresh) and survivals averaged 1.2 ± 0.4 days ($16 \pm 1\%$ of fresh); i.e., our pre-defined plt acceptance criteria were met. Storage for 22 days gave unacceptable results.

Conclusion: Plt concentrates stored at 4°C for up to 3 days have been FDA approved for use in thrombocytopenic patients since the 1970's. These plts had autologous plt recoveries of $40 \pm 5\%$ and survivals of 1.0 ± 0.1 days (S.E.) (Br J Haematol 1976;34:403-419). The FDA has recently approved the use of 3-day 4°C stored apheresis plts for actively bleeding patients. Our data may suggest that plts stored within WB at 4°C for up to 15 days may provide similar post-storage plt viability as plt concentrates or apheresis plts stored for ≤ 3 days. Red cells and plasma derived from CP2D WB stored for 21 days at 4°C are already licensed. Therefore, for actively bleeding patients who need all three components (red cells, plasma, and plts), WB stored for up to 15 days may provide effective quantities of all the needed components.

Table 1.

AUTOLOGOUS YIELDS, RECOVERIES, AND SURVIVALS OF PLATELETS STORED IN WHOLE BLOOD AT 4°C

Storage Time (Days)	N	Rotation	WB PLT COUNTS $\times 10^{10}$		Post-Storage Yield (% of Baseline)	PLT RECOVERIES (%)			PLT SURVIVALS (Days)		
			Baseline	Post-Storage		Fresh	Stored	% of Fresh	Fresh	Stored	% of Fresh
12	7	Mixed at end of storage*	9.4 ± 2.4	4.6 ± 2.0	48 ± 12	50 ± 15	22 ± 8	48 ± 29	8.3 ± 1.6	1.8 ± 0.6	23 ± 10
10	10	Continuously during storage**	9.5 ± 2.1	7.2 ± 1.6	77 ± 10	50 ± 10	26 ± 7	51 ± 7	8.1 ± 1.1	1.3 ± 0.3	16 ± 4
15	10	Continuously during storage**	10.0 ± 1.0	7.6 ± 1.4	76 ± 10	55 ± 11	27 ± 11	49 ± 16	8.0 ± 0.1	1.2 ± 0.4	16 ± 1
22	3	Continuously during storage**	12.9 ± 1.9	9.2 ± 0.7	73 ± 15	56 ± 12	14 ± 4	25 ± 5	7.7 ± 0.6	0.8 ± 0.3	10 ± 3

Data reported as average ± 1 S.D.

*WB units were hand mixed only at the end of storage before plt counting.

**WB units were continuously rotated end-over-end during storage.

Disclosures

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Topics: apheresis, blood platelets, blood tests, centrifugation, concentrate dosage form, hemorrhage, surgical procedures, operative, transfusion, whole blood, wounds and injuries

Author notes

* Asterisk with author names denotes non-ASH members.

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Abstract



BLOOD COMPONENTS

Platelets stored in whole blood at 4°C: in vivo posttransfusion platelet recoveries and survivals and in vitro hemostatic function

Sherrill J. Slichter,^{1,2} Lynda Fitzpatrick,¹ Barbara Osborne,¹ Todd Christoffel,¹ Irena Gettinger,¹ Esther Pellham,¹ S. Lawrence Bailey,¹ Mary Kay Jones,¹ Maryanne C. Herzig,³ and Andrew P. Cap³

BACKGROUND: Ordinarily, whole blood (WB) is separated into components before storage. We assessed the posttransfusion viability and function of platelets (PLTs) if they were stored within WB at 4°C.

STUDY DESIGN AND METHODS: Whole blood was obtained from 30 normal subjects and stored at 4°C without agitation for 12 days and for 10, 15, or 22 days with agitation. After WB storage, a PLT concentrate was prepared, and a fresh PLT sample was obtained from each donor. The stored PLTs were labeled with ¹¹¹In and the fresh with ⁵¹Cr, and both were simultaneously transfused into their donor. Blood samples were obtained after transfusion to determine PLT recoveries and survivals. PLT samples from WB before and after storage were also assayed for PLT function and biochemistry.

RESULTS: After storage for 12 days without WB rotation, poststorage PLT counts averaged only 49 ± 12% of baseline values. After storage for 10, 15, or 22 days with end-over-end WB rotation, PLT counts averaged 76 ± 14% of baseline values. Fifteen-day poststorage radiolabeled PLT recoveries averaged 27 ± 11% (49 ± 16% of fresh), and survivals averaged 1.2 ± 0.4 days (16 ± 6% of fresh). In vitro assays demonstrated marked PLT activation after any storage time, and although PLT function decreased over time, stored PLTs were still considered acceptable.

CONCLUSION: These data suggest that, during rotated WB storage at 4°C for up to 15 days, PLT yields, poststorage PLT recoveries and survivals, and PLT function should be sufficient to support the short-term hemostatic needs of traumatized patients.

Currently, donor platelets (PLTs) are stored at 22°C with agitation for up to 5 days. Although the Food and Drug Administration (FDA) has recently allowed PLT storage times to be extended to 7 days with the use of a point of release bacterial detection assay,¹ this short shelf-life compromises PLT availability and creates other problems: 1) PLTs age during storage and, therefore, are not as functional as fresh PLTs; and 2) storage at room temperature increases the risk of bacterial contamination. There is a need to develop novel methods of preparing and storing PLTs to minimize these issues. This need is particularly acute in the military setting where PLT products are essentially unavailable at far-forward medical facilities, near the point of injury where they would be of greatest utility.

Fractionation of whole blood (WB) into components was developed to more efficiently treat patients' specific cellular and clotting factor deficiencies and to optimize the storage requirements of each component, that is, 4°C for red blood cells (RBCs), freezing for plasma clotting factors, and 22°C for PLTs. US

ABBREVIATIONS: ETP = endogenous thrombin potential; PAI = plasminogen activator inhibitor; PAP = plasmin-antiplasmin complex; R = time to initial clot; TAT = thrombin-antithrombin complex; TEG = thromboelastography; WB = whole blood.

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combat casualty care practice has increased the survival of massively injured soldiers and civilians, in part due to changes in resuscitation practices that include a component transfusion strategy of 1:1:1 (RBCs:plasma:PLTs) and the use of warm, fresh WB from a "walking blood bank" of available donors if the needed components are not available. Such intensive plasma and PLT-based therapy early in resuscitation has led to a 50% reduction in mortality in massively transfused trauma patients.^{2,3} However, if all of the components could be stored as WB at 4°C without the need for component fractionation, this would greatly simplify combat resuscitation.

The major concern regarding the use of unfractionated WB, in providing effective hemostasis, is the effects of 4°C storage on PLT viability.⁴⁻⁶ Posttransfusion PLT recoveries of autologous stored PLT concentrates are similar at either 4 or 22°C for 3 days of storage, but PLT survivals are markedly reduced: 1.0 ± 0.1 days compared to 7.9 ± 0.2 days, respectively.⁶ Furthermore, bleeding time studies have given conflicting results about the superiority of one storage temperature versus the other.⁵⁻⁷

The decision to store PLTs at 22°C rather than 4°C is predicated on the assumption that PLT circulation time should be maximized for chronically thrombocytopenic hematology/oncology patients above all other functional considerations. However, in the treatment of acute hemorrhage, PLTs must circulate and function only long enough for surgical repair. In this clinical setting, hemostatic function supersedes circulation time. The FDA initially licensed PLT concentrates to be stored for 3 days at either 22 or 4°C⁸ and recently allowed 3-day 4°C apheresis PLT storage.^{8,9} The acceptance criteria the FDA established for 22°C stored autologous PLTs was that the lower 95% confidence limit for stored recoveries should be 66% of the same subject's fresh recoveries, and stored survivals should be 58% of fresh. As traumatized soldiers are likely to need only transient PLT support, we suggest that 4°C stored PLT recoveries should average approximately 50% of the same subject's fresh recoveries, and stored PLT survivals should be at least 1 day. The purpose of our studies was to determine how long PLTs could be stored within WB at 4°C while still maintaining reasonable post-storage PLT counts, viability, and function.

MATERIALS AND METHODS

Experimental design

Subject eligibility

Normal subjects 18 years of age and older who met the blood center's eligibility criteria for a 2-unit autologous WB donation were recruited to meet the needs of a 1-unit WB donation and collection of blood samples after radiolabeled PLT infusion. Subjects were excluded if they were taking any PLT-inhibiting drugs or anticoagulants within 7 days before donation. Subjects could not be pregnant (confirmed by pregnancy test) or nursing and both males and females had to use appropriate contraceptive methods during the study. The study was approved by Western

Investigational Review Board, and all subjects signed an informed consent.

WB donation and storage

Subjects donated 500 mL of WB collected in CPD-A2. The WB was not leukoreduced, and the units were stored at 4°C for 10 to 22 days. The 10-day initial WB 4°C storage time was selected to be longer than the currently allowed 7-day 22°C PLT storage time to facilitate far-forward combat PLT availability. The additional 4°C WB storage times of 15 and 22 days were evaluated to see if longer storage times gave comparable results to 10-day storage. Initially, the units were not agitated during storage, but, because of substantial decreases in PLT counts during storage, additional studies were performed with the WB units rotated during storage to improve poststorage PLT counts. A hybridization incubator (Model 400, Scigene) with heat off and door removed was placed in a 4°C walk-in cold room. The collection bag was attached to the rotation device at both ends of the bag to achieve the end-over-end rotation at 2 to 3 rpm.

In vivo measurements

At the end of WB storage, a 140-mL aliquot was withdrawn into a 150-mL transfer pack (Fenwal, Inc.) and centrifuged at $2282 \times g$ for 6.5 minutes using a low-speed centrifuge (Sorval RC3BP+, Thermo Scientific) with brake set at 7. PLT-rich plasma was expressed into another transfer pack for ¹¹¹In radiolabeling. A 43-mL blood sample was drawn from the subject to prepare fresh PLTs for ⁵¹Cr radiolabeling. PLT radiolabeling was done using the BEST method.¹⁰ These radiolabeled aliquots were sequentially infused into the subject. The estimated amounts of the isotopes infused were not more than 15 μ Ci of indium and not more than 20 μ Ci of chromium. Venous samples (approx. 10 mL/draw) were taken within 2 hours after reinfusion and on Days 1, 2, 3, 5, 7 ± 1 , and 10 ± 2 . Calculation of recoveries (extrapolated to $t = 0$ on the survival curve) and survivals were performed as described.¹⁰

Sterility

A 10-mL sample of the WB units for bacterial culture was obtained 1 to 3 days after collection, and the results were evaluated 14 days later or sooner if the blood was stored for a shorter time. A Gram stain was also performed on the isolated 4°C PLTs at the end of storage. All sterility tests had to be negative before PLT reinfusion. Cultures and Gram stains were performed by the University of Washington Microbiology Laboratory (Seattle, WA).

In vitro assays

For all in vitro assays, approximately 60 mL was obtained from the WB unit for prestorage (baseline) and poststorage analyses.

PLT count, biochemistry, and morphology

Platelet counts and mean PLT volume (MPV) were performed on WB using a particle counter (Micros 60, ABX). Blood gases (pH, PO₂, PCO₂, and HCO₃) were measured using a blood gas analyzer (Model 248, Bayer). Glucose and lactate were measured

using a chemistry analyzer (Aeroset, Abbott) at a local private laboratory (Dynacare, Inc.).

Morphology score was performed by the method of Kunicki¹¹ using a phase contrast microscope to determine the percentage of cells that were discoid, spheroid, or dendrites or were spherical PLTs. A numerical value for the cell types was calculated with the maximum being 400.

PLT function

Hypotonic shock response and extent of shape change

An aggregometer (Model 500-CA, Chronolog) was used to optically measure PLT aggregation, hypotonic shock response (HSR), and extent of shape change (ESC) as described by the manufacturer.¹²

PLT aggregation

Platelets were exposed to standard concentrations of ADP (5–10 $\mu\text{mol/L}$), collagen (5 $\mu\text{g/mL}$), and arachidonic acid (0.5 mmol/L ; all from Chrono-Log Corporation), and changes in light transmission were recorded. Data collected included maximum and ending amplitude, slope, lag time, and area under the curve.

Thromboelastography

PLT contribution to clot formation in WB was measured by thromboelastography (TEG), using kaolin-treated WB. Time to initial clot (R), clot formation time (k), alpha angle (rate of clot formation), maximum strength (MA), and clot lysis at 30 min (LY30) were determined on a TEG Hemostasis Analyzer (Model 5000, Haemoscope Corporation).^{13,14}

Thrombin generation test

The calibrated automated thrombogram platform was used to measure thrombin generation in PLT-rich plasma generated from WB samples. Variables for lag time, time to peak, peak thrombin generation (nmol/L), endogenous thrombin potential (ETP), and analysis of area under the thrombin curve were analyzed as described by the manufacturer (Diagnostics Stago, Inc.).¹⁵

PLT activation and apoptotic markers

P-selectin expression and phosphatidylserine externalization

Activated PLTs were measured by P-selectin (CD62) expression on the CD61(+) PLT surfaces with FITC-labeled S-12 antigen and flow cytometry.¹⁶ Phosphatidylserine externalization was detected by annexin V staining and flow cytometry analysis using an apoptosis assay kit (Vybrant 1, V-13240, Molecular Probes). Annexin V was labeled with Alexafluor 488, and dead cells were excluded by Sytox green binding to DNA.

Microparticles

A flow cytometer with a forward scatter photomultiplier tube was used to identify microparticles from PLTs, RBCs, white blood cells (WBCs), and endothelial cells as previously described.¹⁷

Total microparticles and PLT microparticles were analyzed on WB samples at Bloodworks Northwest. All cell types were analyzed from the frozen PLT-poor plasma samples at the USAISR, Ft Sam Houston, Texas. To identify microparticles from different cell types, the following fluorescently labeled antibodies were used: APC-H7-CD41a (GPIIb/IIIa from PLT-derived MP), APC-CD235a (glycophorin A from RBC-derived MP), PE-Cy7-CD45 (WBC-derived MP), PE-CD142 (tissue factor found on MP derived from endothelial cells, monocytes, and PLTs), and FITC-lactadherin (phosphatidylserine), as well as isotype control antibodies. BD TruCount tubes were used for absolute counting of microparticles. Data analysis was performed using computer software (FACS Diva, BD).

Coagulation and complement assays

Coagulation, PLT activation, and complement pathways were measured in citrated plasma by enzyme-linked immunosorbent assay (ELISA). Coagulation protein kits included thrombin-antithrombin complex (TAT; Enzygnost TAT micro, Siemens Healthcare), plasmin-antiplasmin complex (PAP; Imucrone PAP ELISA, American Diagnostica), soluble CD40L (sCD40L, human sCD40L, platinum ELISA extra sensitive, eBioscience), and plasminogen activator inhibitor (PAI-1; Zymutest PAI-1 antigen ELISA, Aniaira). Components of complement activation included C3a, C4d, C5a, and C5b-9a and were assayed using complement ELISA kits (Microvue, Quidel Corporation).

Statistical analyses

Summary statistics (means, standard deviations, confidence limits) were calculated for all in vitro assays and in vivo PLT recoveries and survivals. Comparisons were made between each subject's fresh and stored PLT recoveries and survivals. Two-way repeated-measure analysis of variance (ANOVA) of pre- and poststorage values for all blood unit measurements was used to determine significance ($p < 0.05$). Units with missing data were analyzed by two-way ANOVA. Analyses used computer software (Sigmaplot 12.5, Systat Software, Inc.).

RESULTS

Effects of rotating WB during storage on poststorage PLT counts

Whole blood from seven subjects was stored for 12 days at 4°C without rotation. Predonation hematocrit (Hct) levels averaged $42 \pm 3\%$ and PLT counts $244 \times 10^9 \pm 65 \times 10^9/\text{L}$. Only $49\% \pm 12\%$ of the WB PLTs were recovered poststorage (Table 1). The poststorage WB PLT counts were only $4.6 \times 10^{10} \pm 2.0 \times 10^{10}$, which does not meet the FDA's requirement that a WB PLT concentrate should contain at least 5.5×10^{10} PLTs.¹⁸ However, the WB Hct levels were the same before and after storage, indicating that the differences in pre- versus poststorage PLT counts were not due to inadequate mixing of the WB samples.

By flow cytometry, a nonrotated WB unit was serially tested over 20 days of storage, and there was no increase in microparticles nor PLT aggregates. To reduce potential PLT adherence to the walls of the bag, we used a rotator to produce end-over-end rotation of the WB by holding both ends of the bag. PLT yields averaged between 71% to 76% of baseline values after 10 to 22 days of WB storage with no significant differences based on storage time ($p \leq 0.0001$ compared to 12-day nonrotated units). Poststorage PLT counts met FDA guidelines for PLTs per whole donation, that is, 7.2×10^{10} to 9.2×10^{10} (Table 1).

In vivo fresh and poststorage autologous radiolabeled PLT recoveries and survivals

For PLTs stored for 10, 12, or 15 days, there were no significant differences among stored PLT recoveries or stored recoveries as a percentage of each donor's fresh recoveries whether the WB was rotated or not during storage (Table 2). However, by 22 days of storage, there were significant decreases in PLT recoveries compared to the shorter storage times; that is, poststorage PLT recoveries averaged $25\% \pm 9\%$ for the combined 10, 12, or 15 days of storage versus $14\% \pm 0\%$ for 22 days of storage ($p = 0.02$). PLT survivals did not differ in absolute values or as a percentage of each donor's fresh survivals based on storage duration or whether the WB units were rotated or not during storage (Table 2).

In vitro data

Blood biochemistry

Blood gases, lactate, and glucose levels showed changes during WB storage similar to those seen in stored RBCs (Table 3).¹⁹ pH values decreased with storage, but none of them were lower than 6.6, which is well above the 6.2 to 6.3 levels that are associated with decreases in poststorage viability when PLTs are stored at 22°C.²⁰ Bicarbonate and PO_2 levels were relatively stable during storage while average pCO_2 levels increased from 76 mmHg before to 133 mmHg poststorage ($p < 0.001$). Mean glucose levels decreased from 402 mg/dL before storage to 303 mg/dL after storage ($p < 0.001$), and this was associated with mean increases in lactate levels from 2.1 to 14.7 mmol/L after storage ($p < 0.001$).

Cellular assays, PLT morphology, and microparticles

Pre- and poststorage Hct levels, WBC counts, and MPVs were relatively stable (Tables 1 and 4). As expected, there were significant

changes in PLT morphology after 4°C storage. Kunicki morphology scores were all 200 after storage, reflecting the known disc-to-sphere PLT transformation that occurs at 4°C (Table 4).^{4,11}

There were significant increases in the percentage of total cell marker microparticles before storage versus after storage, whether examining WB samples or PLT-poor plasma samples. Cell-specific markers for both RBC and PLT microparticles showed a significant increase over baseline values ($p < 0.001$). Additionally, PLT microparticles at 22 days of storage were significantly higher than after 10, 12, or 15 days storage ($p < 0.001$). Neither WBC (CD45+) microparticles nor endothelial cell marker microparticles (CD142+, tissue factor) changed significantly (data not shown).

Functional assays

There were decreases in mean pre- versus all poststorage ESC results (pre 20% vs. post 4% [$p < 0.001$]) and HSR responses (pre 54 vs. post 13 [$p < 0.001$]; Table 5). Measuring PLT aggregation responses to standard agonists (ADP, collagen, arachidonic acid) by light transmission aggregometry, there were decreased responses to collagen and arachidonic acid before storage versus after storage ($p < 0.05$; Table 5). However, the ADP responses were more variable. WB hemostatic function as measured by clotting variables during viscoelastic (TEG) measurements showed diminished function with storage; clotting time (R value) was increased while the amplitude of the clot was decreased ($p < 0.001$; Table 6). Clot lysis was also decreased (data not shown). In assays of thrombin generation, although the poststored PLTs had evidence of a significant increase in peak nmol/L thrombin generated in pre- versus poststorage samples and a decreased rate of initiation, the overall thrombin generation or ETP was not significantly changed (Table 6).

Activation assays

Annexin V binding on the PLT increased (mean pre 7% vs. post 54% [$p < 0.001$]) as did P-selectin expression (pre 8% vs. post 76% [$p < 0.001$]; Table 5). WB TAT was measured as a marker of activation of the coagulation system while PAP was measured as a marker of activation of the fibrinolytic system. Through all storage times, no significant changes were seen in TAT or PAP complexes; the TAT/PAP ratios were maintained (Table 7). Activated PLTs release both PAI-1 and sCD40L; both were significantly increased above baseline levels (Table 7, $p < 0.001$) with no significant differences between storage times.

TABLE 1. Total PLT count and Hct data before and after WB storage*

Storage time (days)	Number	Rotation†	PLT yields ($\times 10^{10}$)			Hct (%)		
			Baseline	After storage	% of Baseline	Baseline	After storage	% of Baseline
10	10	Yes	9.5 ± 2.1	7.2 ± 1.6	76 ± 10	34 ± 3	34 ± 3	99
12	7	No	9.4 ± 2.4	4.6 ± 2.0	49 ± 12	36 ± 3	36 ± 3	99
15	10	Yes	10.0 ± 1.0	7.6 ± 1.4	76 ± 14	34 ± 3	33 ± 2	99
22	3	Yes	12.9 ± 1.9	9.2 ± 0.7	71 ± 15	36 ± 4	35 ± 4	99

* Data are reported as mean $\pm$ 1 SD.

† Rotation was end over end during storage.

TABLE 2. Autologous radiolabeled PLT viability: fresh versus after WB storage*

Storage time (days)	Rotation†	Number	PLT recovery (%)			PLT survivals (days)		
			Fresh	Stored	% of Fresh	Fresh	Stored	% of Fresh
10	Yes	10	50 ± 10	26 ± 7	51 ± 7%	8.1 ± 1.0	1.3 ± 0.3	16 ± 4
12	No	6	54 ± 12	21 ± 8	40 ± 22%‡	8.3 ± 1.7	1.7 ± 0.5	22 ± 10
15	Yes	10	55 ± 11	27 ± 11	49 ± 16%	8.1 ± 0.1	1.2 ± 0.4	16 ± 6
22	Yes	3	56 ± 12	14 ± 0	25 ± 5%	7.8 ± 0.6	0.8 ± 0.3	10 ± 3

* Data are reported as mean ± 1 SD.

† Rotation was end over end during storage.

‡ One outlier with stored and fresh PLT recoveries of 28% versus 27%, respectively, was excluded from the results.

In examining markers of complement activation, elevations of C3a, C4d, C5a, and sC5d-9 demonstrated complement activation (Table 8). By two-way RMANOVA, all markers showed significant differences compared to baseline values with no difference between storage times. C5a and sC5d-9 displayed smaller increases over prestorage values; C3a increased approximately two-fold with storage and C4d showed the largest increases; however, no trend with storage time was found. Ten day storage gave a 5.7 fold increase while 20 day storage gave a 4.6-fold increase.^{21,22}

DISCUSSION

The purpose of this Phase I/II clinical trial was to determine the in vivo poststorage viability and in vitro function of PLTs stored in WB at 4°C. It was anticipated that the primary use of 4°C-stored WB would be to provide RBCs, plasma, and PLTs in forward-combat situations without the need to separate the blood into components. Therefore, these PLTs need to provide reasonable posttransfusion PLT increments, survivals of at least 1 day, and evidence of function for at least 6 hours to maintain hemostasis until surgical repair can be performed.

It has been known since the 1970s that PLTs maintain better poststorage viability when stored at 22°C versus 4°C.⁴⁻⁶ Interestingly, poststorage in vitro assays often suggested the PLTs performed better after storage at 4°C than at 22°C. As the survival of 4°C stored PLTs—even after only 1 day of storage—was usually 1 day or less, blood centers uniformly adopted 22°C storage because the majority of PLT transfusions were given to chronically thrombocytopenic hematology/oncology patients where reducing the frequency of PLT transfusions by giving PLTs with longer survivals of 6.5 ± 0.6 at the currently licensed 5 days would be very beneficial.

However, it is recognized that for surgical/trauma patients, as long as the stored PLTs circulate long enough after transfusion to participate in effective clot formation to support surgical repair of the injury, long-term PLT survival is not needed. Furthermore, highly activated PLTs may also promote immediate hemostasis and 4°C-stored PLTs may actually be the best PLT product to meet the needs of these patients.

Our studies have demonstrated that PLTs stored at 4°C within WB for 10 to 15 days must be held by the ends of the bag

and rotated end over end during storage to obtain PLT yields that average 76% ± 14% of baseline values. These rotated units have PLT counts that average $7.6 \times 10^{10} \pm 1.4 \times 10^{10}$, and these post-storage PLT counts are well within the FDA requirements of having 5.5×10^{10} PLT per PLT concentrate made from WB (Table 1). Furthermore, with rotation, in vivo poststored PLT recoveries average 27% ± 11%, and these results were 49% ± 16% of the same subject's fresh PLT recoveries of 55% ± 11%. Poststorage PLT survivals average 1.2 ± 0.6 days, which were 16% ± 1% of the same subject's fresh survivals of 8.1 ± 0.1 days (Table 2). These data compare very well to the longest prior 4°C PLT concentrate storage data of 3 days where stored PLT recoveries averaged 61% ± 7% (SE) and survivals 1.3 ± 0.1 days (SE).⁶ Interestingly, recent studies evaluating 15-day extended 4°C-stored apheresis PLTs showed PLT recoveries of only 12.3% and survivals of 0.8 ± 0.2 days, both significantly less than PLTs stored at 4°C in WB.²³

When the WB was not rotated during 12 days of storage, total PLT counts averaged only $4.6 \times 10^{10} \pm 2.0 \times 10^{10}$, which is well below FDA guidelines for PLT concentrates prepared from WB. Yet both the 12-day poststorage PLT recoveries and the 12-day poststorage survivals were similar to the rotated units stored for 10 or 15 days. However, after 22 days of WB storage at 4°C, in vivo poststorage PLT recoveries (14% ± 0%) were significantly decreased compared to the combined 10-, 12-, and 15-day-stored recoveries (25% ± 9%, $p < 0.001$). However, survivals were similar between the rotated and the nonrotated groups (1.3 ± 0.4-day survivals for the combined 10-, 12-, and 15-day rotated storage data versus 0.8 ± 0.3 days for 22-day storage; $p = 0.07$).

For the in vitro variables measured, microparticles and clot times were increased; PLT function as measured by HSR, ESC, and PLT aggregometry was decreased; and some complement factors were minimally increased, with a maximum 5.7-fold increase of C4d after 10 days of storage and PLT proteins sCD40L and PAI-1 were also increased. However, all these changes were within the norms of stored RBCs or were less than the decreases seen in 22°C-stored PLT concentrates. The observations here are within expected ranges as reported in the literature. The most valid comparisons should consider the function of 5-day-stored 22°C apheresis PLTs and also of reconstituted WB (RBC: fresh-frozen plasma:PLT of 1:1:1). For example, from the literature, it is clear that 22°C-stored apheresis PLTs have lost more than 95% of

TABLE 3. pH, blood gases, glucose, and lactate before versus after WB storage*

Storage time (days)	Number	Rotation†	pH		PO ₂ (mmHg)		PCO ₂ (mmHg)		HCO ₃ (mmol/L)		Glucose (mg/dL)		Lactate (mmol/L)	
			Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
10	10	Yes	7.03 ± 0.3	6.78 ± 0.02	37 ± 6	41 ± 9	79 ± 4	127 ± 10	21 ± 1	18 ± 1	411 ± 54	329 ± 47	2.5 ± 0.7	12.3 ± 0.7
12	7	No	7.06 ± 0.03	6.75 ± 0.17	37 ± 8	42 ± 8	72 ± 9	112 ± 13	20 ± 2	19 ± 2	393 ± 13	320 ± 17	1.9 ± 0.8	11.1 ± 1.1
15	10	Yes	7.06 ± 0.03	6.71 ± 0.04	41 ± 9	46 ± 12	72 ± 7	137 ± 10	20 ± 1	17 ± 1	400 ± 34	296 ± 28	1.7 ± 0.8	15.0 ± 1.6
22	3	Yes	7.02 ± 0.02	6.62 ± 0.01	37 ± 10	38 ± 3	82 ± 9	156 ± 13	21 ± 2	16 ± 1	408 ± 32	266 ± 10	2.4 ± 0.4	20.5 ± 1.5

* Data are reported as mean ± 1 SD.

† Rotation was end over end during storage.

TABLE 4. Cellular assays before and after WB storage*

Storage Time (Days)	Number	Rotation†	WBCs (×10 ⁹ /L)		MPV		Morphology (MP/L × 10 ⁶)		PLT microparticles (%)		RBC microparticles (PLT-poor plasma %)	
			Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
10	10	Yes	4.9 ± 0.9	4.3 ± 1.8	7.4 ± 0.8	7.0 ± 0.7	343 ± 38	200	1.9 ± 0.9	21.7 ± 19.0	5.6 ± 4.9	12.3 ± 4.5
12	7	No	5.8 ± 1.8	5.1 ± 1.7	7.0 ± 0.5	6.5 ± 0.5	375 ± 17	200	2.2 ± 0.9	25.7 ± 17.0	2.7 ± 2.2	11.1 ± 8.8
15	10	Yes	5.7 ± 1.9	4.6 ± 2.0	7.1 ± 0.7	6.7 ± 0.7	382 ± 13	200	1.2 ± 0.9	20.0 ± 10.4	2.9 ± 1.8	8.4 ± 9.6
22	3	Yes	5.1 ± 1.1	3.8 ± 0.8	6.4 ± 0.6	6.2 ± 0.5	377 ± 32	200	1.0 ± 0.8	36.3 ± 6.3	2.5 ± 1.5	4.5 ± 1.1

* Data are reported as mean ± 1 SD.

† Rotation was end over end during storage.

TABLE 5. PLT function and activation assays before versus after WB storage*

Storage time (days)	Number	Rotation†	ESC		HSR		ADP (maximum amplitude)		Collagen (maximum amplitude)		Arachidonic acid (maximum amplitude)		Annexin V binding (%)		P-selectin expression (%)	
			Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
10	10	Yes	24 ± 6	4 ± 3	66 ± 18	19 ± 8	4 ± 4	0.4 ± 0.8	14 ± 5	4 ± 3	6 ± 4	3 ± 2	6 ± 6	32 ± 13	11 ± 11	68 ± 17
12	7	No	23 ± 2	2 ± 1	42 ± 16	4 ± 4	5 ± 10	7 ± 7	29 ± 20	7 ± 5	5 ± 6	8 ± 6	6 ± 3	75 ± 16	6 ± 3	67 ± 17
15	10	Yes	15 ± 6	5 ± 4	51 ± 10	11 ± 10	4 ± 3	2 ± 1	14 ± 3	4 ± 3	7 ± 4	3 ± 3	5 ± 6	51 ± 21	10 ± 11	76 ± 10
22	3	Yes	19 ± 9	4 ± 2	59 ± 9	16 ± 7	0	1 ± 1	10 ± 1	1 ± 2	2 ± 1	0 ± 1	10 ± 8	56 ± 7	7 ± 2	92 ± 1

* Data are reported as mean ± 1 SD.

† Rotation was end over end during storage.

TABLE 6. WB thrombin generation and TEG assays before versus after WB storage

Storage time (days)	Number	Rotation*	TEG R (min)		TEG maximum amplitude (mm)		TGT peak (nmol/L thrombin)		TGT lag time (min)		TGT (ETP mm)	
			Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
10	10	Yes	5.5 ± 1.2	6.4 ± 1.2	64 ± 6	54 ± 6	63 ± 25	177 ± 74	9.1 ± 1.6	6.1 ± 1.5	1166 ± 375	1252 ± 410
12	7	No	4.1 ± 0.6	5.5 ± 0.4	56 ± 6	41 ± 9	81 ± 18	236 ± 58	7.5 ± 2.9	5.3 ± 1.6	1385 ± 264	1387 ± 160
15	10	Yes	4.4 ± 0.8	6.5 ± 0.9	60 ± 6	54 ± 6	83 ± 16	162 ± 37	9.1 ± 3.1	5.1 ± 1.0	1397 ± 308	1243 ± 234
22	3	Yes	6.4 ± 0.7	6.9 ± 0.3	56 ± 14	50 ± 1	109 ± 62	171 ± 54	9.0 ± 0.6	6.5 ± 3.2	1510 ± 614	1355 ± 204

* Rotation was end over end during storage.

TGT = Thrombin Generation Test.

PLATELETS STORED IN WB AT 4°C

TABLE 7. TAT, PAP, PAI-1, and sCD40L before versus after WB storage*

Storage time (days)	Number	Rotation†	TAT (ng/mL)		PAP (ng/mL)		PAI-1 (ng/mL)		sCD40L (ng/mL)	
			Pre	Post	Pre	Post	Pre	Post	Pre	Post
10	10	Yes	231 ± 191	252 ± 186	10.9 ± 7.7	12.0 ± 6.9	24.6 ± 26.5	58.4 ± 45.5	2.7 ± 1.7	3.6 ± 1.7
12	7	No	466 ± 140	504 ± 111	14.5 ± 13.8	10.5 ± 5.1	12.5 ± 7.7	49.2 ± 17.7	2.6 ± 1.2	3.9 ± 1.5
15	10	Yes	405 ± 131	448 ± 119	13.9 ± 10.8	11.2 ± 3.8	7.4 ± 2.4	39.5 ± 9.7	2.0 ± 1.9	3.8 ± 2.1
22	3	Yes	391 ± 21	446 ± 65	8.1 ± 0.9	9.4 ± 2.0	11.0 ± 2.3	75.6 ± 20.8	2.1 ± 1.2	4.4 ± 0.7

* Data are reported as mean ± 1 SD.

† Rotation was end-over-end during storage.

TABLE 8. Complement factors C3a, C4d, C5a, and sC5d-9 before versus after WB storage*

Storage Time (Days)	Number	Rotation†	C3a(ng/ml)		C4d(ng/ml)		C5a(ng/ml)		sC5d-9(ng/ml)	
			Pre	Post	Pre	Post	Pre	Post	Pre	Post
10	10	Yes	128 ± 28	201 ± 53	0.9 ± 0.8	5.4 ± 1.8	7.7 ± 2.5	9.0 ± 2.8	159 ± 35	207 ± 61
12	7	No	155 ± 44	269 ± 160	1.3 ± 0.6	5.5 ± 1.5	7.8 ± 2.7	10.9 ± 4.2	168 ± 45	239 ± 144
15	10	Yes	115 ± 31	260 ± 179	1.3 ± 0.7	6.5 ± 2.4	8.0 ± 4.3	11.3 ± 5.0	129 ± 51	196 ± 199
22	3	Yes	90 ± 19	252 ± 22	1.8 ± 0.7	8.2 ± 0.8	8.2 ± 1.6	10.6 ± 1.4	41 ± 19	81 ± 18

* Data are reported as mean ± 1 SD.

† Rotation was end-over-end during storage.

their response to collagen by 5 days of storage.²⁴ However, after 10 to 15 days of storage in this study, the collagen response of 4°C WB PLTs ranged from 11% to 17% of their initial values. An even better comparison would be of stored WB to reconstituted blood products. In two studies examining WB reconstituted from stored products, it is clear that PLT function measured by aggregometry is nearly nonexistent.²⁵⁻²⁷ The hemostatic function of stored WB by thromboelastometry was within normal parameters up until Day 15,¹⁴ while the reconstituted WB samples composed of an amalgamation of all transfused products given a patient during surgery were just below normal clotting parameters.²⁷

In summary, this study demonstrates that PLTs stored in WB at 4°C for up to 15 days have acceptable in vivo PLT recoveries and survivals that will meet the short-term needs for surgical repair of traumatic injuries. We did not test storage times between 15 and 22 days so, without additional studies, we do not know that 15 days is the longest storage time to give results comparable to 10 days of storage. Furthermore, the viability of PLTs stored in WB at 4°C may be even better than comparably stored 4°C apheresis PLTs.²³ PLT counts decreased in the 4°C-stored blood bags but, by rotating the WB end over end during storage, poststorage PLT counts achieved levels mandated by FDA for PLT concentrates prepared from WB. If rotation is not feasible, PLTs stored in WB for 12 days had comparable poststorage viability and function as rotated units stored for up to 15 days. However, the 4°C-stored WB PLTs suffer decrements in functional tests compared to their baseline or prestorage values. Other in vitro assays suggest that these stored PLTs are highly activated and may be very appropriate to meet the immediate hemostatic needs of injured patients. Cold-stored WB could be a key component to meet far-forward combat hemostatic resuscitation needs.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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Pathogen-Reduced, Platelet Additive Solution, Extended Stored Platelets (PREPS)
 (Previously Pathogen-Reduced, Plasmalyte-Extended Stored Platelets)
 Award Number W81XWH-12-1-0441 Log Number 11105004987987431
 Final Report
 15-SEP-2012 to 14-SEP-2021

Appendix C - Research Projects - #2 Cold Apheresis Platelets in Isoplate or Intersol (CAPI) and #3 Cold Apheresis Platelets in Plasma (CAPP) – Abstracts with Poster Presentation and Publications.

#2a. Military Health System Research Symposium (MHSRS); Kissimmee, FL August 2017

In Vivo Viability of Extended 10-Day 4°C Stored Autologous Platelets

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#2b. Military Health System Research Symposium (MHSRS); Kissimmee, FL August 2018

In Vivo Viability of Extended 20-Day 4°C Stored Autologous Platelets

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#2c. American Association of Blood Banks Annual Meeting; Boston, MA October 2018

Effects of Extended Cold-Storage on Platelet Function, Apoptosis and In Vivo Characteristics in Healthy Human Subjects

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#2d. M. Stolla^{1,2}, L. Fitzpatrick¹, I. Gettinger¹, S. Bailey¹, E. Pellham¹, T. Christoffel¹, S. Slichter^{1,2}

In Vivo Viability of Extended 4°C Stored Autologous Apheresis Platelets. *Transfusion* 2018; 58(10):2407-2413. PMID: 30203458

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#2e. American Association of Blood Banks Annual Meeting; San Antonio, TX October 2019

Effects of Platelet Additive Solution on In Vivo and In Vitro Characteristics in 4°C Stored Platelets. M. Stolla^{1,2}, L. Fitzpatrick¹, S. Bailey¹, L. Fang¹, I. Gettinger¹, E. Pellham¹, T. Christoffel¹

Transfusion 2019; 59(10): (S3):21A.

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#2f. M. Stolla^{1,2}, S. Bailey¹, L. Fang¹, L. Fitzpatrick¹, I. Gettinger¹, E. Pellham¹, T. Christoffel¹

Effects of Storage Time Prolongation on In Vivo and In Vitro Characteristics of 4°C Stored Platelets.

Transfusion 2020; 60(3):613-621. PMID: 32017135

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#2g. S. Bailey, L. Fang, L. Fitzpatrick, D. Byrne, E. Pellham, M. Stolla. Effect of PAS-C on Short-Term Cold-Stored Platelets in Vitro and in Vivo. *Haematologica*. Accepted for publication Dec.2021.

In Vivo Viability of Extended 10-Day 4°C Stored Autologous Platelets

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Abstract: Background: The limited 5-day storage time of room temperature (22° C) stored platelets severely limits platelet availability at far-forward combat medical facilities and rural civilian hospitals. Prior studies comparing 3-day, 22°C versus 4°C stored platelets showed comparable post-transfusion platelet recoveries of 40 ± 3% and 40 ± 5%, respectively, but survival was significantly different 7.9 ± 0.2 days and 1.0 ± 0.1 days (p<0.01). However, for the treatment of actively bleeding patients requiring immediate hemostasis, platelet survival of 1 day or less may be adequate. Methods: Nineteen normal subjects had a 2-unit apheresis procedure. One unit was stored at 4°C in plasma for the FDA approved 3 days ("control unit"). The other "test" unit was stored for 10 days in plasma with either Intersol (n=5) or Isolate (n=3) at 4°C. An additional group was stored for 15 days in plasma (n=5). At the beginning and end of non-agitated storage, samples were drawn for in vitro parameters. At the end of non-agitated storage both units were radiolabeled with ¹¹¹Indium and infused into their respective donors. Pre- and post-infusion samples were drawn to determine platelet recoveries and survival. Results: Platelet counts of the "control" units averaged 3.59 ± 0.31 x 10¹¹ pre storage, and 3.57 ± 0.38 x 10¹¹ post-storage (99 ± 3% of pre-storage). For the "test" plasma, Intersol, and Isolate, and 15 day plasma units, post-storage platelet counts averaged 2.86 ± 0.39 x 10¹¹, 2.82 ± 0.35 x 10¹¹, 2.52 ± 1.28 x 10¹¹, and 2.42 ± 0.36 x 10¹¹, respectively (80 ± 7%, 107 ± 12%, 90 ± 39%, and 72 ± 14% of pre-storage values). Most in vitro parameters did not differ significantly between 10 day stored plasma, Intersol, and Isolate, except for glucose and lactate, which may be due to plasma removal. 15 day plasma storage led to significantly more microparticle formation. For the "control" units, post-storage recoveries averaged 43 ± 11% and survival 2 ± 0.4 days. For the "test" 10 day plasma, Intersol, Isolate, and 15 day plasma units, post-storage recoveries averaged 24 ± 8%, 18 ± 4%, and 8 ± 2%, and 11 ± 3% respectively (55 ± 11%, 43 ± 6%, 21 ± 8%, and 30 ± 3% of the same subject's 3-day data). As a percentage of their 3-day recoveries, both the plasma and Intersol units were significantly greater than the Isolate units (p<0.001 and p<0.05, respectively), but there were no differences between the plasma and Intersol units. Post-transfusion survival for the 10-day platelets stored in plasma, Intersol, Isolate, and 15 day stored in plasma averaged 1.2 ± 0.3 days, 1.1 ± 0.3 days, 0.9 ± 0.8 days, and 0.7 ± 0.2 days respectively (59 ± 12%, 56 ± 8%, 48 ± 42%, and 36 ± 7% of the same subject's 3-day data). There were no significant differences in platelet activation. Platelets stored for 10 days showed the ability to respond to agonists with integrin activation, which required active inside out signaling and integrin conformational change, indicating that they could contribute to hemostasis in vivo immediately upon transfusion.

Platelet Yield

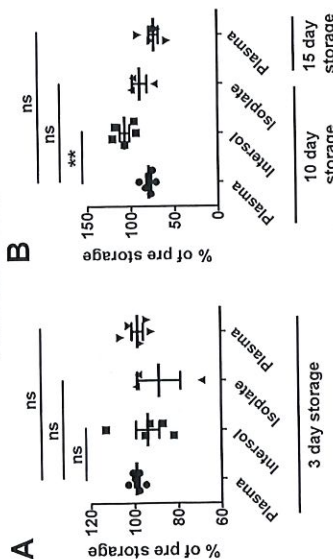


Figure 1: Actual platelet yield [Total platelet yield in the component, calculated by multiplying the platelet count of the sample times the volume of the component (platelet count x component volume = actual platelet yield)]. Platelets were stored in either plasma alone (Plasma, black circles and downward triangles), or in a 65% platelet additive solution, 35% plasma mixture (Intersol, black squares, or Isolate, black, upward triangles). A. Platelets were stored for three days at 4°C. Results are shown as percentage of pre-storage (fresh). No significant differences were seen. B. 10 day and 15 day stored (as indicated) platelets as percentage of 3 day platelets. ns= not significant, **p=0.0012.

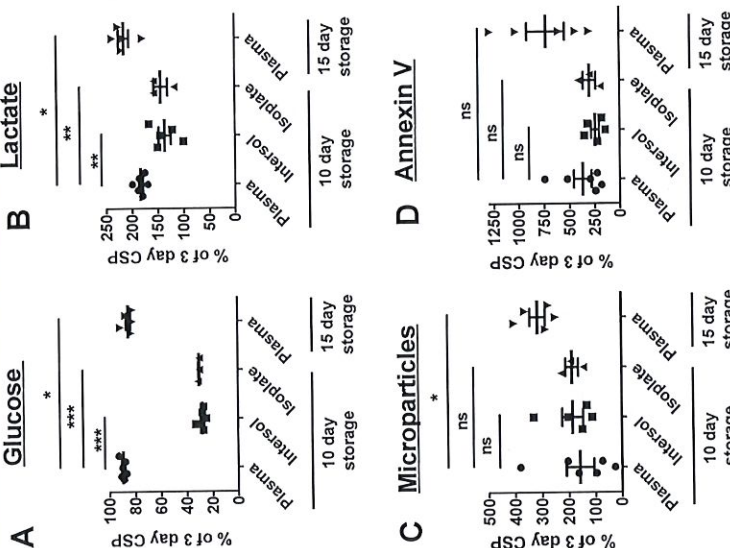


Figure 2: In vitro parameters: Platelets were stored in either plasma alone (Plasma, black circles and downward triangles), or in a 65% platelet additive solution, 35% plasma mixture (Intersol, black squares, or Isolate, black, upward triangles) at 4°C, for either 10 or 15 days as indicated. A, glucose and B, lactate determined by blood gas analysis. C, CD61-positive microparticles determined by flow cytometry. D, Annexin V positive events measured by flow cytometry. E, CD62P-positive (P-selectin) events measured by flow cytometry. All results are shown as percentage of 3 day 4°C stored platelets. *p<0.05, **p<0.01, ***p<0.0001, ns= not significant.

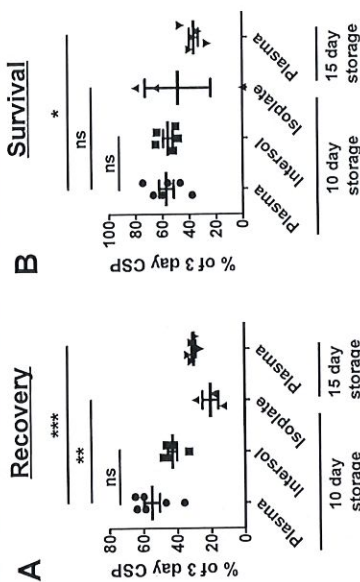


Figure 3: In vivo parameters: Healthy human subjects received autologous platelet transfusions, stored at 4°C. (Plasma, black circles and downward triangles), or in a 65% platelet additive solution, 35% plasma mixture (Intersol, black squares, or Isolate, black, upward triangles). A, Recovery of transfused platelets at 1 hour time point. B, Survival of transfused platelets. All results given as percentage of 3 day 4°C stored platelets. *p<0.05, **p<0.01, ***p<0.0001, ns= not significant.

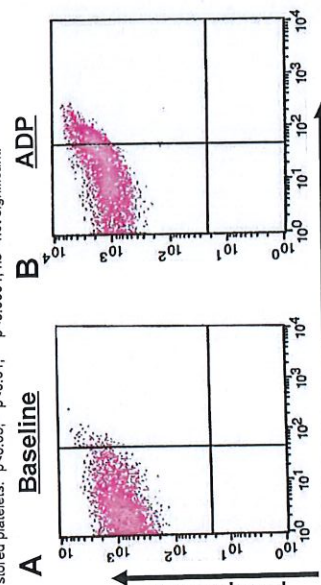


Figure 4: Platelet integrin activation: 4°C-stored platelets were stored for 10 days and either left unstimulated (A, baseline, left panel) or stimulated with 10uM ADP (final concentration) (B, ADP right panel). The activation-dependent alphaIIb beta3-integrin antibody PAC-1 was incubated with both samples along with an activation independent beta3-chain antibody (y-axis, stains all platelets).

Conclusions:

- Storage in Intersol led to a significantly higher platelet yield after 10 day storage compared with plasma.
- Most in vitro platelet activation parameters did not differ significantly between 10d plasma, Intersol, and Isolate. As expected, glucose and lactate were significantly lower in Intersol and Isolate because of plasma removal.
- Post-storage recoveries for platelets stored in plasma or Intersol were significantly greater than for platelets stored in Isolate or stored for 15 days in plasma.
- Platelets, stored for 10 days at 4°C in plasma respond to agonists with inside out signaling and subsequent integrin activation, indicating that they could participate immediately in hemostatic processes.
- Platelet storage for 10 days at 4°C in either plasma or Intersol could be used to expand the available supply of platelets to treat bleeding patients.

The authors have no conflict of interest to disclose

Abstract

Background: The 5-day storage limit of room temperature (22° C)-stored platelets severely constrains platelet availability. Cold (4°C)-stored platelets (CSP) are currently evaluated by the military for active bleeding patients and represent an intriguing storage option for far forward military scenarios and remote hospital locations. We do not know how well platelets circulate and function after extended storage at refrigerated temperatures. In this study, we investigate how cold-storage in plasma for up to 20 days affects platelet viability and function compared to fresh platelets.

Methods: Eighteen healthy human subjects underwent an apheresis platelet collection. The CSP "test" unit was stored for either 5, 10, 15, or 20 days in plasma at 4°C. Platelet samples for in vitro platelet function testing were extracted at baseline (Day 0, "fresh"), and after the designated storage period ("5,10,15,20 days"). After storage, all units were radiolabeled and transfused into their respective donors. All donors came back after a week to provide a fresh sample for radiolabeling and their reinfusion. We did not include the 20 day data in the statistical analysis because this group is currently work in progress and only 2 subjects (n=2) have been completed. The other groups included healthy human subjects as follows: 5 day storage n=5, 10 day storage n=6, and the 15 day storage n=5.

Results: Over storage time, platelet recoveries declined significantly from the respective fresh samples to 34±10% for 5 days (p<0.0001), 18±7% for 10 days (p<0.0004), 13±3% for 15 days (p<0.0001), and 8±0% for 20 days. There was a significant reduction in recovery from 5 to 10 days (p<0.02), but not between 10 and 15 days (p=0.15). As expected, platelet survival was significantly lower compared with their respective fresh samples (all p<0.001) and dropped from 5 to 10 days (1.6±0.5 days versus 0.8±0.2 days, p=0.02), but not between 10 and 15 days. The platelet survival remained stable between 15 to 20 days. The platelet yield dropped significantly when 5,10,15 days were compared to their respective fresh samples (all p<0.01), however remained stable between 5 and 20 days (3.11±0.71x10¹¹ versus 2.21±0.61x10¹¹, p=0.01, 2.11±0.74x10¹¹, p=0.006, 2.17±0.47x10¹¹, p=0.01 and 2.35±0.13x10¹¹ respectively). Glucose concentrations decreased significantly from fresh to 10 and fresh to 15 day stored (both p=0.0003), and decreased significantly from 5 to 15 days and dropped further after 15 days (349±20mg/dl versus 291±22mg/dl, p=0.02, and 267.5±22, respectively). Lactate concentrations increased significantly when compared to respective fresh samples (all p<0.0003), and increased significantly from 5 to 10, and 5 to 15 days (4±0.6 versus 5.6±0.8, p=0.06, and 4±0.6 versus 8.4±1.3, p=0.0001, respectively). Lactate increased further from 15 to 20 days. All markers of platelet activation increased significantly over time at storage in the cold when compared to respective fresh sample (all p<0.05). However, while microparticles and P-selectin increased significantly from 5 to 15 days (5.06±2.18% versus 10.62±4.36%, p=0.04 and 15.65±5.5% versus 45.1±6.5%, p=0.002 respectively), phosphatidyl serine exposure (measured by Annexin V binding) increased from 5 to 10 days (6.8±3.1% versus 16.5±8.4%, p=0.03) and from 5 to 15 days (6.8±3.1% versus 23.4±8.9%, p=0.002). While no statistical analysis was performed with 20 day group yet (n=2), all platelet activation markers markedly increased between 15 and 20 days.

Conclusions: We performed the first reported studies with extended storage cold (4°C)-plasma-stored apheresis platelets up to 20 days with in vitro tests and a fresh comparator. We show that there is continuous loss of recovery up to 20 days of storage, while platelet survival reach a low plateau after 10 days. Platelet yields drop early and do not further decrease over storage time. These data are suggestive of early microaggregate formation without significant increase over prolonged storage. We found that cold-stored platelets show signs of ongoing metabolism even at refrigerated storage conditions. In vitro markers of pre-activation, including P-selectin expression, microparticle release, and phosphatidyl serine exposure increased significantly. Taken together, storage up to 15 days in the cold appears to yield acceptable in vitro and in vivo results.

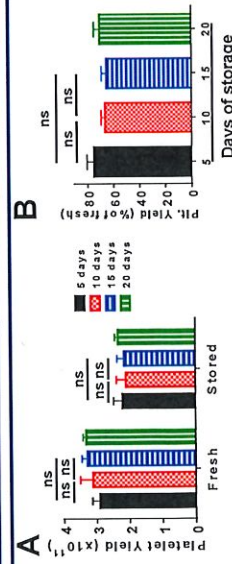


Figure 1: Platelet yields: Total platelet yield in the component was calculated by multiplying the platelet count of the sample times the volume of the component (platelet count x component volume = actual platelet yield). (A) Platelets were sampled at baseline (fresh), or after designated storage period. (B) Results shown as percentage of fresh samples. ns= not significant

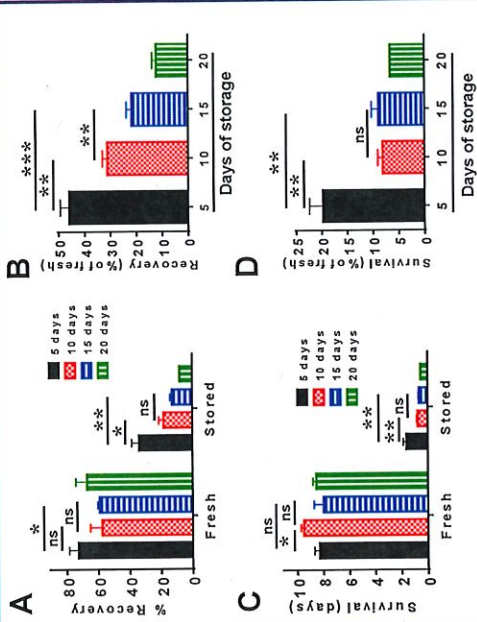


Figure 2: In vivo parameters: Healthy human subjects received either fresh or 4°C-stored autologous platelet transfusions. (A) Recovery of transfused fresh or 4°C-stored platelets at 2 hour time point. (B) Recovery of transfused fresh or stored platelets as percentage of fresh sample. (C) Survival of fresh or stored platelets in days. (D) Survival of transfused platelets as percentage of fresh. *p<0.05, **p<0.01, ***p<0.001, ns= not significant.

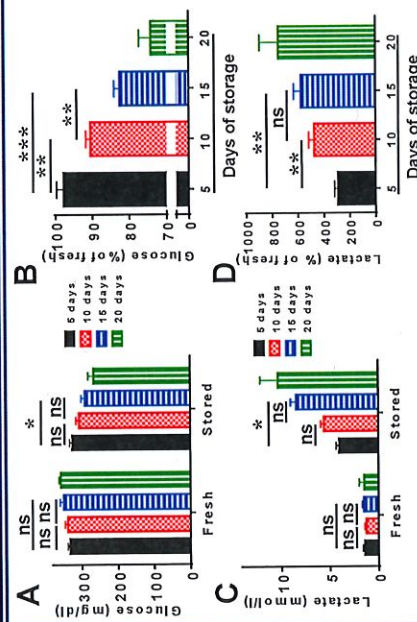


Figure 3: In vitro metabolic parameters: Units were sampled at baseline (fresh) or after designated storage period and tested for glucose or lactate by a commercially available blood gas instrument. (A) Absolute glucose values at baseline (fresh) and after designated storage period. (B) Glucose values after storage as percentage of fresh samples. (C) Absolute lactate values at baseline (fresh) and after designated storage period. (D) Lactate values after storage as percentage of fresh samples. *p<0.05, **p<0.01, ***p<0.001, ns= not significant.

The authors have no conflict of interest to disclose

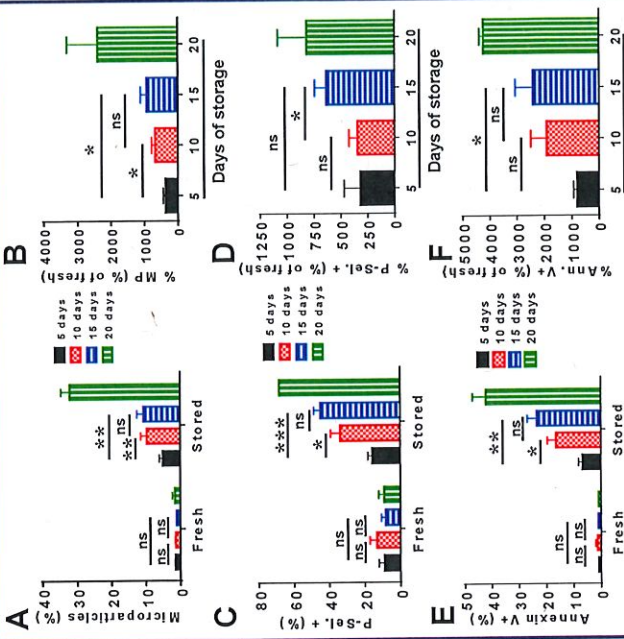


Figure 4: In vitro activation parameters: Platelets were stored in plasma at 4°C and sampled at baseline (fresh) or after designated storage period. (A) CD61-positive microparticles measured by flow cytometry. (B) Microparticles as percentage of fresh samples. (C) P-selectin (CD62P)-positive events measured by flow cytometry. (D) P-selectin events as percentage of fresh samples. (E) Annexin V positive events measured by flow cytometry. (F) Annexin V as percentage of fresh samples. *p<0.05, **p<0.01, ***p<0.0001, ns= not significant.

Conclusions:

- Recovery continues to decline for up to 20 days of storage (maximum tested), while platelet survivals reach a low plateau after 10 days.
- All in vitro platelet activation parameters increase significantly over the course of storage. Our preliminary data of 20 days of storage indicate that there is an especially large increase from 15 to 20 days of storage.
- The metabolic parameters glucose and lactate indicate that there is continuous metabolic activity over 20 days in the cold: Glucose continues to decline from fresh to 20 days, and lactate increases from fresh to 20 days of storage.
- Platelet storage for 15 days at 4°C in plasma could be used to expand the available supply of platelets to treat bleeding patients.

Effects of extended cold-storage on platelet function, apoptosis and in vivo characteristics in healthy human subjects

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Abstract

Background: Cold-stored platelets are currently under investigation for transfusion in actively bleeding patients. The three day storage limit of the FDA variance severely limits cold-stored platelet availability for clinical applications, and far forward military scenarios. The effects of cold-storage on platelet apoptosis have not been investigated thus far. We sought to test which in vitro parameters correlate best with in vivo recovery.

Study Design/Method: Twenty two healthy human subjects underwent apheresis platelet collections. The cold-stored platelet (CSP) "test" unit was stored for either 5, 10, 15 or 20 days in plasma at 4°C. Platelet samples for in vitro platelet tests were extracted on the day of donation and after the designated storage period. After storage, all units were radiolabeled and transfused into their respective donors. All donors came back after a week to provide a fresh sample for radiolabeling and retransfusion.

Results/Findings: Over storage time, platelet recoveries (% of fresh) declined significantly from 5 to 10 days ($p=0.003$), from 10 to 15 days ($p=0.009$), and 15 to 20 days ($p=0.019$). Platelet survival decreased significantly from 5 days to 10 days ($p=0.001$), but not thereafter (all as % of fresh). Measurements of integrin activation revealed a marked preactivation over storage comparable to collagen-stimulated fresh platelets. Of note, stored platelets retained their ability to further activate integrins, although this ability was declining over storage. Mitochondrial membrane integrity decreased only non-significantly over storage time. Similarly, apoptosis measured by activation of the effector caspase 3/7 increased non-significantly over storage time. Microparticles increased significantly from 5 to 10 days ($p=0.042$), P-selectin increased significantly from 10 to 15 days ($p=0.049$), and phosphatidyl serine exposure (measured by Annexin V binding) increased significantly from 15 to 20 days ($p=0.025$). We found best correlations between in vivo recovery (% of fresh) and AHC03, Agglucose, Alactate, Amicroparticles, ΔP-selectin, ΔAnnexin V (all correlation coefficients of at least ± 0.72 , and $p<0.01$).

Conclusion: We performed the first studies with extended storage cold (4°C)-plasma-stored apheresis platelets up to 20 days and a fresh comparator. We show that there is continuous loss of recovery up to 20 days of storage. Overall, continuous platelet activation is a hallmark of cold-stored platelets, while markers for caspase activation and mitochondrial membrane integrity show a delay of apoptosis during cold-storage. Platelet function tests show preserved integrin activation comparable to fresh up to 20 days of storage. Integrin activation and apoptosis markers did not correlate with recovery, but metabolic parameters and in vitro pre-activation parameters did. Taken together, storage up to 15 days in the cold appears to yield acceptable in vitro and in vivo results.

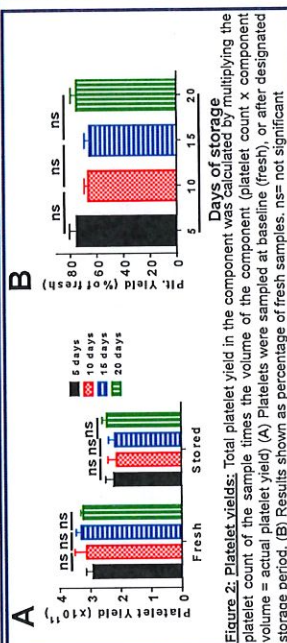


Figure 2: Platelet yields: Total platelet yield in the component was calculated by multiplying the platelet count of the sample times the volume of the component (platelet count x component volume = actual platelet yield). (A) Platelets were sampled at baseline (fresh), or after designated storage period. (B) Results shown as percentage of fresh samples. ns = not significant.

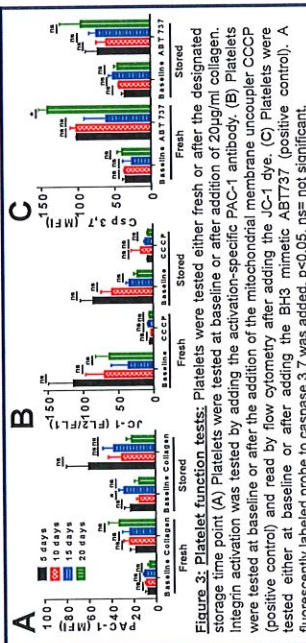


Figure 3: Platelet function tests: Platelets were tested either fresh or after the designated storage time point (A). Platelets were tested at baseline or after addition of 20µg/ml collagen. Integrin activation was tested by adding the activation-specific PAC-1 antibody. (B) Platelets were tested at baseline or after the addition of the mitochondrial membrane uncoupler CCCP (positive control) and read by flow cytometry after adding the JC-1 dye. (C) Platelets were tested either at baseline or after adding the BH-3 mimetic AB737 (positive control). (D) Fluorescently labeled probe to caspase 3/7 was added. $p<0.05$, ns = not significant.

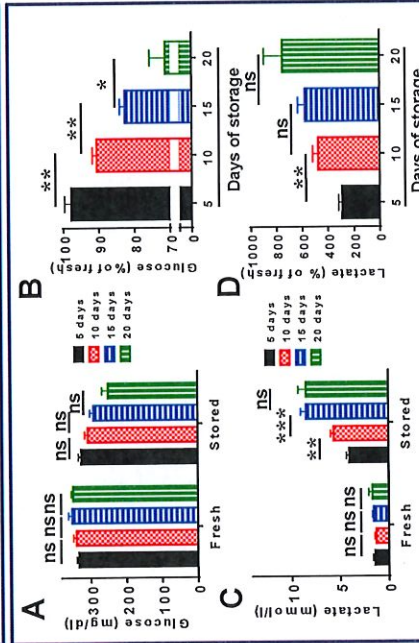


Figure 4: In vitro metabolic parameters: Units were sampled at baseline (fresh) or after designated storage period and tested for glucose or lactate by a commercially available blood gas instrument. (A) Absolute glucose values at baseline (fresh) and after designated storage period. (B) Glucose values after storage as percentage of fresh samples. (C) Absolute lactate values at baseline (fresh) and after designated storage period. (D) Lactate values after storage as percentage of fresh samples. $p<0.001$, $p<0.01$, $p<0.05$, ns = not significant.

The authors have no conflict of interest to disclose

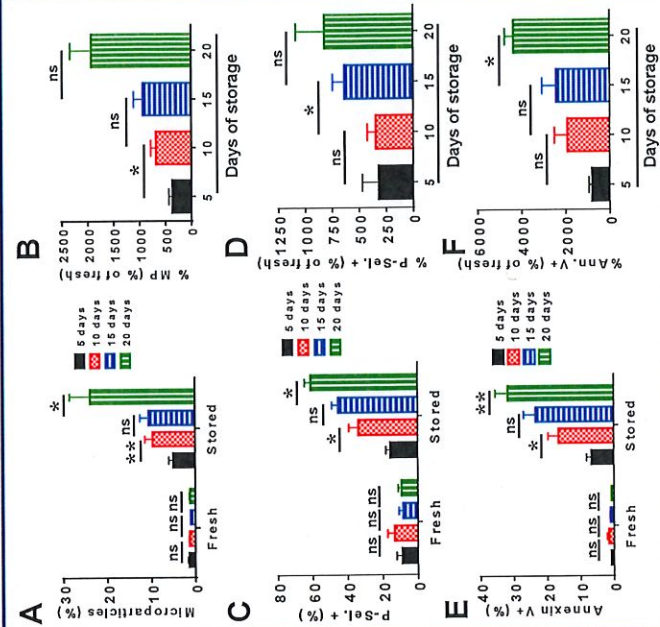


Figure 5: In vitro activation parameters: Platelets were stored in plasma at 4°C and sampled at baseline (fresh), or after designated storage period. (A), CD61-positive microparticles measured by flow cytometry. (B), Microparticles as percentage of fresh samples. (C), P-selectin (CD62P)-positive events measured by flow cytometry. (D), P-selectin events as percentage of fresh samples. (E), Annexin V positive events measured by flow cytometry. (F), Annexin V as percentage of fresh samples. $p<0.05$, $p<0.01$, $p<0.0001$, ns = not significant.

Conclusions:

- Recovery continues to decline for up to 20 days of storage (maximum tested), while platelet survivals reach a low plateau after 10 days.
- All in vitro platelet activation parameters increase significantly over the course of storage. Microparticles show an especially large increase from 15 to 20 days of storage.
- The metabolic parameters glucose and lactate indicate that there is continuous metabolic activity over 20 days in the cold: Glucose continues to decline from fresh to 20 days, and lactate increases from fresh to 20 days of storage.
- In vivo recovery correlated best with metabolic parameters and pre-activation parameters, not with apoptosis and functional marker (integrin activation).
- Platelet storage for 15 days at 4°C in plasma could be used to expand the available supply of platelets to treat bleeding patients.

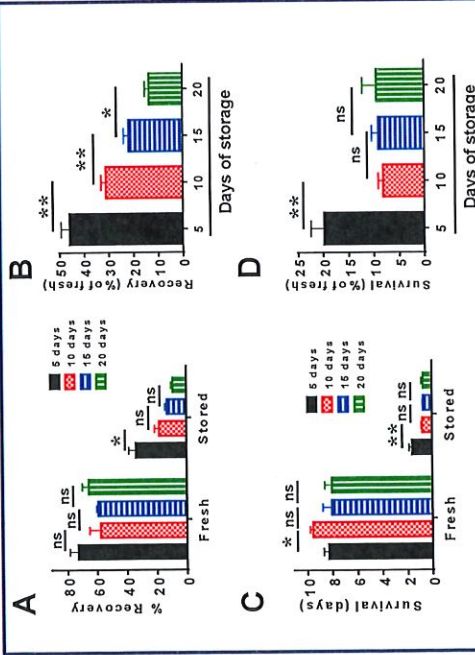


Figure 6: In vivo parameters: Healthy human subjects received either fresh or 4°C stored autologous platelet transfusions. (A) Recovery of transfused fresh or stored platelets at 2 hour time point. (B) Recovery of stored platelets as percentage of fresh sample. (C) Survival of fresh or stored platelets in days (D) Survival of transfused platelets as percentage of fresh. $p<0.01$, $p<0.001$, ns = not significant.

BLOOD COMPONENTS

In vivo viability of extended 4°C-stored autologous apheresis platelets

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BACKGROUND: The current 5-day storage time of room temperature (22°C)-stored platelets (RSPs) severely limits platelet (PLT) availability. Extended cold (4°C)-stored PLTs (CSPs) are currently being investigated for actively bleeding patients. However, we currently do not know how to best store PLTs in the cold for extended periods of time. In this study, we investigate how storage in plasma and PLT additive solutions (PASs) affects PLT viability in vivo.

STUDY DESIGN AND METHODS: Twenty normal subjects had a 2-unit hyperconcentrated apheresis PLT collection. One unit was stored at 4°C in plasma for 3 days ("control unit"), and the CSP "test" unit was stored for 10 or 15 days in plasma or 10 days in 35% plasma with either 65% Intersol or Isoplate. After storage, all units were radiolabeled and transfused into their donors.

RESULTS: For 10-day storage, both the plasma and the Intersol units had significantly better PLT recoveries than the Isoplate units ($24\% \pm 8\%$ vs. $11\% \pm 3\%$ [$55\% \pm 11\%$ vs. $21\% \pm 8\%$ as percentage of control data], $p = 0.002$; and $18\% \pm 4\%$ vs. $11\% \pm 3\%$ [$43\% \pm 6\%$ vs. $21\% \pm 8\%$ as percentage of control data], $p = 0.004$, respectively). There was a trend for lower PLT recoveries with Intersol compared to plasma ($p = 0.056$). PLT survivals and most in vitro measurements did not differ significantly among the units.

CONCLUSIONS: While the in vitro variables suggest largely comparable results between plasma and PASs, in vivo recoveries were higher with plasma compared with both Intersol and Isoplate ($p = 0.057$ and $p = 0.002$, respectively). Whether this difference leads to clinically relevant differences in hemostatic efficacy remains to be determined.

Most US blood banks store platelets (PLTs) at room temperature under gentle agitation with a maximum storage time of 5 days unless additional in vitro bacterial testing is done, which permits 7-day storage. Previous studies demonstrated that storing PLTs in the cold (4°C, CSP) resulted in a significant reduction in both in vivo recoveries and survivals compared with room temperature-stored PLTs (RSPs) stored for the same times.¹ Subsequent studies with CSPs identified a clearance mechanism involving GPIb clustering on the PLT surface and in vivo binding to complement Type 3 receptors with subsequent hepatocyte internalization.^{2,3} In vitro studies suggest that 4°C-stored PLTs have superior functionality compared with RSPs.⁴⁻⁸ Furthermore, storage of PLTs in the cold (4°C) has the advantage of potentially prolonging storage times while reducing posttransfusion infections. Room-temperature storage is limited to 5 days because bacterial growth and septic reactions increase over time.⁹ This limited shelf life leads to periodic shortages on the one hand and frequent outdates on the other.¹⁰ In contrast to that of red blood cells (RBCs), PLT usage is still slightly increasing according to recently published data.¹⁰ Having an additional PLT inventory with extended storage in the cold for specifically targeted

ABBREVIATIONS: CSPs = cold-stored platelets; PAS(s) = platelet additive solution(s); RSPs = room temperature-stored platelets.

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patient populations could lead to both greater PLT availability and fewer outdates.

When first introduced, PLT transfusions were predominantly used for the prophylactic transfusion support of hematology/oncology patients with hypoproliferative thrombocytopenia. These patients benefit from prolonged post-transfusion PLT survivals to decrease transfusion frequency. However, a recent analysis showed that as many as 50% of the PLTs transfused are given to non-hematology/oncology patients (i.e., trauma, surgery, intensive care unit, and general medicine patients) who often require only short-term hemostatic support.¹⁰ CSPs corrected the bleeding time quicker than RSPs in patients with thrombocytopenia.⁸ However, the data on *in vivo* efficacy of 4°C-stored PLTs are not uniformly in favor of 4°C storage. We previously showed that the majority of bleeding time measurements in patients with thrombocytopenia transfused with CSPs remained markedly prolonged, while most measurements in the control group (stored at 22°C) improved.¹¹ In addition, Filip and Aster¹² found that 4°C-stored PLTs were less effective after 72 hours of storage when compared to 22°C-stored PLTs. More studies are needed to conclusively settle the debate if CSPs function better than RSPs *in vivo*.

Two randomized, controlled clinical trials are available suggesting that CSPs are more hemostatically active than RSPs. A trial in pediatric cardiac surgery patients showed that whole blood stored in the cold (4°C) was more effective in reducing blood loss during open heart surgery compared with reconstituted whole blood containing RSPs (22°C). Interestingly, patients who received cold-stored whole blood had significantly better aggregometry responses than RSP-containing reconstituted whole blood.¹³ Preliminary analysis of an ongoing Norwegian randomized, pilot trial in open-heart surgery patients showed that patients who received up to 7-day-stored CSPs in a PLT additive solution (PAS; T-PAS, Terumo BCT) had significantly reduced postoperative blood loss with CSPs compared with RSPs.¹⁴

At present, the Food and Drug Administration (FDA) allows transfusion of 3-day CSPs (whole blood-derived pooled PLTs or apheresis PLTs) only for actively bleeding patients.¹⁵ CSPs have not been widely utilized presumably due to a lack of data suggesting superior efficacy in the target population of bleeding patients. Furthermore, the 3-day storage limit is even shorter than the limit for RSPs, thus offering even less flexibility. Studies to prolong storage time to 5 days and now 7 days with additional bacterial testing were only performed on RSPs because of the reduced *in vivo* viability of CSPs.

PLT storage in PAS have been shown to allow for prolongation of the storage time in RSP storage studies.¹⁶ At present, only two PASs are licensed by the FDA for room temperature storage, and they have not been widely adopted.^{5,14,16} It is unknown how PAS may affect *in vivo* viability of PLTs stored at 4°C. In this study, we investigated the *in vivo* viability of autologous radiolabeled extended CSPs in plasma

versus PAS in normal subjects along with *in vitro* tests currently required by the FDA for PLT licensing.

MATERIALS AND METHODS

Preparation of control and test PLTs

A double hyperconcentrated apheresis PLT unit was collected from 20 healthy adult subjects using an automated blood collection system (Trima Accel, Terumo BCT). After collection, the unit was split into two equal portions, and 1 unit was resuspended in 100% plasma for 3-day 4°C storage (control unit was stored for the longest FDA storage time), and the other unit was stored for an extended period of time at 4°C (test unit). The test unit was suspended in 100% plasma or 35% plasma and 65% PAS (either Isoplate [PAS-F, Terumo BCT] or Intersol [PAS-3, Fenwal, Inc.]). Both control and test units had to achieve a final PLT concentration of approximately 1500×10^9 PLTs/L and they were stored, without agitation, at 4°C. The test units were stored for up to 10 days (plasma or plasma and PAS) or 15 days (plasma).

Radiolabeling of stored PLTs

Nineteen of 20 subjects were available for *in vivo* assessment. One subject did not return for the autologous transfusions. PLTs were radiolabeled as previously described, following the detailed Biomedical Excellence for Safer Transfusion (BEST) Collaborative protocol.¹⁷ In brief, indium (¹¹¹In, Anazao) was used to label both control and test PLTs because per our preliminary experiments, the other available isotope, chromium, demonstrated very poor uptake of ⁵¹Cr by refrigerated PLTs (data not shown). The ¹¹¹In-radiolabeled Day 3 CSPs were largely undetectable by Day 10, and therefore reuse of the same isotope to measure both control and test CSPs was considered feasible. Additionally, we collected a preinfusion radioactivity sample before the "test" transfusion to account for any possible residual ¹¹¹In activity after each subject's control transfusion and adjusted our calculations accordingly.

On Day 3, the subject received an ¹¹¹In-radiolabeled aliquot of their control CSP unit. Follow-up samples from the subject were collected approximately 2 hours postinfusion and on Days 1 (×2, 2-10 hr apart), 2, 3, 4, and 5 to calculate recovery and survival of the subject's 3-day-stored PLTs.

The test CSPs in 100% plasma or PAS and plasma were initially stored for 10 days, and another group of subjects had their PLTs stored in 100% plasma for 15 days. After the test units were stored, an aliquot of their test units was obtained for ¹¹¹In labeling and subsequently transfused. Follow-up samples, as above, were collected to calculate PLT recovery and survival of the test PLTs. These studies allowed direct comparisons of the same subject's control 3-day CSPs versus 10- or 15-day test-stored CSPs. Comparisons were further facilitated as both control and test PLTs were labeled with the same isotope.

VIABILITY OF COLD-STORED PLTs

TABLE 1. PLT yields before and after storage ($\times 10^{11}$)

Storage solution/time	Prestorage	On Day 3	% of Pre	Prestorage	On Day 10	% of Pre
Plasma (100%)/10 days						
	3.26	3.11	95%	3.20	2.44	76%
	3.22	3.26	101%	3.14	2.87	91%
	3.59	3.52	98%	3.41	2.66	78%
	4.01	4.13	103%	3.99	3.18	80%
	3.62	3.55	98%	3.59	2.57	72%
	3.85	3.86	100%	4.09	3.45	84%
Mean	3.59	3.57	99%	3.57	2.86	80%
SD	0.31	0.38	3%	0.40	0.39	7%
Intersol (65%), Plasma (35%)/10 days						
	3.47	2.83	82%	2.45	2.90	118%
	3.53	3.27	93%	2.97	3.17	107%
	3.28	2.86	87%	3.05	2.94	96%
	2.73	3.09	113%	2.38	2.87	121%
	2.74	2.62	95%	2.39	2.24	94%
Mean	3.15	2.93	94%	2.65	2.82	107%
SD	0.39	0.25	12%	0.33	0.35	12%
Isoplate (65%), Plasma (35%)/10 days						
	3.73	2.56	69%	3.54	2.60	73%
	3.66	3.64	99%	3.14	3.11	99%
	4.11	4.03	98%	3.82	3.71	97%
Mean	3.01	2.68	74%	3.52	2.52	90%
SD	1.50	1.48	37%	0.37	1.28	39%
Plasma (100%)/15 days					at Day 15	
	3.18	3.37	106%	3.10	2.82	91%
	3.14	2.95	94%	3.15	2.26	72%
	3.39	3.47	102%	3.35	2.31	69%
	3.51	3.22	92%	3.39	1.95	58%
	3.62	3.50	97%	3.66	2.78	76%
	3.10	3.13	101%	3.20	2.28	71%
Mean	3.32	3.27	99%	3.31	2.40	72%
SD	0.22	0.21	5%	0.21	0.36	14%

In vitro PLT measurements

Platelet counts of collected products were performed on the day after collection to allow PLT disaggregation that might have occurred during collection and at the end of storage using a hematology analyzer (ABX, ABX Diagnostics). PLT yields were calculated by multiplying the PLT count times the volume of the PLT unit. After storage, in vitro measurements of glucose and lactate concentration and pH at 4°C were measured with a commercially available blood gas instrument (ABL Flex 805, Radiometer Medical). Annexin V binding, P-selectin expression, microparticle quantification, and mean PLT volume were performed by flow cytometry (FACSCalibur, Beckman Coulter) as previously described.¹⁸ The following antibodies were utilized: P-selectin CD62P-FITC (BD Biosciences), GPIIb/IIIa-PE (BD Biosciences), and annexin V-FITC (BD Biosciences).

Statistical analysis

Based on our previous study, which evaluated extended room-temperature storage with PASs,¹⁶ we calculated an effect size of approximately 2 for PLT recoveries between

different PAS and plasma and different time points. Based on this a sample size of $n = 5$ was calculated to be required to provide 80% power to detect a significant difference between plasma and PAS (t test, unpaired, two-tailed $\alpha p = 0.05$). We therefore planned to enroll five subjects per group and then assess for significance. We found results in the Isoplate group to be significantly lower with a small standard deviation (SD) and comparable or lower coefficient of variation compared to the other groups and therefore decided to terminate this group early with only three subjects evaluated. Results are reported as mean $\pm$ 1SD, and significance was assessed by unpaired, two-tailed t test, unless otherwise indicated. A p value equal to or less than 0.05 was considered significant.

RESULTS**Total PLT yield after storage**

Poststorage PLT counts of the "control" units averaged $3.57 \times 10^{11} \pm 0.38 \times 10^{11}$ after storage ($99\% \pm 3\%$ of prestorage values). No significant differences in PLT yields between PAS and plasma-stored PLTs were seen after 3 days

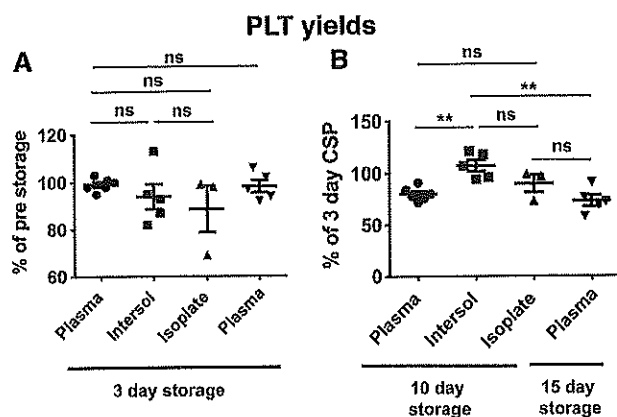


Fig. 1. PLT yields of apheresis units: PLTs were stored either in plasma alone (●, 3-day control units collected during studies on 10-day-stored plasma; ▼, 3-day control units collected during studies on 15-day-stored plasma) or in a 65% PAS, 35% plasma mixture (■, Intersol; ▲, Isoplate). [Total PLT yield in the component, calculated by multiplying the PLT count of the sample times the volume of the component (PLT count $\times$ component volume = actual PLT yield)]. (A) PLTs were stored for 3 days at 4°C. Results are shown as percentage of Day 1 PLT counts. No significant differences were seen between the groups. (B) Ten- and 15-day CSPs (as indicated) as percentage of the same subject's 3-day CSPs. ns = not significant; ** $p < 0.005$.

of storage (Table 1 and Fig. 1A). For the test 10-day plasma, Intersol, Isoplate, and 15-day plasma units, poststorage PLT counts averaged $2.86 \times 10^{11} \pm 0.39 \times 10^{11}$, $2.82 \times 10^{11} \pm 0.35 \times 10^{11}$, $2.52 \times 10^{11} \pm 1.28 \times 10^{11}$, and $2.40 \times 10^{11} \pm 0.36 \times 10^{11}$, respectively (80 $\pm$ 7, 107 $\pm$ 12, 90 $\pm$ 39, and 72% $\pm$ 14% of prestorage values). We found a significant reduction after 10- and 15-day storage in plasma when compared to 10-day PAS Intersol-stored PLTs ($p = 0.001$ and $p = 0.002$, respectively). PLT yields of 10-day plasma-stored, Intersol-stored, and 15-day plasma-stored did not differ significantly when compared to 10-day Isoplate-stored PLTs either absolute or as percentage of 3-day CSPs (Table 1, Fig. 1B).

In vitro PLT measurements

As expected, glucose levels were significantly lower in either Intersol or Isoplate PAS compared to plasma-stored units (Fig. 2A; 10-day plasma, Intersol, Isoplate, 15-day plasma: 321 ± 61 , 99 ± 9 , 82 ± 41 , and 321 ± 81 mg/dL [90 $\pm$ 2, 28 $\pm$ 3, 24 $\pm$ 12, and 86% $\pm$ 4% of Day 3 storage values, respectively]; $p < 0.0001$ for Intersol and Isoplate versus 10-day plasma-stored units) and, inversely, lactate levels were significantly elevated in plasma-stored units compared with either Intersol- or Isoplate-stored units (Fig. 2B; 10-day plasma, Intersol, Intersol, 15-day plasma: 8.1 ± 1.3 , 4.4 ± 0.7 , 4.0 ± 1.9 , and 9.2 ± 1.5 mmol/L [184 $\pm$ 11, 1375 $\pm$ 26, 1195 $\pm$ 54, and 222% $\pm$ 10% of Day 3 storage

values, respectively]; $p = 0.003$ for 10-day plasma vs. Intersol and $p = 0.007$ for 10-day plasma vs. Isoplate). Lactate was also significantly higher in plasma stored for 15 days compared to 10 days of plasma storage, indicating ongoing metabolic activity in plasma (Fig. 2B; $p = 0.012$). Markers indicating membrane orientation changes with phosphatidylserine exposure (% annexin V binding; Fig. 2C) only showed a significant difference between Intersol and 15-day plasma ($p = 0.034$; Fig. 2C—10-day plasma, Intersol, Isoplate, and 15-day plasma: 16.5 ± 11.7 , 16.1 ± 5.1 , 15.8 ± 3 , and 26.5 ± 12.4 [372 $\pm$ 219, 246 $\pm$ 87, 301 $\pm$ 106, and 730% $\pm$ 413% of Day 3 storage values], respectively). There was a trend for lower degranulation as measured by PLT alpha-granule secretion (P-selectin expression) with Intersol when compared with 10-day plasma, which did not reach significance ($p = 0.08$); however, when Intersol was compared to 15-day plasma, we detected a significant difference ($p = 0.004$; Fig. 2D—10-day plasma, Intersol, Isoplate, and 15-day plasma: 31 ± 28 , 21.8 ± 38.6 , 48.6 ± 21.5 , and 40.2 ± 15.3 [203 $\pm$ 76, 123 $\pm$ 53, 267 $\pm$ 118, and 278% $\pm$ 102% of Day 3 storage values], respectively). Microparticles were significantly elevated in 15-day plasma compared to 10-day plasma-stored PLTs ($p = 0.03$; Fig. 2E—10-day plasma, Intersol, Isoplate, and 15-day plasma: $1.7 \times 10^{11} \pm 1.6 \times 10^{11}$, $6.1 \times 10^{10} \pm 3.5 \times 10^{10}$, $7.4 \times 10^{10} \pm 2 \times 10^{10}$, and $4.1 \times 10^{11} \pm 2.1 \times 10^{11}$ /L [158 $\pm$ 127, 1865 $\pm$ 88, 1885 $\pm$ 41, and 561% $\pm$ 605% of Day 3 storage values], respectively), but there was no significant difference between plasma and PASs at 10 days of storage. For all PLT activation variables there was either a trend or a significantly higher value in 15-day plasma-stored units compared with 10-day plasma-stored units indicating continuous in vitro activation of PLTs in plasma.

In vivo PLT viability

For the 3-day control units ($n = 19$), poststorage PLT recoveries averaged 43% $\pm$ 11% and survivals 2 $\pm$ 0.4 days. For the test 10-day plasma, Intersol, Isoplate, and 15-day plasma units, poststorage recoveries averaged 24 $\pm$ 8, 18 $\pm$ 4, 8 $\pm$ 2, and 11% $\pm$ 3%, respectively (55 $\pm$ 11, 43 $\pm$ 6, 21 $\pm$ 8, and 29% $\pm$ 3%, respectively, of the same subject's 3-day control data; Fig. 3A). We found the recovery of 10-day Isoplate and 15-day-stored plasma PLTs to be significantly lower compared with both 10-day plasma and Intersol-stored PLTs (Fig. 3A; $p = 0.002$ and $p = 0.004$ for 10 day Isoplate vs. 10-day plasma and Intersol, respectively, as well as $p = 0.001$ and $p = 0.002$ for 15-day plasma vs. 10-day plasma and Intersol, respectively). Interestingly, there was no significant difference between the recoveries of 10-day Intersol- and plasma-stored PLTs, although there was a trend for a lower recovery with Intersol (Fig. 3A; $p = 0.057$). Notably, Isoplate-stored PLTs showed significantly lower recoveries compared with Intersol-stored PLTs ($p = 0.004$). Posttransfusion survivals for the 10-day CSPs in

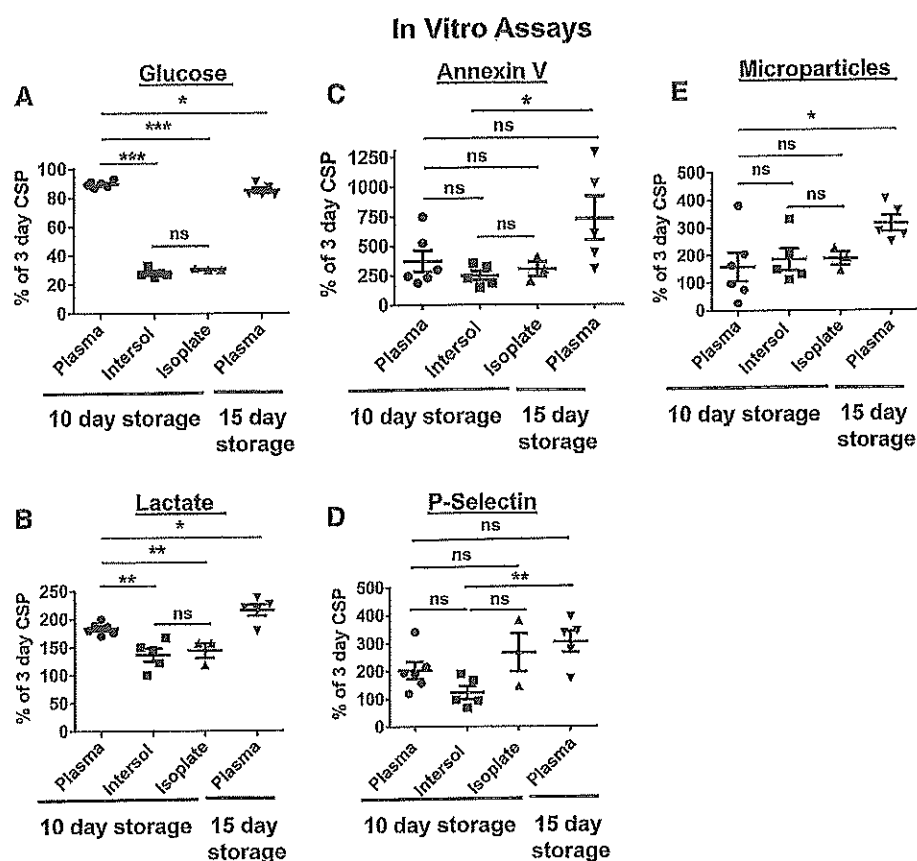


Fig. 2. In vitro PLT measurements: PLTs were stored either in plasma alone (●, 10-day storage; ▼, 15-day storage) or in a 65% PAS, 35% plasma mixture (■, Intersol; ▲, Isoplate) at 4°C, for either 10 or 15 days as indicated. (A) Glucose and (B) lactate determined by blood gas analyses, (C) annexin V-positive events, (D) CD62P-positive (P-selectin) events, and (E) CD61-positive microparticles were determined by flow cytometry. All results are shown as percentage of the same subject's 3-day 4°C-stored PLTs. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; ns = not significant.

plasma, Intersol, Isoplate, and 15 days in plasma averaged 1.2 ± 0.3 , 1.1 ± 0.3 , 0.9 ± 0.8 , and 0.7 ± 0.2 days, respectively (59 ± 12 , 56 ± 8 , 48 ± 42 , and $37\% \pm 7\%$, respectively, of the same subject's 3-day data; Fig. 3B). PLT in vivo survival studies showed a significantly lower survival with PLTs stored in plasma for 15 days compared to any of the 10-day-stored PLTs (Fig. 3B; $p = 0.01$), but there were no significant differences among the 10-day-stored PLT groups.

DISCUSSION

Our study has four major findings: First, the PLT yield is significantly lower in plasma compared with PLTs stored in Intersol. Second, all CSPs are metabolically active and consume glucose, produce lactate, and show signs of increasing preactivation. Third, different PASs can yield significantly different in vivo results which cannot be predicted by their in vitro results. In this study, Isoplate showed a significantly lower recovery and a significantly lower PLT yield compared with Intersol, even though both have in vitro variables

mostly in the same range. Finally, PLT recovery appears to be better in plasma compared with PASs, even though the PLT yield in vitro is significantly higher with Intersol compared with plasma.

In vivo survival beyond Day 1 is likely not a major factor for CSPs, since the current target patient population for use of these PLTs are those with active bleeding, who are in need of PLTs to facilitate immediate localized hemostasis. PLT survivals did not differ among any of the 10-day CSPs in either plasma or PAS but was significantly less at 15 days in plasma.

Previous in vivo animal imaging studies suggest that hemostasis and local clot formation are processes that require minutes to hours and are not accomplished within seconds.^{19,20} In addition, the time frame required to complete major surgery itself is likely several hours. We therefore speculate that the 2-hour in vivo PLT recovery and the 1-day survival time point are likely the most important readouts for CSPs targeted for actively bleeding patients. One of our major findings is that there are significant differences between PASs for 10-day cold storage. While PLTs collected in Intersol showed recoveries that were not significantly

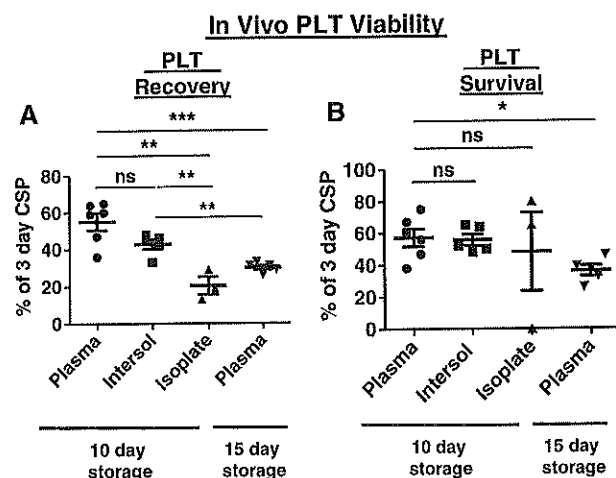


Fig. 3. In vivo PLT measurements: healthy human subjects received their autologous radiolabeled PLTs after storage at 4°C (plasma—●, 10-day storage; ▼, 15-day storage) or in a 65% PAS, 35% plasma mixture (■, Intersol; ▲, Isoplate). (A) Recovery of transfused PLTs at the 1-hour time point. (B) Survival of transfused PLTs. All results given as percentage of the subject's 3-day autologous radiolabeled 4°C-stored control PLTs.

* $p < 0.05$; ** $p < 0.01$; *** $p = 0.001$; ns = not significant.

different from that of plasma ($p = 0.057$), Isoplate showed significantly lower recoveries ($p = 0.002$). Previous studies suggest that PLT function is better preserved during cold storage in PAS and that there is less PLT aggregate formation in PAS compared with plasma.⁵ We saw a lower PLT yield in plasma compared with PASs, but overall PLT recoveries were *better* in plasma compared with PAS. This either suggests that any potential aggregates that pass the bedside transfusion filter disaggregate in vivo and are of no clinical significance or that the aggregates are filtered out by the transfusion filter and PLTs that pass the bedside transfusion filter have a higher recovery compared with PAS-stored PLTs. It should also be emphasized that while one group reported visible aggregates in PLT units stored in plasma at 4°C,⁵ we did not see any visible aggregates in our plasma-stored units. The only evidence for microaggregates in our study is based on the lower PLT yield in plasma versus PAS. However, we have preliminary data to suggest that this loss is in part due to loss of PLTs that adhere to the bag walls (data not shown).

We found glucose significantly lower in PAS plus glucose solutions compared with plasma alone. The removal of plasma (which contains glucose) during preparation of the PAS plus plasma units was likely the reason for lower glucose levels. Lower glucose in PAS was also likely the reason for the lower lactate, since removal of plasma with glucose deprived them of the essential energy source. It is also possible that PLTs in PAS are less metabolically active compared with PLTs stored in plasma; however, this will require further investigation.

A recently presented randomized Norwegian pilot study showed overall less blood loss in cardiac surgery

patients after chest closure with CSPs stored in the PAS T-PAS (Terumo BCT) compared with RSPs.¹⁴ We did not investigate T-PAS since it is currently not available in the United States. Better in vivo function measured by reduced blood loss in surgery patients has yet to be demonstrated with plasma-stored CSPs.

Should CSPs be considered for transfusion support in massively bleeding patients, the additional plasma in the plasma-stored unit may be of importance. Recent studies have suggested that a higher plasma-to-RBC ratio (1:1:1; plasma, PLTs, and RBCs, respectively) could be beneficial in trauma patients.²¹ PLT units in plasma are simpler to prepare than units that require replacement of plasma with PAS in a very specific ratio, a significant factor given that these PLTs are currently targeted as a future primary choice for blood banks in remote locations and far forward military scenarios. Patients who are in need of functional PLTs are frequently coagulopathic as well and could benefit from the additional plasma in the PLT unit, and the minor PLT loss due to microaggregates during storage in plasma may not outweigh the loss of plasma when opting for the replacement with PAS. More studies are needed to further investigate PASs for cold storage that are currently licensed for room temperature (Isoplate and Intersol) and newer PAS solutions including T-PAS, which is on a path to licensure in the United States.

Our study has limitations, including the collection of PLTs in Intersol and Isoplate on the Trima system (Trima is licensed for use with Isoplate, while Intersol requires collection with the Fenwal Amicus system [Fresenius Kabi]). While it is possible that this has influenced our findings, we believe that it is highly unlikely that this was a major determinant in this study. Another limitation is that we use recovery and survival studies and in vitro PLT variables and cannot make any claims about in vivo function. We need large clinical trials or bleeding time studies in humans with a room-temperature comparator to settle the debate if the in vivo function of CSPs is indeed better at preventing and treating blood loss. Finally, one limitation arises from a lack of a current standard-of-care (room temperature-stored) control; however, recovery and survival studies have been performed extensively on RSPs and in vivo recovery and survival are known to be superior to CSPs.

In summary, our study is the first to compare recovery and survival of CSPs in PAS and plasma in healthy human subjects. We found that both 65% Intersol/35% plasma and 100% plasma CSPs have potential advantages over the other; however, more studies especially in actively bleeding patients to demonstrate hemostatic efficacy are needed.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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ABSTRACT

21A

Conclusions: Our study demonstrates that normal ROTEM EXTEM CT results occur in the vast majority of ESLD patients with minimally elevated INRs and the institution of ROTEM was associated with a reduction in FP usage in liver transplant patients with no increase in RBC usage intraoperatively. These findings suggest that minimally elevated INRs are not an appropriate trigger for FP transfusion in ESLD patients and that EXTEM CT may be more appropriate.

OA4-SN2-16

Repeat Serotonin Release Testing for Heparin-Induced Thrombocytopenia After Initial Negative or Indeterminate Evaluation: A 3-Year Retrospective Study of Results from a US National Reference Laboratory

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Background/Case Studies: The unfractionated heparin (UFH) serotonin release assay (SRA) is a functional assay for the diagnosis of heparin-induced thrombocytopenia (HIT). Test results should be obtained only if the clinical suspicion is considered intermediate or highly probable by Warkentin's 4T score. Published sensitivity and specificity of the UFH SRA for HIT is 88% to 100% and 89% to 100%, respectively. However, little is known about repeat testing in patients with initial negative or indeterminate results. We conducted a retrospective 3-year review of repeat serotonin release testing after an initial negative result within the previous 2 weeks.

Study Design/Methods: The serotonin release assay in the laboratory involved combining patient serum with UFH (at concentrations of 0.1, 0.5, and 100 U/mL) and fresh donor washed 14C-serotonin labeled platelets with subsequently testing for 14C-serotonin release per standard laboratory protocol. Negative testing was characterized by <20% release at either low dose UFH (bovine source) concentrations. In contrast, a positive result was defined as >20% release with either low-dose (0.1 or 0.5 U/mL) UFH and >50% suppression of release at high dose (100 U/mL) UFH. An indeterminate result was defined as >20% release at either low-dose UFH but without >50% suppression at the high-dose UFH. Positive SRA was further stratified as either strong (both low-UFH concentrations >20% release, with one >90%), moderate (both low-UFH concentrations >20% release, with one between 50 and 90%), mild (both low-UFH concentrations >20%, with one 21% to 49%), and weak (only one low concentration >20%).

Results/Findings: Over a 3-year period, a total of 2538 patients with repeat testing within a 2-week period were identified. Among these patients, initial results were negative in 2107 (83.0%), indeterminate in 248 (9.8%), and positive in 183 (7.2%). Repeat testing results were positive in 13 (5.2%) patients with initial indeterminate results and 53 (2.5%) patients with initial negative results. Of patients with initial indeterminate results, 61.5% had strong, 15.4% had moderate, 7.7% had mild, and 15.4% had weak 14C-serotonin release. Of patients with initial negative results, the repeat positive testing demonstrated strong (49.0%), moderate (35.8%), mild (7.5%) and weak (7.5%) 14C-serotonin release. Of the strong 14C-serotonin release results, most patients in both the initial negative (61.5%) and indeterminate (87.5%) groups had greater than 90% release in both low UFH concentrations. The median number of days between initial and repeat testing was 5 (range, 0-14) for patients with initial negative and 6 (range, 1-9) for patients with initial indeterminate results.

Conclusions: A small percentage of patients who initially test negative or indeterminate by SRA can test positive within 2 weeks. Most of these results are either strongly or moderately positive, justifying repeat testing in these patients if HIT is clinically suspected. A change to a positive test result may be caused by initial testing during acute HIT, during which functional antigenic/antibody complexes are not high enough to be detected by the SRA. This population in which a result changes from negative or indeterminate to positive could be studied further to improve current clinical scoring tools or develop more sensitive functional assays for HIT.

OA5-SN2-16

Effects of Platelet Additive Solution on In vivo and In vitro Characteristics in 4°C-Stored Platelets

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Background/Case Studies: Cold-stored platelets are currently under investigation for actively bleeding patients. Some groups reported a high

failure rate and increased wastage due to aggregate formation in 3-day, 100% plasma, cold (4°C)-stored platelets. Other groups have shown prevention of aggregate formation when platelet additive solutions (PASs) were added to cold-stored platelets. We hypothesized that cold storage of platelets in PAS improves platelet function, protects from apoptosis, and improves recovery in vivo.

Study Design/Methods: Ten healthy human subjects underwent apheresis platelet collection. We tested platelets stored for 5 days in either 100% plasma at 4°C, or in 65% PAS-C (InterSol) and 35% plasma at 4°C, and 5 days in 100% plasma at 22°C (room temperature, current clinical standard, historic control group). Platelet samples for in vitro testing were extracted on the day of donation and after 5 days. After storage, all units were radiolabeled and transfused into their respective donors. All donors came back after a week to provide a fresh sample for radiolabeling and reinfusion.

Results/Findings: No significant differences were observed for in vivo recovery and in vivo survival between platelets stored at 4°C in PAS-/plasma or pure plasma. Both 4°C groups showed significantly lower recovery and survival than the 5-day room temperature control group. Platelet yields were significantly higher in PAS/plasma stored units compared to plasma alone at 4°C. Five-day room temperature-stored platelets showed the highest yields; however, they were not significantly different compared to 5-day PAS/plasma at 4°C. Glucose levels did not drop significantly in PAS/plasma and pure plasma-stored platelets at 4°C, while at 22°C, levels dropped significantly when compared to prestorage values. Consistently, 5-day PAS/plasma storage led to significantly lower lactate values compared with both 100% plasma groups at 22°C and 4°C. Cold-stored platelets in 100% plasma showed significantly lower lactate values compared with 22°C-stored 100% plasma platelets. There was a trend for lower integrin activation in 4°C PAS/plasma-stored platelets after stimulation with collagen compared to 100% plasma, 4°C-stored platelets; however, this did not reach significance. Both pure plasma and PAS/plasma stored at 4°C were significantly more reactive when compared to 22°C, 100% plasma-stored platelets. Early apoptosis, measured by mitochondrial membrane potential, did not differ significantly between PAS/plasma and pure plasma at 4°C, but both cold-stored groups showed a better preserved mitochondrial membrane potential compared to room temperature storage. PAS/plasma-stored platelets showed significantly more caspase 3/7 activation after stimulation with ABT 737 compared to pure plasma storage at 4°C and 22°C, indicating an increased capacity for late apoptosis.

Conclusions: We found PAS/plasma storage as a viable option for cold-stored platelets; however, clinical trials will have to show hemostatic efficacy compared to 100% plasma stored platelets at 22°C and 4°C.

Oral Abstract Presentations: New Innovations, New Techniques and New Technologies

OA1-SN5-36

Scalable in vitro Platelet Production from Cord Blood-Derived Hematopoietic Stem Cells

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Background/Case Studies: Approximately 7000 units of platelets are required by patients daily, and demand is projected to continually rise. This demand coupled with a short shelf life and the source limitations of volunteer donors has contributed to platelet shortages. In vitro manufactured platelets are being investigated as a donor-independent solution. This study demonstrates the ability to produce functional platelets that are scalable to transfusable numbers starting with CD34+ hematopoietic stem cells (HSCs) isolated from cord blood mononuclear cells (CBMCs).

Study Design/Methods: Cord blood derived CD34+ HSCs were expanded for 7 days using supplemented, commercially available serum-free media. Expanded CD34+ HSCs were differentiated into megakaryocytes (MKs) with media containing thrombopoietin, stem cell factor, interleukin-6, interleukin-9, and other cytokines. Rho-associated protein kinase inhibitor, Y-27632, promoted maturation and polyploidization of MKs, increasing the number of higher ploidy (>8N) platelet-producing MKs. Mature MKs were subjected to culture under shear conditions that promoted the production of platelet-like particles (PLPs). Characterization of the in vitro-produced platelets was conducted using scanning electron microscopy (SEM), flow cytometry, and functional assays.



BLOOD COMPONENTS

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 Christoffel¹

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 $\alpha IIb\beta 3$ 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; $22 \pm 2^\circ\text{C}$). RT storage maximizes platelet recovery and survival in transfused recipients¹; 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.

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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 $700\text{--}2100 \times 10^3$ 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), GPIIb α -PE (BD Biosciences), and Annexin V-PE (BD Biosciences). PAC-1 (BD Pharmingen) binding was tested by flow cytometry as previously described¹⁵ at baseline and after stimulation with 20 μ g/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 $\pm$ 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 ($75 \pm 2\%$ vs. $98 \pm 1\%$, 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 ($75 \pm 4\%$ vs. $98 \pm 1\%$, $p = 0.0012$).

In vivo platelet viability

Calculated as percentage of fresh, the recoveries of all time points were significantly lower compared to the respective previous steps ($46 \pm 3\%$ vs. $31 \pm 2\%$, $p = 0.003$; $31 \pm 2\%$ vs. $22 \pm 2\%$, $p = 0.009$; $22 \pm 2\%$ vs. $13 \pm 1\%$, $p = 0.008$), and the recovery of 7-day RT-stored platelets was significantly higher compared to 5-day cold-stored platelets ($70 \pm 7\%$ vs. $46 \pm 3\%$, $p = 0.017$) (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 ($51 \pm 8\%$ vs. $20 \pm 3\%$, $p < 0.0001$) and 5-day cold and 10-day cold ($20 \pm 3\%$ vs. $8 \pm 1\%$, $p = 0.0014$), 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 ($65 \pm 2\%$

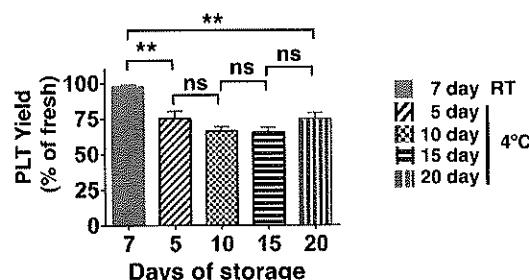


Fig. 1. In vitro platelet yields: (Total Platelet Yield in the Component [Platelet Count $\times$ Component Volume = Platelet Yield]) Platelets were stored in plasma for either 7 days at 22°C (solid gray bars), 5-day 4°C-stored (white with black diagonal stripes), 10-day 4°C-stored (red with white squares), 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]

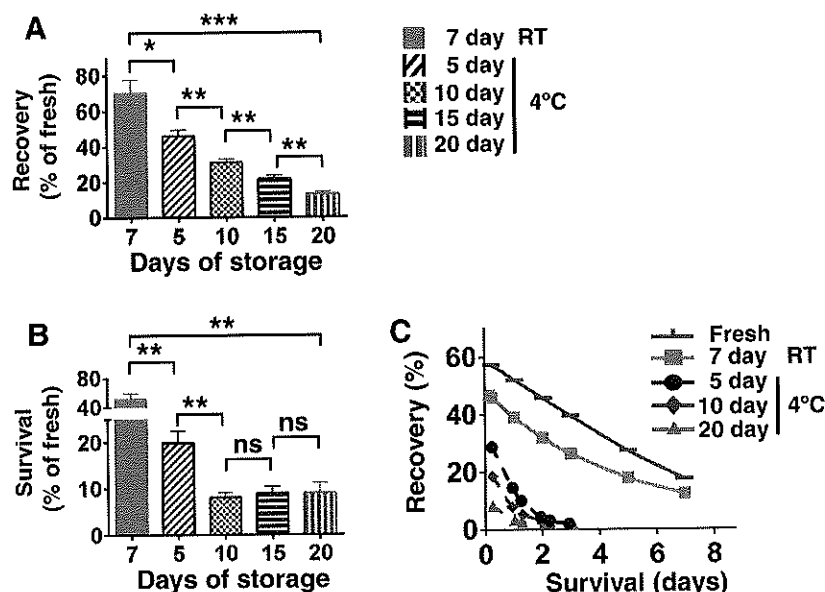


Fig. 2. In vivo platelet characteristics: Healthy human subjects received autologous radiolabeled platelets either fresh or after storage at 4°C or RT (22°C). Seven-day RT-stored (gray bars), 5-day 4°C-stored (white with black diagonal stripes), 10-day 4°C-stored (red with white squares), 15-day 4°C-stored (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. $98 \pm 2\%$, $p < 0.0001$) (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 ($98 \pm 2\%$ vs. $90 \pm 1\%$, $p = 0.007$; and $90 \pm 1\%$ vs. $83 \pm 2\%$, $p = 0.003$ 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 ($830 \pm 134\%$ vs. $292 \pm 26\%$, $p = 0.003$), and we saw a stepwise, significant increase from 5 to 10 days ($292 \pm 26\%$ vs. $473 \pm 41\%$). 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).

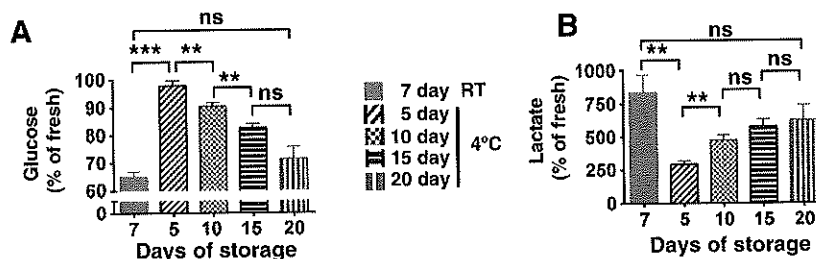


Fig. 3. In vitro platelet metabolism parameters: Glucose and lactate levels measured by blood gas analyzer. Seven-day RT-stored (gray bars), 5-day 4°C-stored (white with black diagonal stripes), 10-day 4°C-stored (red with white squares), 15-day 4°C-stored (blue with horizontal white stripes), 20-day 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 ($2326 \pm 567\%$ vs. $235 \pm 32\%$, $p = 0.0095$).

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 ($315 \pm 151\%$ vs. $1013 \pm 368\%$, $p = 0.119$). 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 ($841 \pm 215\%$ vs. $1013 \pm 368\%$, $p = 0.685$) (Fig. 4B).

Platelet α IIb β 3 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 α IIb β 3 integrin.¹⁹ Platelet α IIb β 3 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 phosphatidyl 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 hydrazone (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 ($2 \pm 1\%$ vs. $184 \pm 62\%$, $p = 0.009$

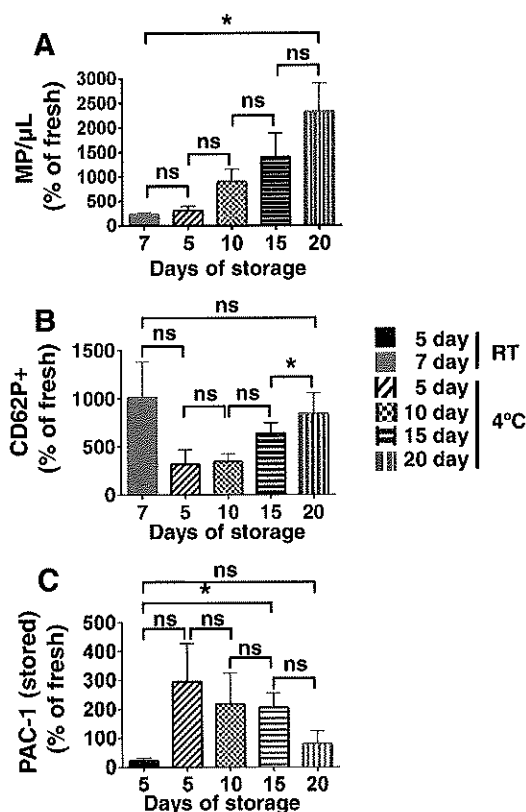


Fig. 4. In vitro platelet activation parameters: Five- or 7-day RT-stored platelets (black and gray bars respectively), 5-day, 4°C-stored (white with black diagonal stripes), 10-day 4°C-stored (red with white squares), 15-day 4°C-stored (blue with horizontal white stripes), 20-day 4°C-stored (green with 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 α IIb β 3 integrin activation was measured by PAC-1 antibody binding. PAC-1 antibody binding was measured after stimulation with the agonist collagen at 20 μ g/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 ($2 \pm 1\%$ vs. $55 \pm 16\%$, $p = 0.004$) (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

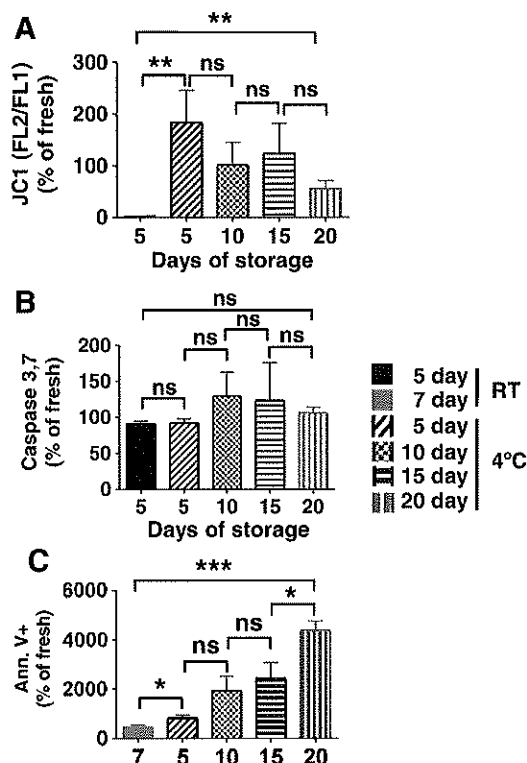


Fig. 5. In vitro platelet apoptosis parameters: Platelet mitochondrial membrane potential measured as JC-1 red (FL2) to green (FL-1) ratio. Five-day 4°C-stored (white with black diagonal stripes), 10-day 4°C-stored (red with white squares), 15-day 4°C-stored (blue with horizontal white stripes), 20-day 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 $\pm$ 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 ($817 \pm 118\%$ vs. $478 \pm 76\%$, $p = 0.043$), 15 days were compared to 20 days ($2431 \pm 646\%$ vs. $4376 \pm 390\%$, $p = 0.025$), and 20 days to 7 days at RT ($4376 \pm 390\%$ vs. $478 \pm 76\%$, $p < 0.0001$).

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

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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 α IIb β 3 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 α IIb β 3 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 α IIb β 3 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 α IIb β 3 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 suggest 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.

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Effect of PAS-C on short-term cold-stored platelets in vitro and in vivo

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Esther Pellham, and Moritz Stolla

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Contributions: S.L.B. analyzed the data, established assays, and performed experiments, E.P., D.B., and L.Y.F performed experiments. LF performed apheresis collection procedures. MS designed the study, analyzed the data and wrote the manuscript.

Letter to the editor

Effect of PAS-C on short-term cold-stored platelets *in vitro* and *in vivo*

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Clinical Trial Information

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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) are 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 re-evaluated for bleeding patients, where immediate function matters more than long circulation time. An added benefit is that bacterial growth is markedly reduced at 4 °C. However, how to best store CSP 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 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 groups were stored for 5 days. We collected a standard single apheresis platelet unit from 6 healthy subjects stored in either 100% plasma at 22°C or 4°C, or 65% PAS-C 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 for healthy human volunteer's risk and 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 US and we previously obtained *in vivo* data favored PAS-C over PAS-F for CSP.(9) All three groups (PAS-CSP, P-CSP, and P-RSP) were comprised of different cohorts (no matching between groups). The Annexin V and P-selectin data were obtained from a separate group of four volunteers by apheresis and stored in mini storage bags to clarify a role of these platelets activation parameters.

All apheresis units were collected and RT-stored bags 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 (WIRB), and all human participants gave written informed consent. The study was conducted following the Declaration of Helsinki. We assessed statistical significance by One Way Anova with correction for multiple comparisons (Tukey). To minimize biologic variability, and following recommendations by Murphy et al., we present the normalized stored data (percentage of fresh values). Supplemental Figure 1-3 contain the absolute data.

As previously described, CSP counts were significantly lower than P-RSP counts. PAS-CSP counts were significantly higher than P-CSP, corroborating findings from others and our group (Figure 1 A).(1, 11) Consistent with high metabolic activity at RT, 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 1 B). While P-RSP showed the highest lactate levels, cold storage reduced lactate production, with a trend for lower levels with PAS-CSP than P-CSP (Figure 1 C). 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 2 A). We observed a trend for prolonged survival in PAS-CSP than P-CSP (Figure 2 B). To obtain more insights into the biological health of stored platelets, we tested for mitochondrial membrane potential as an early marker for apoptosis. We observed significantly better-preserved membrane potential in P-CSP than P-RSP. PAS-CSP and P-CSP did not differ significantly as percentage of fresh, but the absolute data showed a significantly better preservation in PAS-CSP (Figure 3 A, and Supplemental Figure 2 D). All cells responded appropriately to the uncoupler CCCP (2-[2-(3-Chlorophenyl)hydrazinylydene]propanedinitrile) (Supplemental Figure 2 C, D). There was a trend for more caspase activation in P-CSP, but overall, we did not see significant differences in this late apoptosis marker (Figure 3 B). Adding ABT 737 induced caspase activation before and after storage, indicating that platelets had the capacity to undergo apoptosis (Supplemental Figure 2 E, F). We did not find significant differences in procoagulant activity, but there was a trend for higher levels at RT (Figure 3 C). Similarly, we observed higher P-selectin levels at RT, but this was significant when compared to PAS-CSP, but not P-CSP (Figure 3D).

Integrin activation was largest in P-CSP, significantly more than P-RSP. PAS-CSP integrin response was lower than P-CSP but did not differ significantly (Figure 3 E-G). The largest difference between P-CSP and PAS-CSP was after stimulation with arachidonic acid (Figure 3 F).

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 (Supplemental Figure 3 C). 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 aggregate detection (Supplemental Figure 3 D). Other investigators attempted to stored 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, lactate in PAS-CSP stored for five days was lower than 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 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 compared to baseline up to day 15 in some apoptosis markers.(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 a shorter 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 compared to 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 GPIIb α -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 over the next years.

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Figure Legends

Figure 1: In Vitro Platelet Characteristics P-RSP (Plasma, 22 °C, solid black bars), P-CSP (Plasma, 4 °C, striped bars), PAS-CSP (PAS-C/plasma, 4 °C, solid grey bars) were tested fresh and on day 5 after storage. **(A)** Platelet count measured by ABX Hemanalyzer, P-RSP n=18, P-CSP n=5, PAS-CSP n=6, **(B)** Glucose measured by blood gas analyzer, P-RSP n=6, P-CSP n=5, PAS-CSP n=6, **(C)** Lactate measured by 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 + SEM, *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 or 5 day stored P-RSP (Plasma, 22 °C, solid black bars), P-CSP (Plasma, 4 °C, striped bars), or PAS-CSP (PAS-C/plasma, 4 °C, solid grey 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 + SEM, ***p<0.001, ns = not significant, n=5-21

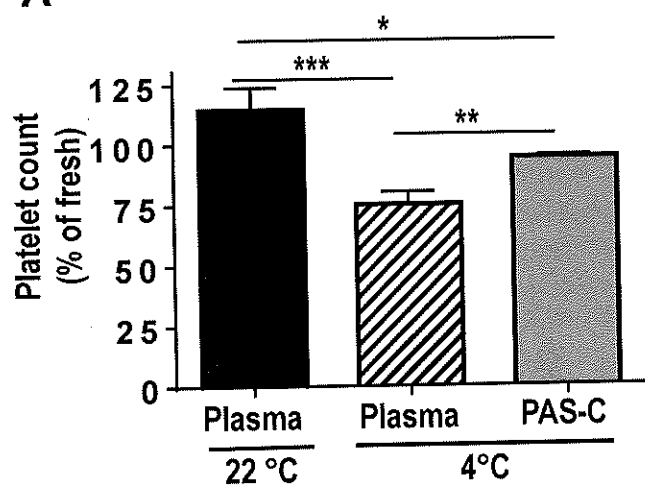
Figure 3: In Vitro Apoptosis and Activation Parameters P-RSP (Plasma, 22 °C, solid black bars), P-CSP (Plasma, 4 °C, striped bars), or PAS-CSP (PAS-C/plasma, 4 °C, solid grey bars) were tested on fresh platelets or stored for 5 days. **(A)** Platelet mitochondrial membrane potential measured by JC-1 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 was measured by Annexin V binding by flow cytometry. P-RSP n=4, P-CSP n=4, PAS-CSP n=4. (D) α -degranulation was measured by CD62P binding by flow cytometry. P-RSP n=4, P-CSP n=4, PAS-CSP n=4. Platelet α IIb β 3 integrin activation was measured by PAC-1 antibody binding by flow cytometry at baseline (BL) and after stimulation with (E) collagen (C), P-RSP n=5, P-CSP n=7, PAS-CSP n=5. (F) arachidonic acid (AA), P-RSP n=5, P-CSP n=5, PAS-CSP n=5. or (G) ADP (A) P-RSP n=5, P-CSP n=5, PAS-CSP n=5. (A-C) data shown as percentage of fresh mean + SEM, (E-G) shown as absolute data. Mean + SEM, *p<0.05, **p < 0.01, ***p $\leq$ 0.001.

Figure 1

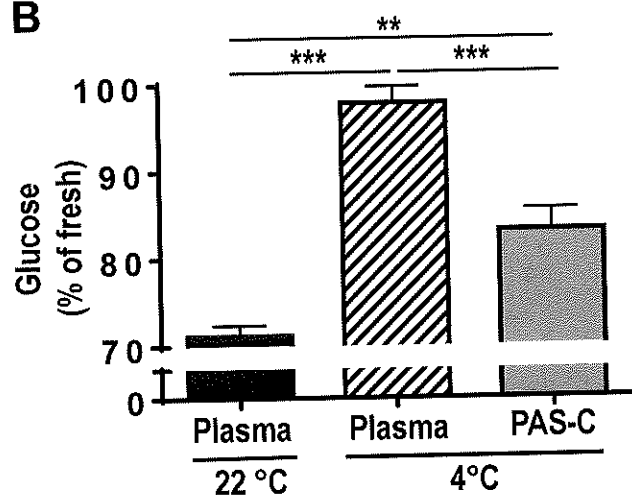
A

Platelet count



B

Glucose



C

Lactate

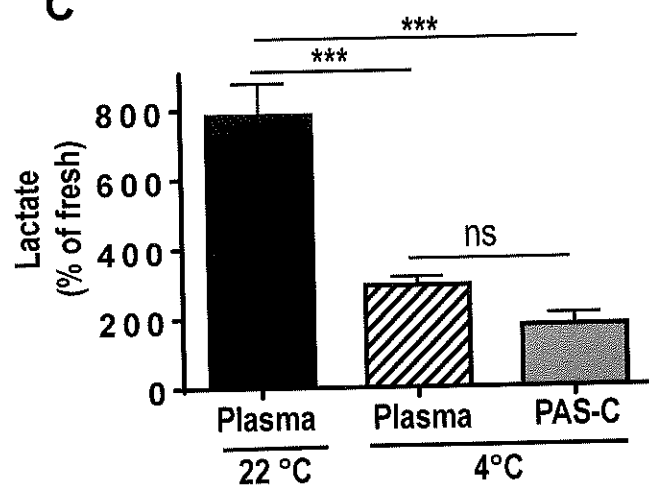


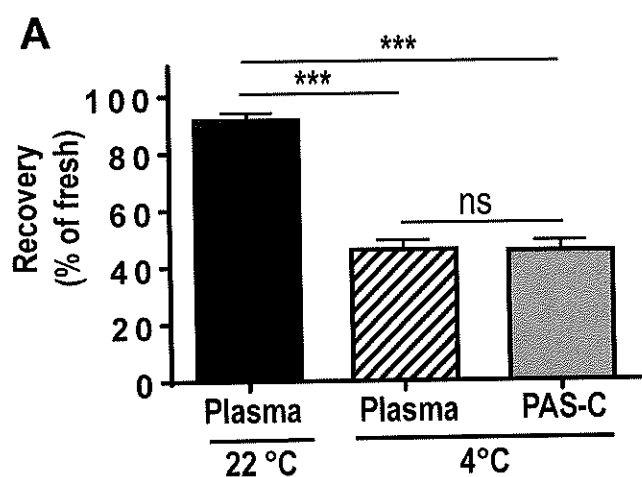
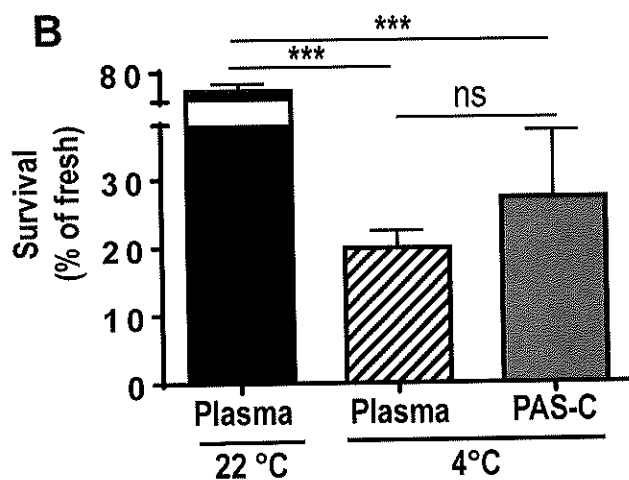
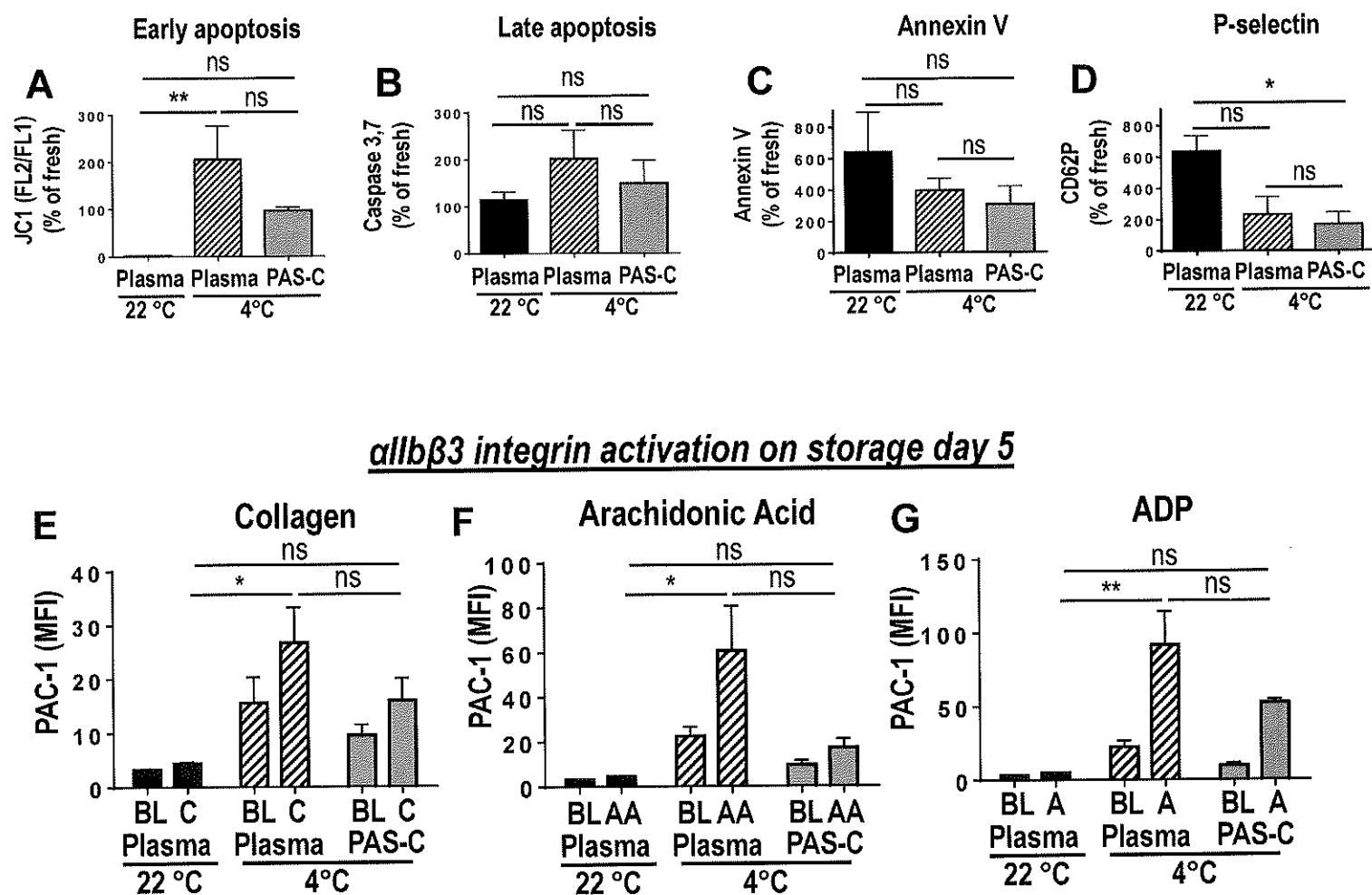
Figure 2***In vivo recovery******In vivo survival***

Figure 3

Supplemental Figure Legends

Supplemental Figure 1: In vitro platelet characteristics (absolute data)

P-RSP (Plasma, 22 °C, solid black bars), P-CSP (Plasma, 4 °C, diagonally striped bars), PAS-CSP (PAS-C/plasma, 4 °C, solid grey bars) were tested fresh (*F*) or after storage for 5 days (*S*). **(A)** Platelet count measured by ABX Hemanalyzer. (P-RSP n=18, P-CSP n=5, PAS-CSP n=6) **(B)** Glucose P-RSP n=6, P-CSP n=5, PAS-CSP n=6, and **(C)** Lactate measured by blood gas analyzer P-RSP n=6, P-CSP n=5, PAS-CSP n=6. Data shown as mean + SEM.

Supplemental Figure 2: In vivo platelet characteristics and in vitro apoptosis parameters (absolute data)

P-RSP (Plasma, 22 °C, solid black bars), P-CSP (Plasma, 4 °C, diagonally striped bars), PAS-CSP (PAS-C/plasma, 4 °C, solid grey bars) were tested fresh (*F*) or after storage for 5 days (*S*). **(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. Data shown as percentage of fresh mean + SEM, (P-RSP n=21, P-CSP n=5, PAS-CSP n=5). **(C)** Platelet mitochondrial membrane potential measured by JC-1 dye red (FL2) to green (FL-1) ratio tested on fresh platelets, (P-RSP n=9, P-CSP n=5, PAS-CSP n=6) **(D)** Platelet mitochondrial membrane potential measured by JC-1 dye red (FL2) to green (FL-1) ratio tested on 5 day-stored platelets (P-RSP n=7, P-CSP n=5, PAS-CSP n=5), **(E)** Caspase 3,7 activation measured by flow cytometry on fresh platelets (P-RSP n=5, P-CSP n=5, PAS-CSP n=6) **(F)** Caspase 3,7 activation measured by flow cytometry on 5 day-stored platelets (P-RSP n=5, P-CSP n=5, PAS-CSP n=5). Data shown as percentage of fresh mean + SEM.

Supplemental Figure 3: In vitro activation (absolute data) and aggregate storage data

P RSP (Plasma, 22 °C, solid black bars), P-CSP (Plasma, 4 °C, diagonally striped bars), PAS-CSP (PAS-C/plasma, 4 °C, solid grey bars) were tested fresh or after storage for 5 days. **(A)** Platelet phosphatidylserine exposure, measure by Annexin V binding by flow cytometry (all groups n=4). **(B)** Platelet alpha-degranulation measured by P-selectin binding by flow cytometry (all groups n=4). **(C)** Platelets were stored at 4 °C for 10 days either with (black bars) or without agitation (grey bars), and the platelet count was measured by ABX. n=2, Data shown as mean $\pm$ SEM **(D)** Occurrence of visual aggregates (black bars) or no visual aggregates (grey bars) in resting or manipulated units during 4 °C storage. "Resting" included units without any interference during storage time (no sampling/rewarming) (n=23) (reference (11)). "Manipulated" included units with frequent sampling and inadvertent rewarming (n=9).

Pathogen-Reduced, Platelet Additive Solution, Extended Stored Platelets (PREPS)
(Previously Pathogen-Reduced, Plasmalyte-Extended Stored Platelets)
Award Number W81XWH-12-1-0441 Log Number 11105004987987431
Final Report
15-SEP-2012 to 14-SEP-2021

Appendix D - Research Project #4 - Reversal of Aspirin Antiplatelet Therapy with Cold Stored Platelets (RASP) – Abstract with Oral Presentation.

#3a. American Society of Hematology Annual Meeting; Atlanta, GA December 2021.

Safety and Efficacy of 14-Day Cold Stored Platelets in Reversing Effects of Aspirin.

V. J. Li, MD^{1,2}, S. Bailey, BS², M. Bawcom-Randall², D. Byrne², J. Miles², J. Corson, RN² and M. Stolla, MD^{2,3}
Blood 2021 138(Suppl 1): 353 NOV2021. (Abstract and Oral Presentation – Slides attached)

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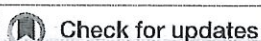
³Department of Medicine, University of Washington, Seattle, WA



401.BLOOD TRANSFUSION | NOVEMBER 5, 2021

Safety and Efficacy of 14-Day Cold Stored Platelets in Reversing Effects of Aspirin

Valery J Li, Shawn Lawrence Bailey, Morgan Bawcom-Randall, Daire Byrne, Jeffrey Miles, Jill Corson, Moritz Stolla

*Blood* (2021) 138 (Supplement 1): 353.<https://doi.org/10.1182/blood-2021-148217>

Split-Screen



Abstract

Background: Aspirin is an antiplatelet therapy used to reduce the risk of vascular occlusive events. However, this therapy is associated with an increased risk of bleeding for which there is no antidote currently. Transfusion of 5-day room stored platelets (RSP) at 22°C can reverse the effect of aspirin but surprisingly, the recent randomized PATCH trial showed increased morbidity and mortality for patients who received RSP transfusion for intracranial hemorrhage while on aspirin. Prior studies have shown that cold stored platelets (CSP) at 4°C are mildly activated and may participate in clot formation immediately, thus may have the potential to reduce blood loss more rapidly than RSP in acutely bleeding patients. CSP also have the added advantages of decreased risk of bacterial contamination and longer shelf-life up to 14 days per current FDA variance. However, the function of 14-day CSP in plasma after transfusion is unclear and lacks high quality data. We aimed to evaluate the post-transfusion safety and efficacy of 14-day CSP in reversing the effects of aspirin therapy compared to that of 7-day RSP.

Methods: Seven healthy human subjects were included in the analysis of this randomized, controlled, crossover study comparing transfusion of autologous 14-day CSP to 7-day RSP. Each subject participated in two study periods, which crossed over from one storage product to the other (CSP vs. RSP) according to randomization. For each study period, subjects underwent an apheresis platelet collection for autologous transfusion. Platelets were stored for either 14 days for CSP or 7 days for RSP. Subjects received a loading dose of aspirin 24 hours prior to transfusion. Blood was drawn at baseline, immediately pre-transfusion, at 1-hr, 4-hr, and 24-hr post-transfusion for an array of platelet function testing. After a washout period of 10-28 days, second study period commenced with similar sequence of events as the first study period using the other platelet storage product. The primary endpoint is the VerifyNOW Aspirin Reaction Units (ARU) at 1-hr after autologous

transfusion. Secondary endpoints include ARU at 4-hr and 24-hr post transfusion, light transmission aggregometry in response to arachidonic acid and collagen, and the corrected count increment. Paired t-tests were used for statistical analysis between the two groups and, where appropriate, the change from pre-transfusion values were analyzed.

Results: Transfusion of 14-day CSP and 7-day RSP units were well-tolerated by all subjects. Storage of platelets in the cold led to a non-significant trend for decreased platelet count, and the total platelet yield at the end of storage was significantly less in 14-day CSP compared to 7-day RSP ($p=0.02$). However, the corrected count increment did not differ significantly at 1-hr after transfusion. Platelet aggregation using the agonists, arachidonic acid 0.5mM and collagen 2.5ug/mL, did not reveal any significant difference between the two groups at any time points. The primary endpoint, platelet function testing by VerifyNOW, showed a larger change in platelet responsiveness at 1-hr post-transfusion in RSP than in CSP ($p=0.03$). Surprisingly, only RSP transfusion resulted in a significant change from the pre-transfusion VerifyNow measurements. Later time points showed a slight trend for improved platelet function as measured by VerifyNow with transfusion of both platelet products, but none were statistically significant.

Conclusion: We report the first safety and efficacy data for 14-day cold stored platelets in healthy humans. While prior in-vitro studies have demonstrated possible hemostatic superiority of cold stored over room temperature stored platelets, we observed inferior reversal of aspirin at early time points with CSP. This was in contrast to the results from our previous study, where 5 day-stored CSP were equivalent to RSP at early post transfusion time points. Further studies are needed to evaluate the maximal storage that provides functional equivalency between CSP and RSP. In addition, studies in actively bleeding patients are needed.

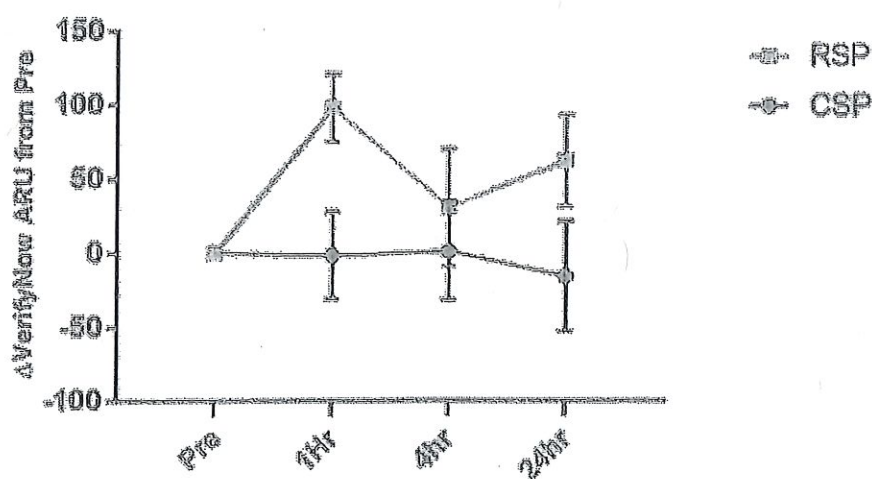


Figure 1. Change in VerifyNow ARU from pre-transfusion at 1-hr RSP vs. CSP ($p=0.03$); no other time points are significantly different.

Safety and Efficacy of 14-Day Cold Stored Platelets in Reversing Effects of Aspirin

Valery Li, MD
Hematology Fellow



UNIVERSITY of WASHINGTON



FRED HUTCHTM



American Society of Hematology



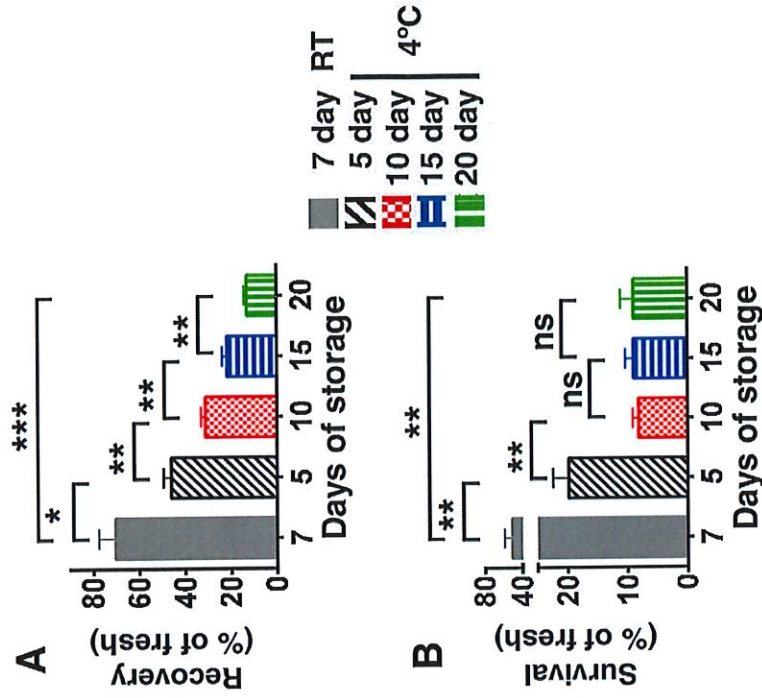
Disclosure

- None.

Cold Stored Platelets (CSP) at 4°C



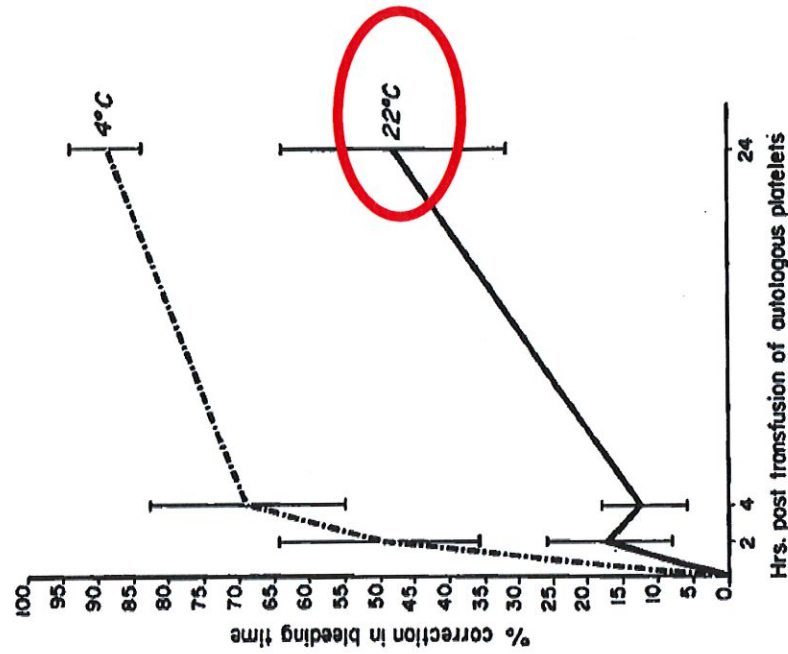
- Decreasing PLT recovery & survival with increasing CSP storage length.
[Stolla et al. Transfusion 2020.](#)
- Advantages of limiting bacterial growth while prolonging storage time.
 - Up to 14-day outdate per FDA variance





Function of CSP

- CSP is more activated and corrected bleeding times faster than RT PLTs in aspirin-treated subjects.
Becker et al. Transfusion 1973.
Valeri CR. NEJM 1974.
- In-vivo function of 14-day CSP is not well studied.





Objective

- Evaluate the safety and efficacy of extended CSP in healthy human volunteers on aspirin.

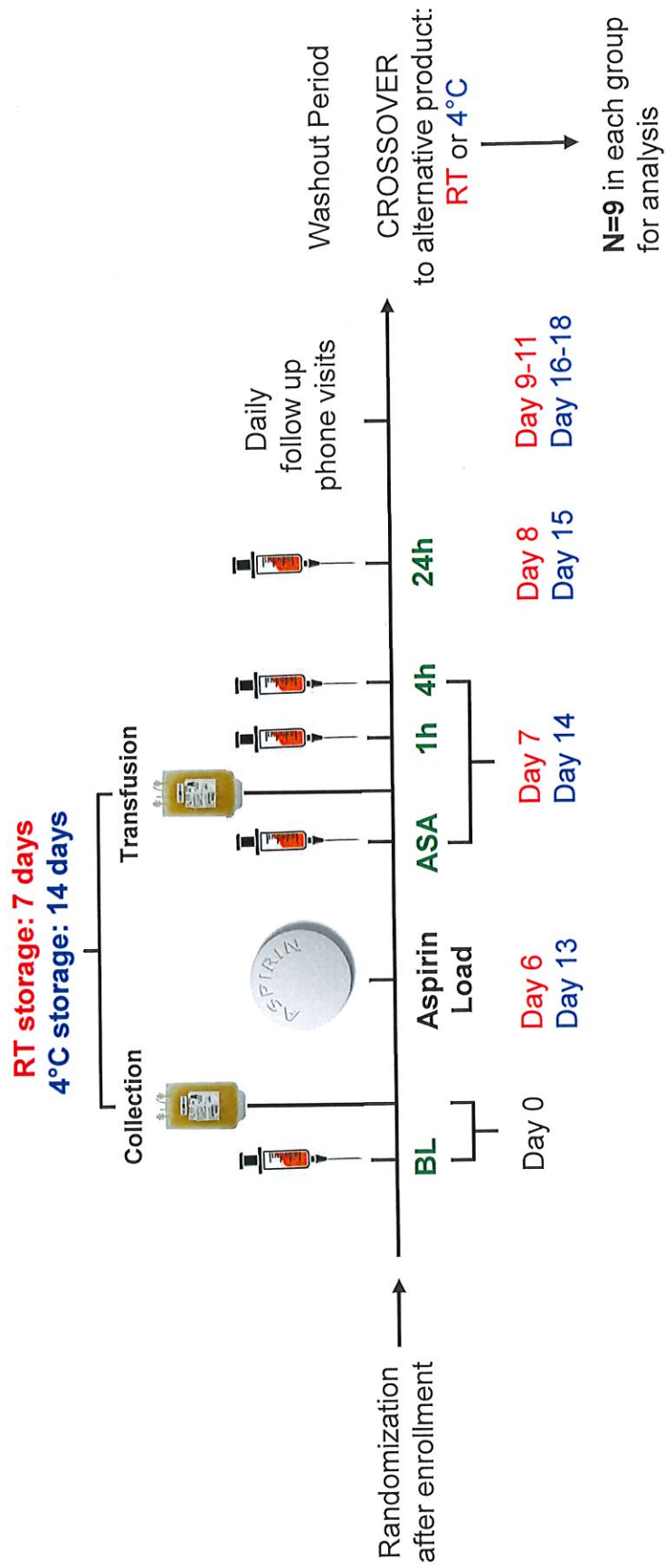
Hypothesis

- CSP is equivalent to RT-stored PLT in reversing effects of aspirin in healthy human volunteers.



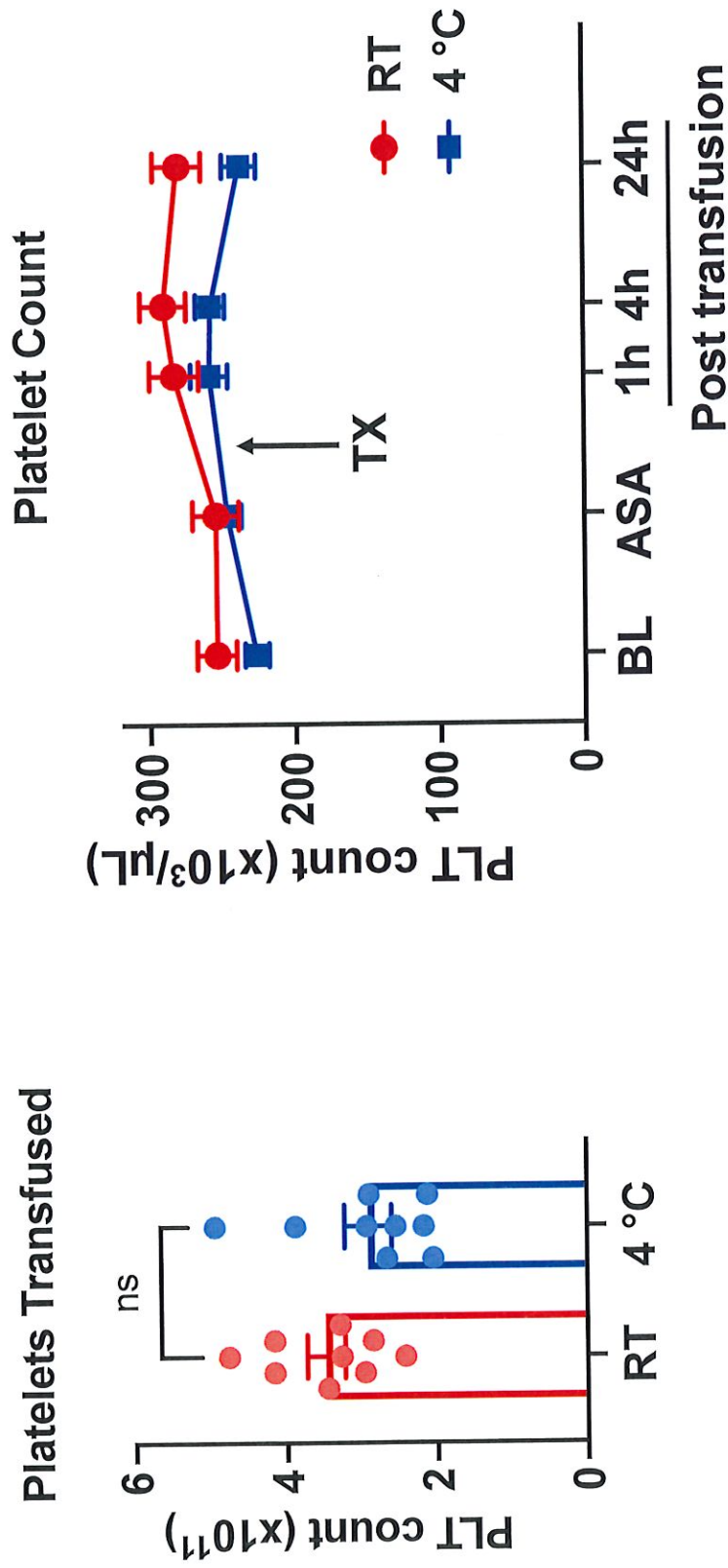


Study Design & Method



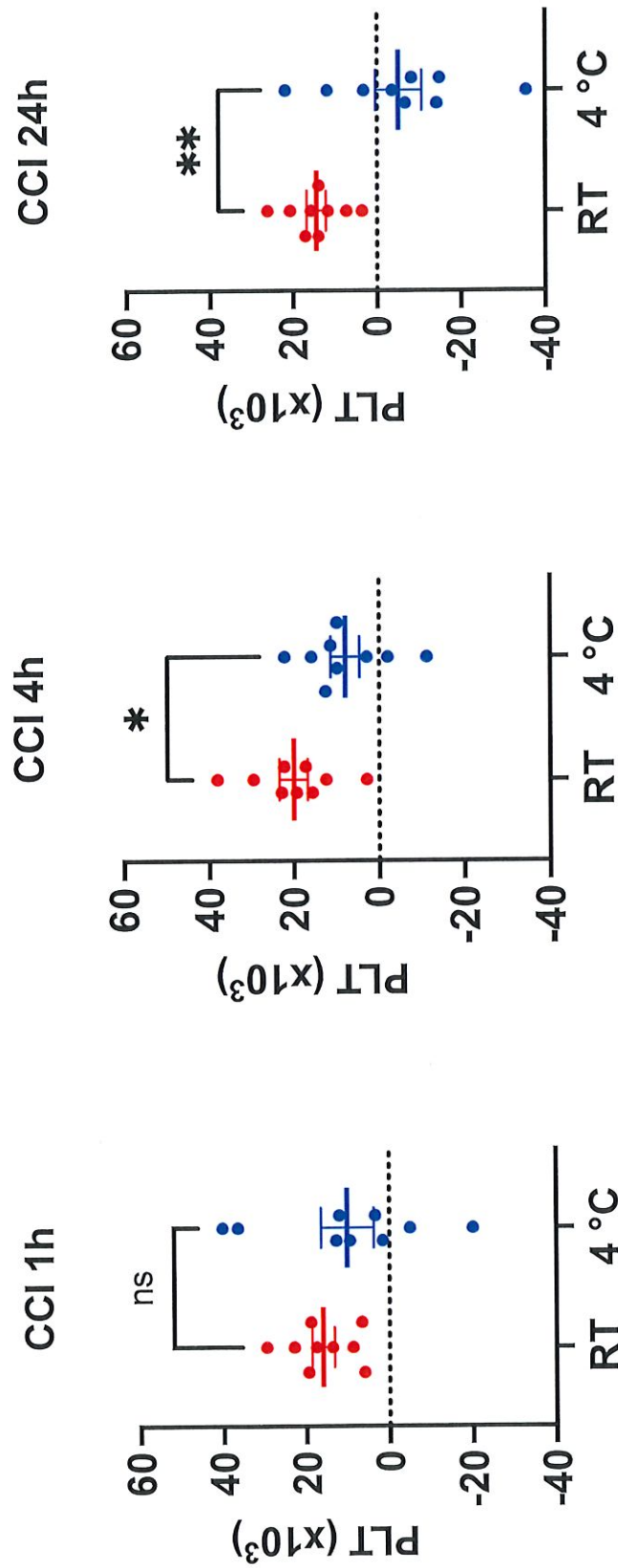


Platelet Transfusion Characteristics





Platelet Transfusion Characteristics

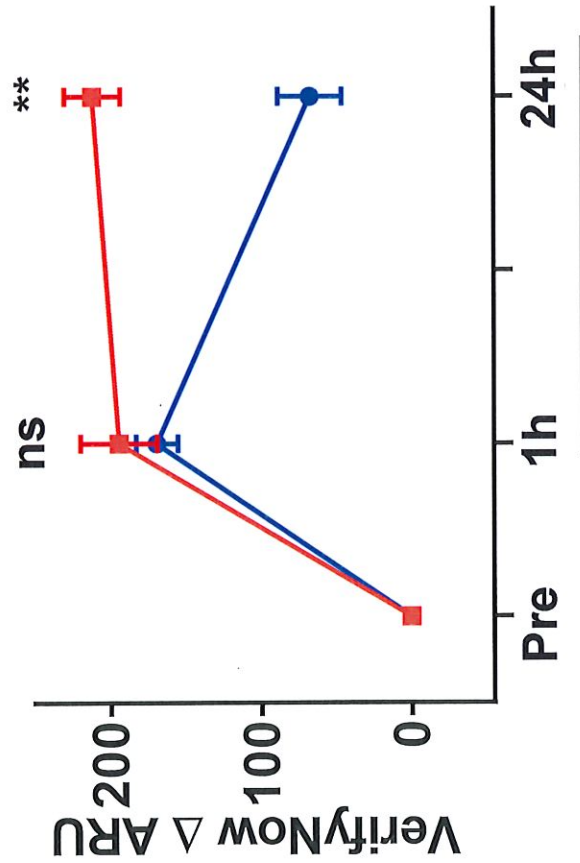




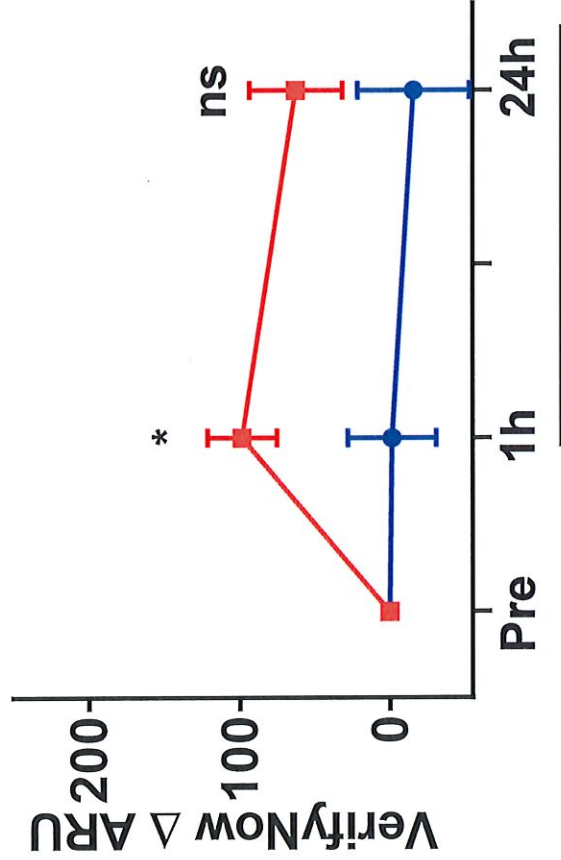
Reversal of Aspirin with CSP



● 5 day CSP ■ 5 day RSP ● 14 day CSP ■ 7 day RSP



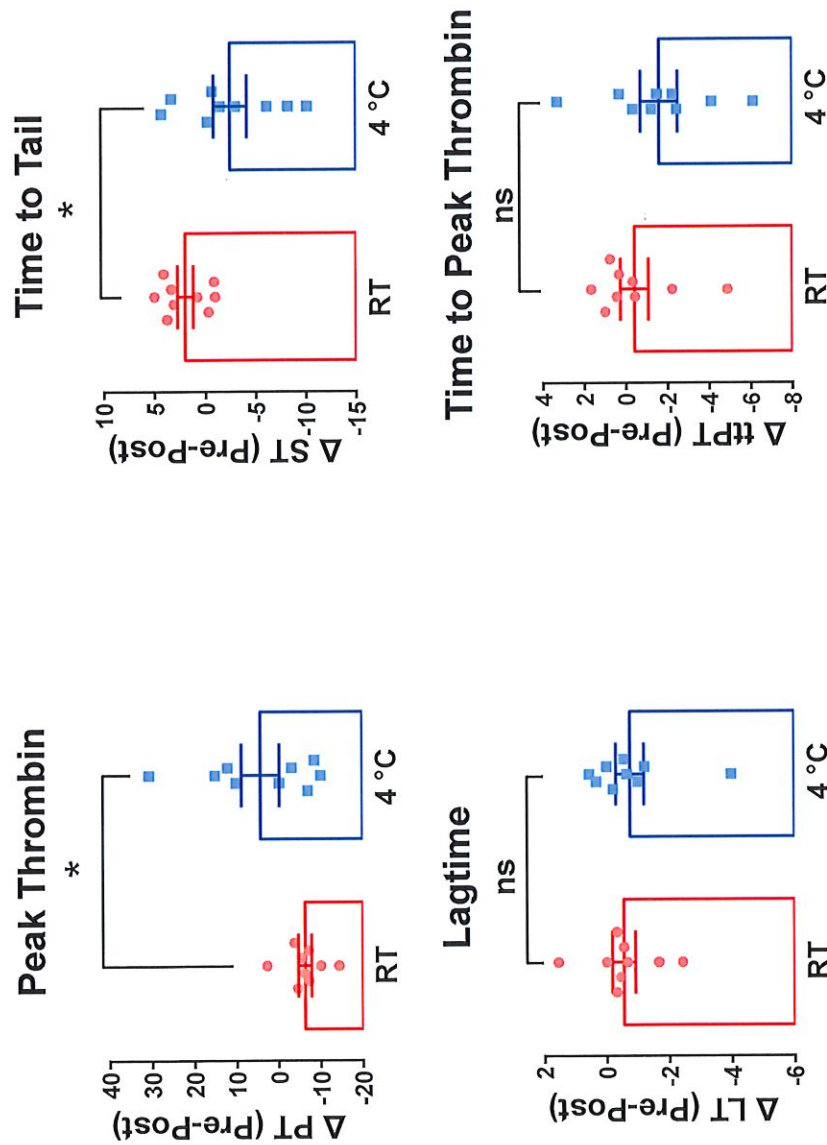
Post transfusion



Post transfusion

Miles J. et al. Blood Adv. 2021

Post-transfusion function: Thrombin Generation





Summary & Future Explorations

- First safety and efficacy data for 14-day CSP in healthy humans treated with aspirin.
- Inferior reversal of aspirin with 14-day CSP vs. 7-day RSP.
 - Maximal storage for CSP to achieve similar function as RSP?
 - Efficacy in actively bleeding patients?
- Peak thrombin generation higher in CSP- mechanism?
 - Increased PLT microparticles in CSP



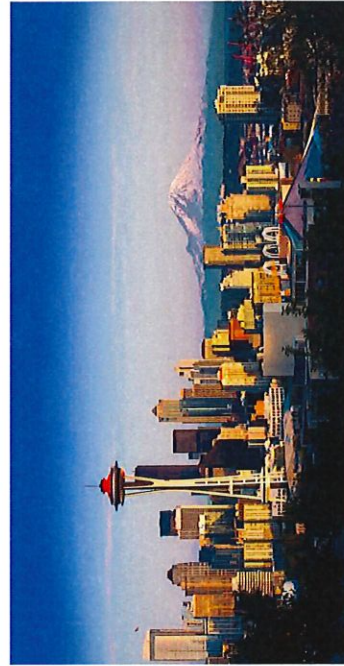
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- Pat Klotz
- Olga Yakovenko
- Anastasiia Stratiievska

Funding:



Pathogen-Reduced, Platelet Additive Solution, Extended Stored Platelets (PREPS)
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Award Number W81XWH-12-1-0441 Log Number 11105004987987431
Final Report
15-SEP-2012 to 14-SEP-2021

Appendix E - QUAD CHART: Dated 13DEC2021 FINAL.



Pathogen-Reduced, Extended Platelet Storage in Platelet Additive Solution (PAS) EDMS 5570/11105004 W81XWH-12-1-0441

PI: Moritz Stolla MD

Org: Bloodworks Northwest

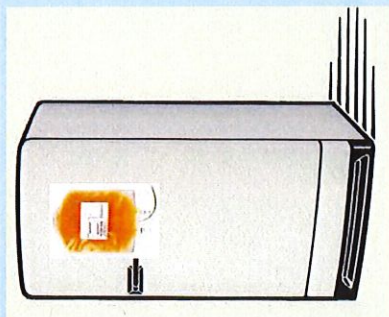
Award Amount: \$4,100,464 Includes funds sub-contracted to Terumo of \$1,402,000 (\$884,163 spent) and \$517,837 was return to BWNW.

Study/Product Aim(s)

- Research related to cold storage of platelets derived from whole blood and apheresis
- 4° C of platelets in Platelet Additive Solution (PAS)
- 4° C extended storage of platelets in plasma
- 4° C stored platelets to measure reversal of aspirin antiplatelet therapy

Approach

This is a non-clinical, exploratory study of apheresis platelets stored in the cold (4° C). We are exploring a variety of elements to determine optimum storage criteria and functionality for cold (4° C) stored platelets. Experimental elements include; extending the storage time of platelets in the cold from 3 - 20+ days and using a Platelet Additive Solution versus plasma for storage solution. We will be gathering in vivo data using radiolabeled recovery and survival in healthy volunteer studies. Various invitro tests will be performed at time points after collection, during and at the end of storage to evaluate the platelets. Also, we will be exploring the reversal of antiplatelet therapy with cold (4° C) stored platelets through a variety of projects including RASP-ECSP study.



Refrigerated platelets in plasma-stored for up to 20 days

Accomplishment: Publications and presentations on studies performed to evaluate apheresis platelets at 4° C.

Timeline and Cost

Activities	CY	15	16	17	18	19 NCE	20 NCE	21 NCE
Study evaluating platelets in WB at 4° C								
Development and regulatory approval of apheresis platelets in PAS at 4° C study								
Apheresis platelets in PAS at 4° C study enrollment, data collection and analysis								
Apheresis platelets in plasma at 4° C study enrollment, data collection and analysis								
Begin RASP-ECSP study								
Estimated Budget Spent (\$K)		\$1,124	\$1,592	\$1,745	\$2,698	\$2,698	\$2,968	\$4,100

Date: 13DEC2021 FINAL

Goals/Milestones

- CY 18 Goal** – Continued enrollment, data collection and analysis
- ☑ Complete analysis of apheresis platelets stored in plasma for 3-20 days at 4° C to same subject's fresh platelets
 - ☑ Complete approvals, conduct enrollment, data collection and analysis of apheresis platelets stored in Isoplete/plasma or InterSol/plasma for 3-20 days at 4° C to same subject's fresh platelets
 - ☑ Complete analysis of apheresis platelets stored in plasma for 20 days at 4° C to same subject's fresh platelets
 - ☑ CY 19 Goal – Continued publication and presentation on projects performed under this award.
 - ☑ CY 20/21 Goals – Submission and enrollment for a new study entitled; 'Reversal of Aspirin Antiplatelet Therapy with Extended Cold Stored Platelets (RASP)'.
 - ☑ CY 21 Goals – RASP enrollment completed and data analysis continuing funded by BWNW PI.
- Budget Expenditure to Date**
- Projected: \$4,100,464 (\$2,698,464 to Bloodworks + \$1,402,000 to Terumo)
- Actual Expenditure: Bloodworks & Terumo - \$4,100,464. Left to spend \$00