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

PRINCIPAL INVESTIGATOR: Moritz Stolla, MD

CONTRACTING ORGANIZATION: Bloodworks Northwest

Seattle, WA 98104

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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)

Grant Number 11105004

Annual Report

15-SEP-2018 to 14-SEP-2019

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Pathogen-Reduced, Platelet Additive Solution, Extended Stored Platelets (PREPS)
(Previously Pathogen-Reduced, Plasmalyte-Extended Stored Platelets)
Grant Number 11105004
Annual Report
15-SEP-2018 to 14-SEP-2019

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, 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.

Study activity has been limited this year to data analysis and submission of abstracts/manuscripts for publication. No additional subject enrollment or data has been collected since the submission of the last annual report 22OCT2018.

Training and professional development over the past year has included presentations on Cold Stored Platelets by Dr. Stolla at the following academic institutions:

- 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

Disseminating results to communities of interest have been performed through the following publications:

- ‘In Vivo Viability of Extended 4°C-storage Autologous Apheresis Platelets”. StollaM et.al., Published in Transfusion 10-2018.
- ‘Effects of storage time prolongation on in vivo and in vitro characteristics of 4°C-stored platelets.’ StollaM et.al. Publication submitted to Transfusion 05/2019 and is currently under review.

- Effects of extended cold-storage on platelet function, apoptosis and in vivo characteristics in healthy human subjects. Stolla, M. Poster presentation at AABB 2018, October 2018, Boston MA.

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: Nothing to report.

PRODUCTS: Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS: Terumo Corporation is a subcontractor on this award and will be submitting a separate annual report as required by the sponsor.

Below is a list of individual who have worked more than one person hour on this grant over this reporting period.

Name:	*Moritz Stolla, MD*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*<1*
Contribution to Project:	*Design, oversight and conduct of research study.*
Name:	*Shawn Bailey, BS*
Project Role:	*Lab Technologist*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*<1*
Contribution to Project:	*Radiolabeling, laboratory testing, unit processing, data capture, data entry.*
Name:	*Lydia Fang, BS, MS*
Project Role:	*Lab Technologist*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*<1*
Contribution to Project:	*Laboratory testing, unit processing, data capture, data entry.*
Name:	*Jill S Corson, RN*
Project Role:	*Study Coordinator/Research Nurse*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*<1*
Contribution to Project:	*Data capture and recording. Regulatory document maintenance.*
Name:	*Barbara Osborne, RN*
Project Role:	*Study Coordinator/Research Nurse*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*<1*
Contribution to Project:	*Subject enrollment. Apheresis collection. Radiolabeled infusion. Data capture and recording.*

REFERENCES: None

APPENDICES:

- Statement of Work - Novel approaches to storage of platelets for transfusion
- Protocol - Cold Apheresis Platelets in Plasma (CAPP)
- 'In Vivo Viability of Extended 4°C-storage Autologous Apheresis Platelets". StollaM et.al., Published in Transfusion 10-2018.
- 'Effects of storage time prolongation on in vivo and in vitro characteristics of 4°C-stored platelets.' StollaM et.al. Publication submitted to Transfusion 05/2019 and is currently under review.
- Effects of extended cold-storage on platelet function, apoptosis and in vivo characteristics in healthy human subjects. Stolla, M. Poster presentation at AABB 2018, October 2018, Boston MA.

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.

Cold Apheresis Platelets in PAS (CAPP)
Platelet Additive Solution (PAS)

I. PROTOCOL INFORMATION
Title: Cold Apheresis Platelets in PAS (CAPP)
Phase of Study: Phase I/II. Proof of Principle.
Version/Date of Protocol: Version 4/May 17, 2018

II. SPONSOR INFORMATION
The study is being sponsored by the Department of Defense (DOD) Congressionally Directed Medical Research Program (CDMRP).

III. PRINCIPAL INVESTIGATOR'S INFORMATION
PI Name: Moritz Stolla, MD
Title: Director Platelet Transfusion Research
Name & Address of Research Institution: BloodworksNW (formerly Puget Sound Blood Center)
Phone #: 206-689-6541
FAX #: 866-791-4098
Email: mstolla@BloodWorksNW.org

IV. ROLES AND RESPONSIBILITIES
Principal Investigator (PI): The PI will have overall responsibility for the study. He will ensure compliance with the protocol, institutional policies, and all applicable regulations. The PI will supervise the use of the test articles and review study data at regular intervals. The PI will permit and comply with audits and monitoring requirements. The PI will report all unanticipated problems involving risk to subjects or others to the Research Monitor, appropriate regulatory bodies, including the University of Washington Human Subjects Division and the USAMRMC, ORP, HRPO.

Study Coordinator (SC): The SC will assist in the preparation of the protocol, Institutional Review Board (IRB) applications and amendments, required quarterly reports, and other regulatory documents as needed. The SC will manage implementation of the research protocol under the supervision of the Principal Investigator. She will identify and recruit eligible subjects, review information on source documents to ensure data are complete and correct, and assist in rectifying discrepancies. She will maintain study records and logs and assist in evaluating study results. In addition the SC may perform all tasks ascribed to the Clinical Research Staff (below).

Clinical Research Staff (CRS) will perform research-related interventions under the direction of the PI and/or the SC. CRS will ensure that subjects have read and understand the informed consent document and have all questions appropriately answered and that informed consent documents are properly signed and dated. CRS will schedule study subject visits; explain study procedures; assess and document study subject's clinical status as required by research protocol; collect apheresis units; obtain subject blood samples; administer radiolabeled platelets as required by the protocol (only trained Registered Nurse CRS will perform this task); monitor study subject's progress and report adverse effects to the PI.

Laboratory Research Staff will perform research-related laboratory testing and platelet radiolabeling in accordance with the study protocol. Laboratory Research Staff will perform data entry into the study data base. Upon occasion Laboratory Research Staff may also collect blood samples from subjects. BloodworksNW Staff (either research or non-research) will collect follow-up blood samples and hold them for pick up and processing by Laboratory Research Staff.

Research Monitor: The Research Monitor will act as the safety advocate for study subjects. The Research Monitor will review all unanticipated problems involving risk to subjects or others and will provide an unbiased written report of the event to appropriate regulatory bodies, including the University of Washington Human Subjects Division and the USAMRMC, ORP, HRPO.

V. SITE INFORMATION

All study activities with the exception of the laboratory tests noted below will occur at BloodworksNW (formerly Puget Sound Blood Center) under the direction of Dr. Moritz Stolla. Bacterial testing and Gram Staining will be conducted by the University of Washington (UW) Microbiology Laboratory in Seattle, or by LabCorp in Seattle. All samples sent to outside microbiology laboratories will be stripped of all personal identifiers and labeled with a study ID number only.

VI. STUDY INFORMATION

Type of Research: Biomedical

VII. STUDY DESIGN

Background

Platelets are transfused to prevent bleeding and induce hemostasis, and can thus be critical in saving lives following trauma and in supporting thrombocytopenic cancer patients. Currently, platelets collected from volunteers are stored at room temperature. Room temperature storage has been demonstrated to maximize platelet recovery and survival in transfused patients; however it also increases the opportunity for bacterial growth in the platelet unit. The FDA limits the shelf life of platelets to 5 days or less to minimize this bacterial risk. Recently, the FDA has allowed 7 day storage with additional point-of-release bacterial testing. Nonetheless, transfusion associated sepsis remains the principal lethal risk associated with platelet transfusion.

Cold storage (4°C) is known to reduce post transfusion platelet recoveries but the effect is no more than 10% to 20% after 3 days of platelet concentrate storage. However, survivals are reduced to 1 to 2 days compared to an average survival of 4 to 5 days at 22°C storage[1-3]. In addition, there is controversy regarding the ability of 4°C stored platelets to correct bleeding times in thrombocytopenic patients compared to 22°C stored platelets[1]. However, we have demonstrated, in preliminary studies that platelets stored within whole blood for 15 days have radiolabeled autologous recoveries of 27±11% (49% of the same donor's fresh autologous recoveries) and survivals averaging 1.2±0.4 days (16% of the same donor's fresh autologous survivals)[4]. These data suggest that 4°C storage of apheresis platelets, as proposed in this study, may clearly show similar or even better platelet viability as platelet storage within whole blood.

Platelet Additive Solution (PAS)

Platelet Additive Solutions are isotonic solutions used to replace a portion of the plasma to store leukocyte reduced apheresis platelets. Several of these solutions are FDA approved for storage in a 65% PAS/35% plasma mixture for up to 5 days at 20-24°C with continuous agitation. Some FDA licensures associate the particular PAS with a specific collection devices or storage bag. None are approved for use

with platelets stored in the cold. We will be using these storage solutions in an off-label manner as regards to the collection device and storage conditions. However, all of the collection devices and storage solutions are FDA approved for platelet collection and storage.

Cold Stored Platelets

While not conducive to maintaining circulating platelet counts in thrombocytopenic cancer patients, transfusion of refrigerated platelets for deployed military medical units might provide adequate platelet hemostatic capacity to bleeding trauma patients and improve platelet availability for such patients. Based on recent in vitro studies of 4°C versus 22°C stored platelets, clot strength, platelet aggregation and sheer induced platelet aggregation are all better maintained at 4°C[6-8].

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. In this research proposal, we are interested in evaluating metabolic, functional and viability changes to apheresis platelets preserved in an additive solution and stored at 4°C. We will also determine the recovery and survival of these platelets by radiolabeling an aliquot of the apheresis platelets and re-infusing it into the donor/subject.

Current Research Approach

Cold stored platelets suspended in a mixture of PAS and plasma as well as cold stored platelets suspended in plasma without PAS will be evaluated.

For PAS suspended platelets. a single hyperconcentrated apheresis platelet unit (target platelet yield 3.0 X 10^{11}/unit and concentration of ~4000 x 10^3 platelets/µL) will be collected from a healthy adult volunteer subject using the Trima Accel® Automated Blood Collection System. Concurrent plasma (150 mL) will also be collected. After collection the unit will be re-suspended in a platelet additive solution (either InterSol or Isoplate) and plasma. The ratio of PAS to plasma will be 65% PAS: 35% plasma.

For plasma suspended platelets, a standard single apheresis platelet unit (target platelet yield 3.0 X 10^{11}/unit and concentration of ~1500 x 10^3 platelets/µL) will be collected as described above. Fifty milliliters of concurrent plasma will also be collected.

Regardless of the storage medium, each unit will achieve a final platelet concentration of 700 – 2100 x 10^6 platelets/µL, as per allowable bag parameters. The unit will be stored for a predetermined period of up to 20 days at 4°C. Various in vitro assays (see "In Vitro Tests Performed on Test Units") will be performed on the day of or day after collection.

At the end of the storage period, the subject will return to receive an ^{111}Indium Oxine (Indium 111, In-111) radiolabeled aliquot of their 4°C stored platelets. Follow-up samples from the subject will be collected approximately 2 hours post-infusion and on Days 1 (2X), 2 (2X), and 3 to calculate recovery and survival of the subject's 4°C stored platelets. The Day 1 and Day 2 the sample draws will be 2 - 10 hours apart.

In addition to radiolabeled platelet recovery and survival measurements, the same in vitro assays that were performed on the unit on the day of or day after collection will also be performed on the unit at the end of 4°C storage.

Seven to 14 days after the infusion of the radiolabeled aliquot, the subject will return to receive a second radiolabeled aliquot of fresh platelets. To facilitate this, on the morning of the second infusion, the subject will return for collection of a 43 mL blood sample. The blood will be processed to obtain a fresh sample of the subject's platelets to serve as a control comparator. The platelets will be radiolabeled with In-111. The subject will return later in the day for infusion of the radiolabeled fresh 'control' comparator aliquot. Follow-up blood samples will be drawn at ~2 hours after the infusion on Day 0 and then on Days 1, 2, 3, 4 or 5, and 6 or 7 day post infusion to calculate recovery and survival of the subject's fresh vs. stored platelets.

VIII. INCLUSION / EXCLUSION CRITERIA

Inclusion Criteria

The subject is in good health, is taking no excluded medications and meets platelet donor suitability requirements aimed at assuring donor safety. Recipient safety restrictions (e.g. travel and sexual contact) do not apply for this study. No infectious disease testing will be performed.

Specific inclusion criteria are:

- Weight: ≥110 pounds
- Hematocrit: ≥38% for females, ≥39% for males, but not >55% *
- Platelet count ≥150X10^3/mm^3 *
- Temperature: ≤99.5°F
- Resting blood pressure: systolic ≤ 180 mmHg; diastolic ≤100 mmHg
- Resting heart rate: 40 to 100 beats per minute
- Subjects must be ≥ 18 years old, of either sex
- Subjects must be able to read, understand and sign the informed consent document and commit to the study follow-up schedule. The ability to read and speak English is required for participation.
- Subjects must have good veins for apheresis platelet collection and drawing blood samples.
- Subjects of child-bearing potential (either male or female) must agree to use an effective method of contraception during the course of the study. The following methods of contraception will be considered 'effective' when self-reported by subject; abstinence, intrauterine contraception devices, hormonal methods, barrier methods or history of sterilization.

* The CBC will be run in duplicate from a single sample and the results averaged. Results > 10% difference from each other will be repeated.

Exclusion Criteria

Healthy subjects will be excluded from the study for any of the following reasons:

- Unable to achieve target platelet yield of 3.0 X 10^{11}/unit per Trima Accel (apheresis machine) configuration parameters.
- Ever received radiation therapy.
- Already participated in 4 research studies involving radioisotopes within the current calendar-year.
- Taken aspirin, non-steroidal anti-inflammatory, or other platelet affecting drugs within 72 hours of collection or infusion. Subjects who have ever been prescribed anti-platelet medications (e.g. clopidogrel) will be excluded from study participation regardless of the interval to their last dose.

- Currently pregnant or nursing as assessed during interview. A urine pregnancy test prior to radioisotope infusion is required for women of childbearing potential.
- Unable to comply with the protocol in the opinion of the investigator.
- Donated granulocytes within the last 2 days.
- Donated whole blood within the last 7 days.
- Donated platelets or plasma within the last 28 days.

IX. SUBJECT RECRUITMENT & SCREENING

The study will advertise for healthy adult volunteers on websites, newspapers and/or bulletin boards. Prospective subjects will be asked to contact the Study Coordinator by email or phone. Email inquiries will be answered, by the Study Coordinator, with a summary email along with attachments of study documents (study consent, HIPAA policy, directions to BloodworksNW and a schedule of study visits). The subject will be encouraged to call the Coordinator to discuss the study by phone before making a screening appointment. The Study Coordinator may reference *Talking Points for Volunteer Inquiries* during the phone conversation.

Prospective subjects responding by phone will speak with the Study Coordinator, as described above, and will be offered an email with attached study documents.

Individuals who wish to make an in person appointment for consent and screening will make those arrangements by phone or email with the Study Coordinator. An email confirmation and reminder will be sent by the Study Coordinator. Contact information from people who do not make appointments will not be retained.

A total of 90 subjects with evaluable complete data sets may be enrolled. Allowing for screenfails and withdrawals this may require consenting of as many as 190 subjects.

X. INFORMED CONSENT PROCESS

At the time of the recruitment visit, Clinical Research Staff, usually the Study Coordinator will review the consent with the study subject in a private space at the BloodworksNW. The purpose of the study, the study procedures, the risks and options to not participate or to withdraw will be discussed. The number of venipunctures, the radioisotope exposure and the time demands of multiple blood draws will be emphasized. Throughout the process the subject will be encouraged to ask questions or make comments.

Subjects will sign the consent form in the presence of the staff administering the consent and that person will also sign the consent. The subject will be given a copy of the consent and HIPAA document.

After the subject has given informed consent eligibility screening will be performed. See Study Procedures section below. Screening questions are related to establishing that the subject is in good health. See Section 8, Inclusion/Exclusion Criteria.

All Clinical Research Staff have been trained and are certified in the Protection of Human Research Subjects.

XI. STUDY PROCEDURES

Screening

An abbreviated version of blood donor screening will be performed including completion of a study specific health history questionnaire, check of vital signs and a blood draw to obtain a 2 mL sample for a complete blood count (CBC) to obtain the hematocrit and platelet count. Only criteria aimed at assuring donor safety will apply. Recipient safety restrictions (e.g. travel and sexual contact) do not apply for this study. No infectious disease testing will be performed. If the subject meets eligibility criteria an appointment for apheresis platelet collection, within the next 35 days, will be made.

Apheresis Platelet Collection

Prior to apheresis, the pre-apheresis health history questionnaire and check of vital signs will be completed. The subject's platelets will be collected using the Trima Accel Automated Blood Collection System which is licensed by the FDA for this purpose. A venipuncture site will be selected and cleaned using standard BloodworksNW procedures. A needle will be placed in one of the subject's arms at the antecubital area. A CBC sample is obtained using an inline diversion pouch. Whole blood is drawn into the apheresis machine and the blood components are separated by centrifugation. Platelets and plasma are collected into the Terumo ELP storage bags and the red blood cells are returned to the subject. Along with the return of the subject's red blood cells the subject receives approximately 350 mL of ACD (citrate) anticoagulant during the collection process. The platelet apheresis collection lasts ~2 hours. Subjects are observed throughout the collection by a nurse or technician specifically trained in apheresis.

Suspension in PAS and Cold Storage

Immediately after apheresis collection, using sterile technique, research laboratory staff may suspend the platelets in a mixture of PAS/plasma at a 65%/35% ratio or leave the platelets in the attendant plasma, depending upon the predetermined storage medium being tested. After suspension units will rest for 1-24 hours at room temperature prior to sampling for in vitro assays. Units are weighed to calculate platelet yield. The units are placed in a locked cage in a refrigerator at 4±2°C and are not agitated during storage.

Temperature monitors will record temperatures and trigger alarms for out of range conditions. End of storage will be defined as the date and time when the aliquot for radiolabeling and infusion is removed from the stored unit.

Autologous infusion of radiolabeled platelets

Radiolabeling will be done according to a modified Biomedical Excellence for Safer Transfusion (BEST) method. In the BEST method, a concurrent, dual, fresh and stored label using two different isotopes is used to achieve an evaluable survival and recovery calculation. This approach is not practical as the error corrections inherent in the BEST method arrive at numerous irrational data outputs when comparing products of very different signal strengths. Therefore, Indium-111, will be used for both test and control platelets. In-111 infusions will be separated by at least one week. The In-111 administered on Day 0 will be undetectable by Day 7 and therefore re-use of the same isotope to measure both cold stored (test) and fresh (control) platelets is valid. To confirm this, we will collect a pre-infusion radioactivity sample to account for any residual In-111, and adjust our calculations accordingly.

Prior to infusion the subject's health will be reassessed via interview. If the subject feels unwell, has flu-like symptoms, or has any significant negative change to his or her health status, then he/she will be considered ineligible for the radiolabeled infusion and will exit the study. Pre-menopausal female

subjects who do not report sterilization will have a urine pregnancy test to confirm that they are not pregnant prior to the infusion. Any subject with a positive pregnancy test will be ineligible to continue with the infusion and will exit the study. Vital signs (temperature, pulse and blood pressure), height and weight will be assessed and recorded. Prior to infusion, microbiological tests (bacterial testing and Gram stain) of the platelet unit will be verified as negative.

Height and weight will be measured at the time of infusion. After venous access has been established, a blood sample (20 mL) will be obtained to determine baseline radioactivity. Approximately 10 mL (2-10 mL) of autologous cold-stored Indium-111 labeled platelets will be infused into the subject. During each platelet infusion, the subject will be carefully monitored for adverse reactions; i.e., fever, chills, dyspnea, urticaria or pain (infusion site, chest pain or other). Any adverse reactions will be recorded and reported to the study investigator.

After infusion, the line will be flushed with saline and removed. The subject will remain at, or return to, BloodworksNW for the Day 0 post-infusion blood sample, which will be collected ~2 hours after the infusion.

Seven to 14 days after the 1st radiolabeled aliquot is infused, the subject will return to have a 43 mL sample of whole blood collected to obtain a 'fresh' sample of the subject's platelets. The whole blood sample will be processed using a soft centrifugation to obtain platelet-rich-plasma (PRP). The PRP will be hard spun to produce a fresh sample of concentrated platelets. The fresh platelets will be radiolabeled with ^{111}Indium Oxine and infused into the subject as described in the above paragraphs.

Follow-up

The subject will return to BloodworksNW for sample collection (10 mL of blood) for measurement of radioactivity to calculate platelet-survival curves; Day 0, Day 1 (twice, 2-10 hours apart), Day 2 (twice, 2-10 hours apart), and Day 3 post infusion #1 for a total of 6 sample draws. After the second infusion the subject will return for the same 10 mL blood sample collections on Day 0, Day 1, Day 2, Day 3, Day 4 or 5 and Day 6 or 7 post infusion #2 for a total of 6 sample draws. (See Schedule of Events below). These samples will be used to determine platelet recovery and survival using computerized modeling of a multiple hit decay function.

Schedule of Events

Study Day	Study Procedures
1 – 35 days before apheresis	Informed consent process
	Screening (including collection of a 2 mL blood sample for hematocrit and platelet count) and enrollment
Apheresis platelet collection day	Pre-apheresis vital signs and health assessment
	Apheresis platelet collection
	In vitro testing on platelet unit (some testing may occur day after collection)
	Platelet unit reconstituted with 65% PAS/35% plasma or left on attendant plasma with no PAS , placed into storage at 4±2°C (may occur 1 -24 hours after collection)
1 day after apheresis	Bacterial culture sample collected from platelet unit and sent to UW microbiology laboratory
Day 0 Infusion day (3-20 days after apheresis platelet collection day)	Platelet storage ends
	Aliquot removed from stored platelets and processed for ^{111}Indium radiolabel (test)
	In vitro testing on stored unit
	Bacterial cultures evaluated
	Gram stain on stored unit sent to UW microbiology laboratory and evaluated
	Pre-infusion ID check, vital signs and health assessment. Urine pregnancy test if woman subject of childbearing potential.
	20 mL blood sample from subject for baseline radioactivity
	Infusion of 4°C stored radiolabeled platelet aliquot
	Post infusion recovery and survival (R&S) sample from subject (≥2 hours post infusion)
Post Infusion Day 1	Post infusion R&S sample from subject (twice, 2 - 10 hours apart)
Day 2	Post infusion R&S sample from subject (twice, 2 - 10 hours apart)
Day 3	Post infusion R&S sample from subject
Day 7 (visit can occur 7 – 14 days after the Day 0 infusion. All subsequent visits, below, will be pushed out accordingly)	43 mL fresh blood sample collected from subject and processed for ^{111}Indium radiolabel (control)
	Pre-infusion ID check, vital signs and health assessment. Urine pregnancy test if woman subject of childbearing potential.
	20 mL blood sample from subject for baseline radioactivity
	Infusion of fresh radiolabeled platelet aliquot
	Post infusion recovery and survival (R&S) sample from subject (≥2 hours post infusion)
Day 8	Post infusion R&S sample from subject
Day 9	Post infusion R&S sample from subject
Day 10	Post infusion R&S sample from subject
Day 11 or 12	Post infusion R&S sample from subject
Day 13 or 14	Post infusion R&S sample from subject
	Subject exits study

Total Volume of Blood Collected

The total amount of blood loss during the course of the study is approximately 290 mL. This includes CBC (2 mL), diversion pouch sample (~25 mL), apheresis platelets (~60 mL residual in disposable kit), immediate pre-infusion for baseline radioactivity (20 mL, twice),fresh whole blood sample on morning of infusion for fresh platelet control comparator (43 mL), and post infusion blood samples (10 mL each X 12) to determine circulating radioactivity.

In addition to the above volumes, 75 mL – 200 mL apheresis platelets in plasma and 50 - 150 mL of concurrent plasma will be collected.

In Vitro Testing Schedule

In addition to the in vivo platelet viability measurements after re-infusion, a number of in vitro laboratory measurements will be performed. Samples for these experiments will be obtained from the apheresis unit on the day of or day after collection and at the end of storage. These tests will be performed using standardized methods.

A sample from the platelet product will be sent for bacterial culture to an outside microbiology laboratory one day after the platelet collection. At the end of the storage period, a sample from the stored platelet unit will be sent to the University of Washington Microbiology Lab for a Gram stain. If either test is positive, the subject's stored platelets will not be reinfused and the subject will be withdrawn from the study.

The following table provides a list of the tests that will be performed on the apheresis platelet unit at the end of storage. These are the standard in vitro assays that the FDA requires for platelet licensing.

In Vitro Tests Performed on Stored Apheresis Unit at the End of Storage

Test Type	Day of or day after collection testing	End of storage testing
Platelet Concentration	✓	✓
Volume	✓	✓
Platelet yield	✓	✓
Blood Gases (pH and pCO2, PO2, HC03)	✓	✓
Glucose and Lactate	✓	✓
P-selectin	✓	✓
Morphology	✓	✓
Annexin V binding	✓	✓
Extent of Shape Change	✓	✓
Hypotonic Shock Response	✓	✓
Platelet Microparticles	✓	✓
Swirling	✓	✓
Mean Platelet Volume (MPV)	✓	✓
Mitochondria assay: JC-1 (±)	✓	✓
Apoptosis assay: Caspase 3/7 activation (±)	✓	✓
Integrin PAC-1 activation (±)	✓	✓
Flow chamber adhesion and aggregation assay: collagen and vWF (±)	✓	✓
Bacterial Culture*	✓	✓
Gram stain		✓

All samples will be discarded once testing is complete and no residual radiation is detectable.

*Bacterial Culture sample removed from unit 1 day after collection and evaluated at end of storage.

(±) May or may not be performed depending on staffing and reagent availability.

Adverse Event (AE) Assessments

During apheresis collection and infusion of platelets, the subject will be carefully monitored for adverse reactions; e.g., fever, chills, dyspnea, urticaria, or pain (infusion site, chest pain or other). Adverse reactions will be recorded in the study file and reported to the study investigator. Subjects will be instructed to report changes in health condition over the course of the study to the study coordinators. Minor AEs that are associated with venipuncture and blood collection, such as minor bruising at the needle site, will not be recorded as AEs, unless they worsen over time (e.g., become infected, etc.).

XII. DATA and ANALYSIS

Laboratory and other evaluable results will be transcribed from source documents (e.g. lab result print-outs) into an electronic database.

Summary statistics (means, medians, standard deviations, interquartile range) will be calculated for all in-vitro assays.

Tables of recovery and survival summary statistics will display values by group from fresh and stored platelets. Recovery and survival of stored platelets as percentage of corresponding fresh platelets will be plotted against days stored. Regression methods will be used to determine if there is evidence of any trend in the mean storage or recovery of 4°C stored platelets with respect to storage time as a percentage of each subject's fresh platelet results. Histograms of recovery and survival as percentage of 4°C stored platelet measurements will be plotted, and corresponding confidence intervals will be calculated.

XII. LABELING & STORAGE OF DATA & SPECIMENS

Study records, samples, and test results will be identified with a unique identifier and access will be limited to sponsor authorized personnel, the investigator, site research staff, and authorized regulatory authorities, including representatives of the FDA.

An alpha-numeric code that is unique to this study will be used as study identifiers. The study ID number will be associated with the subject's name on a study ID log. That log and the study database will be kept in separate folders on an electronic network at BloodworksNW. BloodworksNW uses Active Directory NT Authentication along with Access Control Lists (ACL's) for all network folders. File and folder access is logged on network shares. Security is enforced by the Information Technology Department. A network firewall is used to prevent unauthorized access to the network from outside entities.

Source paper documents will be kept in the Study Coordinator's office at BloodworksNW which is a security-card-restricted-access-building. The door to the coordinator's office is kept locked. Any documents not needed for source documentation will be shredded using a secure records-destruction service.

The link between the subject's identify and their study data will be destroyed/deleted when the research ends and any required monitoring of the study is finished, which will be no later than December 31, 2025. Consents will be destroyed six years after the conclusion of data analysis.

BloodworksNW utilizes an independent waste management contractor to dispose of research samples. The waste management contractor is contractually obligated to be in compliance with all applicable regulations regarding the pick-up, transport and treatment of regulated medical waste.

Subject samples that are radioactive at the time of collection are stored on a secure-access floor until such time as they have no detectable residual radiation. This is generally about 2 weeks. At that point they are disposed of as described above.

XIII. RISK AND INJURY

Apheresis-Related Risks and Precautions

Risks associated with standard platelet-product apheresis procedures are listed below. A single apheresis procedure typically lasts about 2 hours.

- Venipuncture-related risks: Venipuncture may lead to apprehension, discomfort, pain, bruising or infiltration at the venipuncture site. A vasovagal response, such as lightheadedness or fainting, nausea, or vomiting may occur. There is a very small risk of infection at the venipuncture site.
- Citrate infusion related risks (hypocalcaemia): Citrate (Acid-Citrate-Dextrose) is added to the apheresis circuit as an anticoagulant. This may result in perioral tingling or paresthesias. Non-specific mild symptoms of hypocalcaemia include headaches, nervousness, irritability, lightheadedness, flushing, shivering, nausea, vomiting, chest discomfort and abdominal cramping. Slowing the collection rate, pausing the collection and/or administering oral calcium (TUMS) will effectively address these symptoms. Rarely, intravenous calcium is administered when symptoms do not resolve. If allowed to progress citrate toxicity could potentially manifest as muscle cramps, tremors, tetany, laryngospasm, seizures and life threatening cardiac arrhythmias.
- Blood Loss: In rare and unusual circumstances, blood loss has occurred due to inability to complete the procedure.

The following precautions will be taken: The subject's pre-apheresis vital signs (blood pressure, heart rate, temperature) and pre-apheresis hematocrit will be determined. Subjects will be visually monitored for signs of distress during all procedures by trained and experienced staff. Citrate reactions will be treated according to the standard treatments at the site, which includes oral or, rarely, intravenous calcium supplementation, and/or slowing, pausing or stopping the procedure.

Radioisotope Infusion-Related Risks and Precautions

The radiation dose in this study is less than annual background radiation (3 mSv) and is not known to be associated with any health hazard. The amount of the isotope that will be infused is ≤30 μCi of Indium-111. The total radiation dose is approximately ≤30 μCi for a splenic dose of 8 mGy and a total body effective dose equivalent of 0.8 mSv. The risks of radiation exposure to a fetus are unknown. Therefore, women of childbearing potential will have a pregnancy test performed prior to the radiolabeled platelet infusion.

BloodworksNW's Platelet Transfusion Research Department will maintain a record of each subject's participation and will limit the number of studies any one individual can participate in to four studies in a calendar year. Patients who have received radiation therapy will be excluded from the study.

Platelet Transfusion-Related Risks and Precautions

Risks associated with receiving any blood product include chills, fever, hives, itching, immune response against blood cells, and/or blood infection from bacterial contamination. There is a rare risk of receiving the wrong subject's cells upon infusion, which could cause symptoms similar to those listed above.

The following precautions will be taken: In this study, subjects will be infused with their own cells; confirmation of identification will be done by two person verification of the infusion material. To prevent bacterial contamination, the product will be bacterially screened before infusion and sterile technique will be used for all manipulations of the study platelets.

Venipuncture-Related Risks and Precautions

Risks associated with venipuncture for blood sampling are apprehension, pain, discomfort, venospasm, fainting, bleeding, or bruising or infiltration at the venipuncture site.

The following precautions will be taken: Trained and experienced phlebotomists will perform the venipuncture procedures so that discomfort of the subject should be minimal.

XIV. BENEFIT(S)

There is no direct benefit to the study subject. Real benefits are altruistic in nature: subjects participating in this study will assist the scientific and medical communities in gathering important information to improving the availability of platelet transfusions.

XV. COMPENSATION

Subjects will receive $900.00 at the conclusion of the study for their time involved in study participation. If the subject is unable to complete the entire study or has to be withdrawn from the study, they will receive partial payment for their time involved in the study. The partial payment scale is the following (number in parentheses equals the number of times each procedure occurs during the course of the study):

Initial screening (Day -35 to Day -1, one visit during this time period)		$30
Apheresis collection (Day 0)		$200
Infusion of radiolabeled platelets, including pre-infusion sample draw (Day 0 and Day 7 - 14. Two separate infusions)	$100 (x2) =	$200
Collection of 43 mL of whole blood on morning of 2nd infusion day		$35
Follow-up blood sample, platelet recovery and survival calculation	$35 (X12) =	$420
End of study exit		$15
Total for completing all study procedures		$900

XVI. CONFIDENTIALITY

BloodworksNW considers all data and information collected during this study confidential. All data used in the analysis and summary of this study will be anonymous, and without reference to specific subject names. Study records, samples, and test results will be identified with a unique identifier and access will be limited to sponsor authorized personnel, the investigator, site research staff, and authorized regulatory authorities, including representatives of the FDA.

XVII. USAMRMC REPORTING REQUIREMENTS FOR SAE

All unanticipated problems involving risk to subjects or others will be promptly reported by telephone (301-619-2165), by email (usarmy.detrick.medcom-usamrmc.other.hrpo@mail.mil), or by facsimile (301-619-7803) to the Human Research Protection Office (HRPO). A complete written report will follow the

initial notification. In addition to the methods above, the complete report will be sent to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RP, 810 Schreider Street, Fort Detrick, Maryland 21702-5000.

XX. LITERATURE REVIEW

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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% ± 8% vs. 11% ± 3% [55% ± 11% vs. 21% ± 8% as percentage of control data], $p = 0.002$; and 18% ± 4% vs. 11% ± 3% [43% ± 6% vs. 21% ± 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 Aster[12] 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 (^{111}In, 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 ^{51}Cr by refrigerated PLTs (data not shown). The ^{111}In-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 ^{111}In activity after each subject's control transfusion and adjusted our calculations accordingly.

On Day 3, the subject received an ^{111}In-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 ^{111}In 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.

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%
					at Day 15	
Plasma (100%)/15 days	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), GPIbα-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 α 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 ± 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% ± 3% of prestorage values). No significant differences in PLT yields between PAS and plasma-stored PLTs were seen after 3 days

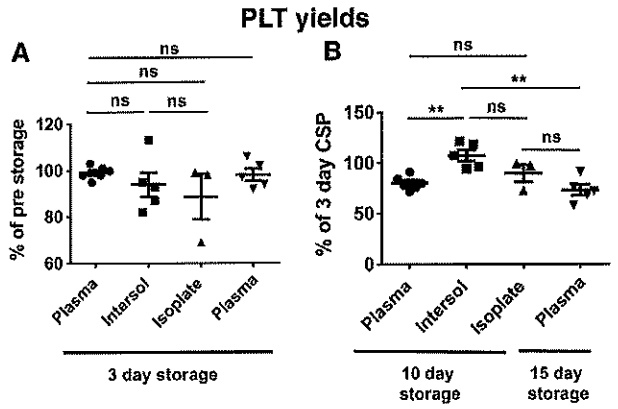

Fig. 1. PLT yields of apheresis units: PLTs were stored either in plasma alone (●, 3-day control units collected during studies on 10-day-stored plasma; ▼, 3-day control units collected during studies on 15-day-stored plasma) or in a 65% PAS, 35% plasma mixture (■, Intersol; ▲, Isoplate). [Total PLT yield in the component, calculated by multiplying the PLT count of the sample times the volume of the component (PLT count × 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 ± 7, 107 ± 12, 90 ± 39, and 72% ± 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 ± 2, 28 ± 3, 24 ± 12, and 86% ± 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 ± 11, 1375 ± 26, 1195 ± 54, and 222% ± 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 ± 219, 246 ± 87, 301 ± 106, and 730% ± 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 ± 76, 123 ± 53, 267 ± 118, and 278% ± 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 ± 127, 1865 ± 88, 1885 ± 41, and 561% ± 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% ± 11% and survivals 2 ± 0.4 days. For the test 10-day plasma, Intersol, Isoplate, and 15-day plasma units, poststorage recoveries averaged 24 ± 8, 18 ± 4, 8 ± 2, and 11% ± 3%, respectively (55 ± 11, 43 ± 6, 21 ± 8, and 29% ± 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

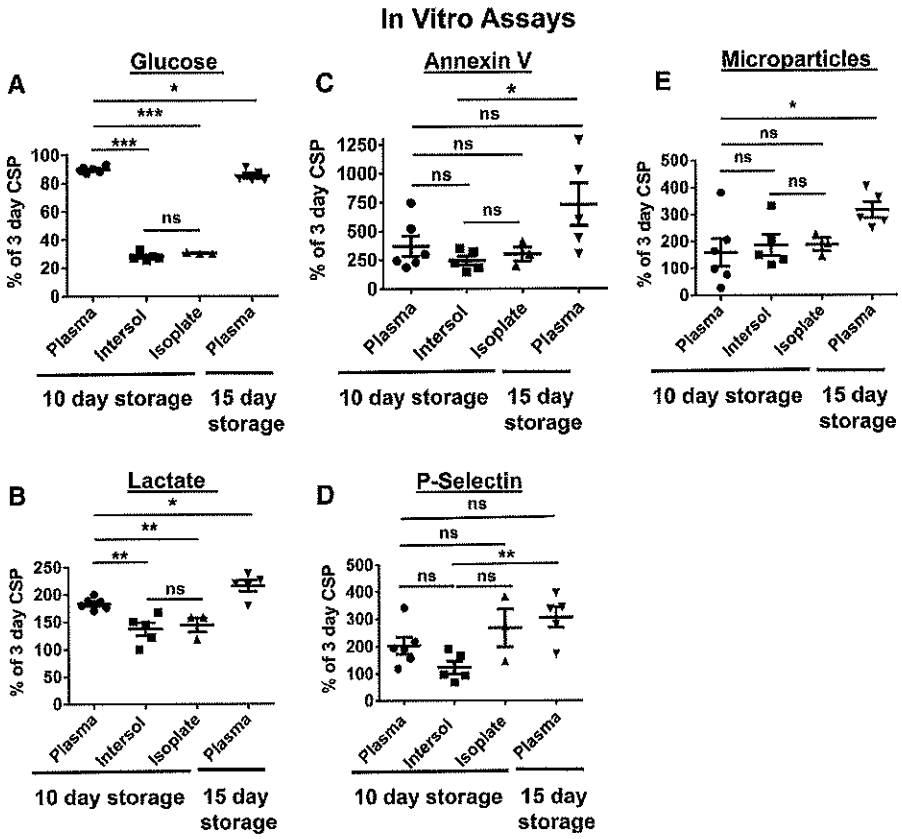

Fig. 2. In vitro PLT measurements: PLTs were stored either in plasma alone (●, 10-day storage; ▼, 15-day storage) or in a 65% PAS, 35% plasma mixture (■, Intersol; ▲, Isoplate) at 4°C, for either 10 or 15 days as indicated. (A) Glucose and (B) lactate determined by blood gas analyses, (C) annexin V–positive 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% ± 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

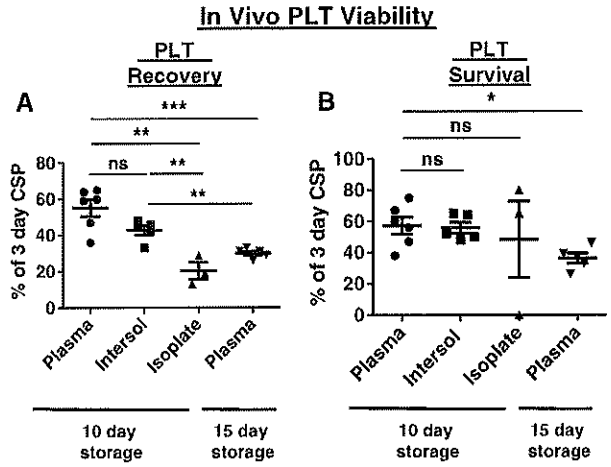

Fig. 3. In vivo PLT measurements: healthy human subjects received their autologous radiolabeled PLTs after storage at 4°C (plasma—●, 10-day storage; ▼, 15-day storage) or in a 65% PAS, 35% plasma mixture (■, Intersol; ▲, Isoplate). (A) Recovery of transfused PLTs at the 1-hour time point. (B) Survival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

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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

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Short running head: Viability of extended storage refrigerated platelets

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ABSTRACT

Cold (4°C)-stored platelets (CSP) are currently under investigation for transfusion in actively bleeding patients. The historic three day storage limit severely limits CSP availability for clinical and military applications. It is currently unknown how long CSP can be stored for clinical application.

In vivo, we found a continuous decline in platelet recovery from 5 to 20 days. Platelet survival reached a low plateau at 10 days of storage. Ex vivo, the maximum platelet αIIbβ3 integrin response to collagen was observed at 5 days of cold-storage, and a continuous decline was seen thereafter. However, platelet integrin activation and mitochondrial membrane preservation remained significantly better at 20 days in the cold compared to 5 days at room temperature. All platelet metabolic and functional parameters suggest comparable or better results in 20 day CSP compared to 5 day RT-stored platelets.

In summary, we performed the first studies with extended storage CSP apheresis platelets in plasma for up to 20 days with a fresh comparator. Storing CSP 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.

Key words: 4°C stored platelets, cold-stored platelets, extended platelet storage, platelets stored in plasma

INTRODUCTION

Platelet transfusions can be life-saving by facilitating hemostasis in trauma and surgical patients and can be used for bleeding prophylaxis in thrombocytopenic cancer patients. Platelets for transfusion are currently collected from volunteer donors and stored at room temperature (22 ± 2°C). Room temperature storage maximizes platelet recovery and survival in transfused recipients[1]; however, it also increases the opportunity for bacterial growth in the platelet bag. The FDA limits the shelf life of platelets to 5 days to reduce this bacterial risk. The limited storage time leads to outdates and wastage and subsequent times of shortage especially in rural areas in the US and in far forward military scenarios. Some laboratories can extend the shelf life with an additional bacterial point of care test to 7 days.[2] Recently, refined guidelines by the FDA increased the requirement for bacterial testing or alternatively, requested pathogen reduction. These new guidelines will make room temperature-stored platelets more costly and labor intensive. These recent efforts by the FDA to reduce bacterial contamination highlight the magnitude of the problem for the transfusion medicine community.[3] 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]

Cold-storage of platelets could alleviate these issues because of very limited bacterial growth at 4°C. Unfortunately, refrigeration of platelets is known to reduce platelet circulatory lifespan to 1 to 2 days compared to an average lifespan of 4 to 5 days at 22°C storage.[1] Previous reports suggested superior *in vitro* performance of platelets stored in the cold.[4,5] However, earlier studies about bleeding time corrections after transfusion of cold-stored platelets in thrombocytopenic patients yielded contradictory results. In

addition, they were performed in the 1960s and 1970s and require confirmation in contemporary platelet transfusion settings including clinical trials.[4,6,7] 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 US can currently apply for a FDA variance, which allows for 3 day cold-storage in plasma for actively bleeding patients, which is based on a historic 3 day storage time limit.

For licensing purposes the FDA requires recovery of 66% and survival of 58% of a healthy human subjects' fresh platelets for room temperature stored platelet products.[8-10]

If these guidelines need to be revised for alternative platelet/hemostatic products is currently under discussion.

One potential problem of platelets in plasma exposed to cold is the formation of micro- and macro-aggregates, as reported by some groups.[11,12] Aggregate formation occurs presumably due to platelet αIIbβ3 integrin activation with crosslinking of platelets and plasma proteins. If these aggregates are clinically relevant and/or removed by the bedside transfusion filter remains to be investigated. Using a rigid 3 day storage time limit, and plasma as storage medium, one group reported wastage of up to 20%, due to outdates and aggregates.

Aggregate formation can be prevented by the replacement of plasma with platelet additive solutions (PAS).[11]

PAS are balanced electrolyte solutions with the intent to replace plasma components, including allergens, ABO antibodies, or pathogens.[13] Cold-stored platelets are predominantly intended for actively bleeding surgery and trauma patients, and no hemostatic benefit can be expected from PAS.[14] Trauma and surgery patients can require replacement of the entire blood volume by transfusion products, therefore the reduction of plasma in favor of PAS can further dilute coagulation factors possibly to critical levels.[15,16]

We have previously shown that CSP stored in plasma show a better recovery *in vivo* compared with platelets stored in PAS licensed in the US.[17]

Based on these results we concluded that platelets stored in plasma are the best way to move forward since they (i) yield a higher recovery compared with US-licensed PAS[17], (ii) are intended for actively bleeding trauma and surgery patients, and (iii) are easier to manufacture in far forward military scenarios.

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 day room temperature comparator. 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 better understand changes in platelet biology during storage at 4°C and to further characterizing the cold lesion.

Our studies shed light on in vivo and in vitro features of platelets stored up to 20 days in plasma. Twenty day storage essentially quadruples the current platelet shelf-life for room

temperature-stored platelets. Our *in vitro* data suggests that 20 day CSP are not inferior and may even be superior in trauma or surgery patients compared with 5 day RT platelets.

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 Control and Test platelets

A standard single apheresis platelet unit (target platelet yield 3.0 X 10^{11}/unit and concentration of ~1500 x 10^3 platelets/μL) was collected from 23 healthy subjects using the Trima Accel Automated Blood Collection System (TerumoBCT, Denver, CO).

Each unit achieved a final platelet concentration of 700 – 2100 x 10^6 platelets/μL, as per allowable bag parameters. The unit was stored for a predetermined period of up to 20 days at 4°C, or up to 5 days at RT (22°C).

At the end of the storage period, the subject returned to receive an ^{111}Indium Oxine (Indium 111, In-111) radiolabeled aliquot of their 4°C stored platelets. Follow-up samples from the subject were collected approximately 2 hours post-transfusion (recovery) and on Days 1 (2X), 2 (2X), and 3 to calculate recovery and survival of the subject's 4°C stored platelets. The Day 1 and Day 2 sample draws were 2 - 10 hours apart.

Seven to 14 days after the infusion of the radiolabeled aliquot, the subject returned to receive a second radiolabeled aliquot of fresh platelets. To facilitate this, on the morning

of the second infusion, the subject returned for collection of a 43 mL blood sample. The blood was processed to obtain a sample of the subject's platelets to serve as a "fresh" or pre-storage control comparator. The platelets were radiolabeled with In-111. The subject returned later in the day for infusion of the radiolabeled fresh 'control' comparator aliquot. Follow-up blood samples were drawn at ~2 hours after the infusion on Day 0 and then on Days 1, 2, 3, 4 or 5, and 6 or 7 day post infusion to calculate recovery and survival of the subject's fresh vs. stored platelets.

All room temperature data are taken from a previously performed and reported study, which was done under conditions which match this current report in terms of collection concentration, platelet count and storage. The only difference was that for the fresh comparator Indium 111 was used and the radiolabeling compound for the stored platelets was Cr-51. These data have been reported previously, albeit in a different format, to show correlation with platelet metabolites in the storage bag.[18] Re-utilization of these data avoids unnecessary repetition of experiments in healthy human subjects who have virtually no medical benefit and expose themselves to risks for these procedures. The 5 day RT time point is a common control group for many studies, therefore we consider it ethically irresponsible to repeat this group with every study as long as collection, storage, and test parameters do not change.

Radiolabeling of stored platelets

Please see supplemental material for details.

In vitro platelet measurements

Please see supplemental material for details.

Healthy human subjects research

The research was approved by the Western Institutional Review Board (WIRB) and all human participants gave written informed consent. MS, SB, and LF analyzed the data and all authors had access to primary clinical trial data. The clinical trial registration number for this study was NCT02754414 and the entry name: Cold Apheresis Platelets in PAS (CAPP). The study was conducted in accordance with the Declaration of Helsinki.

Statistical analysis

Based on our previous study which evaluated extended room temperature-storage with platelet additive solutions,[19] we calculated an effect size of ~2 for platelet recoveries between different time points. Based on this a sample size of n=5 was calculated to be required to provide 80% power to detect a statistically significant difference between the different plasma time points (t-test, unpaired, two-tailed, α err. prob. 0.05). We therefore planned to enroll 5 subjects per group and then assess for statistical significance. No additional data points were obtained after the study was concluded and tests for significance were run. Results are reported as mean ± standard error of the mean and statistical significance was assessed by unpaired, 2-tailed Student *t* test, unless otherwise indicated. A *P* value equal or less than 0.05 was considered significant.

RESULTS

Total platelet yield after storage

Post storage platelet yields averaged 3.36 ± 0.12 x 10^{11} for 5 day room temperature-stored platelets. A significantly lower platelet yield was observed for platelets stored for 5 days in the cold (2.21 ± 0.27 x 10^{11}, p=0.0003). After 5 days no significant differences were observed between 5 to 10 days, 10 to 15 days, and 15 to 20 days (Figure 1A) either as absolute or percentage of fresh. Five day 4°C-stored platelets showed a significantly lower yield compared to 5 day 22°C (114 ± 9% of pre-storage values vs. 75 ± 5%, p=0.046) (Figure 1B).

In vivo platelet viability

There was a significant step-wise decrease in recovery of stored platelets from 5 day to 10 day 4°C (34 ± 5% vs. 18 ± 3%, p=0.016), and 15 to 20 day 4°C (13 ± 1% vs. 9% ± 1%, p=0.041) (Figure 2A, E). Cold storage of platelets led to a reduction of recoveries when compared to room temperature storage which was significant after 5days of storage (53 ± 2% vs. 34 ± 5%, p=0.0007). When we calculated the percentage of fresh results, all steps were significantly lower compared to the respective previous step, and 5 day RT was significantly lower compared to 5 day CSP (92 ± 3% vs. 46 ±3%, p<0.0001, 46 ±3% vs. 31 ± 2%, p=0.003, 31 ± 2% vs. 22 ± 2%, p=0.009, 22 ± 2% vs. 13 ± 1%, p=0.008) (Figure 2B).

Platelet survival studies showed a significantly lower survival of refrigerated platelets compared to room temperature-stored platelets. Interestingly, the maximum drop

occurred early (between 0 and 10 days) and after 10 days a low plateau was reached. This was reflected in both the absolute analysis and the percentage of pre-storage analysis which both showed significant drops between 5 day RT and 5 day cold (6.6 ± 0.3 days vs. 1.6 ± 0.2 days, $p<0.0001$, 74 ± 3% vs. 20 ± 3%, $p<0.0001$) and 5 day cold and 10 day cold (1.6 ± 0.2 days vs. 0.8 ± 0.1 days, $p=0.004$, 20 ± 3% vs. 8 ± 1%, $p=0.001$), but no significant further decrease thereafter (Figure 2C, D, E).

Platelet metabolism markers

Glucose levels dropped quickly and significantly in room temperature-stored samples when compared to 5 day cold-stored samples (326 ± 7mg/dl vs. 378 ± 6mg/dl, $p=0.003$) (Figure 3A). In contrast, there was a step-wise decrease in cold-stored platelets from 5 to 20 days of storage, which was not significant (when calculated comparing step by step) in the absolute analysis, but significant between 5 and 10 day, and 10 and 15 days in the percentage of pre-storage analysis (98 ± 2% vs. 90 ± 1%, $p=0.007$, and 90 ± 1% vs. 71 ± 4%, $p=0.05$ respectively) (Figure 3B). Correspondingly, lactate increased more rapidly at RT and was significantly higher than 5 day cold-stored platelets (9.9 ± 0.5mg/dl vs. 4 ± 0.3mg/dl, $p<0.0001$). Lactate in cold-stored platelets mirrored the glucose measurements with a step wise, significant increase from 5 to 10 days (4 ± 0.3mg/dl vs. 5.6 ± 0.3mg/dl, $p=0.0064$), and 10 to 15 days (5.6 ± 0.3mg/dl ± 8.6 ± 0.4mg/dl, $p=0.0009$), but after 15 days a high plateau was reached.

Platelet viability markers in vitro

Platelet αIIbβ3 integrin activation requires inside out signaling and is critical for platelet participation in hemostasis at the site of vascular injury. Therefore we tested platelet

activation in response to collagen, an extracellular matrix protein which can cause platelet adhesion under flow and platelet aggregation via the ITAM receptor GPVI and the αIIβ1 integrin.[20] Platelet αIIbβ3 integrin activation was measured with the PAC-1 antibody which specifically binds the activated conformation of the integrin.[21]

All samples responded appropriately to collagen before storage (fresh) (Supplemental Figure 1A). As described previously, we observed pre-activation of αIIbβ3 integrin at 5 days of cold-storage (Figure 4A), when compared to pre-storage (Supplemental Figure 1A). No further increase of pre-activation was noted between 5 and 20 days (Figure 4A). The pre-activation level was higher in 5 day chilled platelets compared to five day room temperature-stored platelets (3.2 ± 0.22 vs. 15.7 ± 4.1, p=0.038). Five day refrigerated platelets showed a significantly greater response to collagen compared to 5 day room temperature-stored platelets (26.7 ± 6.6 vs. 4.4 ± 0.3, p=0.018) (Figure 3A). The integrin activation in response to collagen was significantly lower in 5 day room temperature stored platelets compared to cold-stored platelets even after 20 days of storage (21.3 ± 4.8 vs. 4.4 ± 0.3, p=0.012) (Figure 4A). When the activation response of the corresponding fresh platelets was taken into account a significantly greater response was noted in 15 day cold-stored platelets compared to 5 day room temperature-stored platelets (Figure 4B). The corresponding scatter plots and histograms showed a clear increase in PAC-1 binding in refrigerated platelets which was comparable to fresh platelets and a lack of PAC-1 binding in RT-stored platelets comparable to unstimulated samples (Figure 4 C,D).

Platelet α-degranulation is a hallmark of platelet storage lesion. We tested for P-selectin expression over time in the storage bag (without agonist stimulation). We found that room

temperature storage leads to a significantly greater P-selectin expression at 5 days (47.6 ± 7.4 vs. 15.7 ± 2.5, p=0.005) compared to 5 days cold-storage. However, at refrigerated temperatures, there was a continuous increase in degranulation and by day 20 the level was similar, and not significantly different compared to 5 day RT-stored platelets, neither in absolute numbers or as percentage of fresh (47.6 ± 7.4 vs. 61 ± 3.5, p=0.134, and 457 ± 98% vs. 841 ± 215%, p=0.135) (Figure 4E,F).

To see how early apoptosis events compare between cold-storage and room temperature-storage we tested for mitochondrial membrane integrity and caspase 3,7 activation. Mitochondrial membrane integrity was assessed by flow cytometry utilizing the JC-1 dye. When the dye is highly concentrated in mitochondria it forms J-aggregates with an emission maximum at ~590nm (FL2); when more diluted in cytoplasm, the dye emits at ~530nm (FL1). We utilized the proton gradient uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP) as positive control, to show successful disruption of the electron transport chain. All fresh samples showed a significant drop of the FL2/FL1 ratio after CCCP was added to the sample (Supplemental Figure 1B). When testing stored samples we found a markedly reduced FL2/FL1 ratio in 5 day room temperature-stored platelets, compared to cold-stored platelets at 5 days (109.5 ± 25.9 vs. 1.1 ± 0.5, p=0.0005) (Figure 4A). Surprisingly, this difference remained significant for the maximum storage time tested (20 days), indicating a significant advantage of cold-storage for mitochondrial preservation (22.7 ± 4.5 vs. 1.1 ± 0.5, p=0.0003) (Figure 4A, C). Of note, cold platelets responded appropriately to CCCP and a reduction in FL2/FL1 ratio was observed up to 20 days of storage, while the ratio was already too low at baseline to appreciate a reduction in RT-stored platelets (Figure 4A). The percentage of fresh

analysis corroborated the absolute analysis and significant differences were found between 5 day RT and 5 day 4°C and 5 day RT and 20 days of cold-storage (1.7 ± 0.8 vs. 183.5 ± 62.4, p=0.009, and 1.7 ± 0.8 vs. 55.3 ± 15.9, p=0.004, respectively) (Figure 4B).

During cell stress mitochondrial membrane disruption is followed by caspase 3,7 activation.[22] Surprisingly, this sequence was not found in 5 day room temperature, 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 cold-storage in the percentage of fresh analysis, no significance was observed for any time point between the groups (Figure 2). Importantly, all 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) (Figure 4D).

For this study we did not systematically address the occurrence of micro and macroaggregates in cold units. Overall, over twenty 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.

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.[17] 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 the current clinical standard.

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 very low plateau, 3) platelet yield is significantly lower at 5 day 4°C storage, compared to 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.

Cold-stored platelets are evaluated for trauma and surgical patients with active bleeding and therefore platelet survival is not likely to be of high importance. However, since CSP represent a hemostatic transfusion product it is vitally important to characterize its fate *in vivo*. We sought to investigate how long CSP stay in circulation 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.

Our αIIbβ3 integrin activation data suggests that platelet activation after 20 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 storage lesion.

In fact, the maximum αIIbβ3 pre-activation during cold-storage was achieved at the earliest time point (5 days). 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 cold-storage time suggesting that there are limits for platelet storage at 4°C. Our studies using mitochondrial marker and caspase enzyme induction show a peculiar pattern: While 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 well with αIIbβ3 activation response to collagen suggesting a causal relationship between mitochondrial health and integrin activation.

Our findings contradict a previously published study by Li et al. who show caspase 3 activation in room temperature-stored platelets after 7 days.[23] While Li et al. see activation already at 3 and 5 days, a different group reports that caspase activation during platelets storage is a relatively late event.[24] Therefore it is possible that we did not store platelets long enough at both RT and 4°C to detect caspase activation. The successful caspase

activation by ABT737 however indicates that the lack of response was not an intrinsic problem with the assay itself.

We found continuous decrease in glucose and increase in lactate over storage time indicating that metabolism continues even though it is markedly slowed when compared to room temperature storage. Interestingly, 20 day 4°C–storage showed comparable glucose levels to 5 day 22°C storage suggesting a metabolism slowed to approximately one quarter of the metabolism at room temperature. Also in this regard 20 day cold-storage appears to be comparable to 5 day room temperature storage.

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 pre-activated αIIbβ3 integrins on platelets. Our data suggest that the maximum aggregation happens early during storage (between 0 and 5 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 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 in response to antiplatelet therapy has often been suggested by some[6,25,26], but is criticized by others due to a lack of correlation with surgical bleeding.[27] Fecal occult blood is often only present in thrombocytopenic patients and antiplatelet reagents do not

reliably lead to detectable fecal blood loss.[28] Furthermore the short term effect of a hemostatic product like CSP is likely hard to measure in a fecal occult blood test.

Another limitation is clearly that we show in vivo and in vitro characteristics, but the exact maximum storage time has yet to be determined and should focus on the preservation of hemostatic efficacy in vivo. We found a continuous decrease in recovery to approximately 15% of the recovery of room temperature-stored platelets. This could suggests that storing platelets at 20 days in the cold already reduces recovery to an extent that hemostatic efficacy is compromised. Ultimately, the balancing act of storage time prolongation, in vitro and in vivo function, and in vivo viability will have to be addressed in future studies in thrombocytopenic and/or actively bleeding patients.

In summary, our study is the first to compare cold-stored platelets up to 20 days to fresh, and 5 day room temperature-stored platelets in plasma in healthy human subjects. We found that 20 day storage in the cold share many similarities with 5 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.

Author contributions

MS designed the study, analyzed the data and wrote the manuscript, SB, IG, TC, EP and LF performed experiments and analyzed the data, LF recruited healthy human subjects, performed apheresis procedures and analyzed the data.

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Figure Legends

Figure 1: Platelet Yields: [Total platelet yield in the component (platelet count x component volume = platelet yield)] Platelets were stored in plasma for either 5 days at 22°C (solid black 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). F= Fresh (pre-storage) sample, S= stored sample. **A**) Results are shown as mean ± SEM of absolute counts. ns= not significant,***$p<0.001$. **B)** Results are shown as percentage of the respective fresh sample. ns= not significant,*$p\leq0.05$, n= 21 for RT samples, n=5-7 for cold-stored groups.

Figure 2: In Vivo Platelet Characteristics: Healthy human subjects received autologous radiolabeled platelets either fresh (F), or after storage (S) at 4°C or room temperature (22°C). Room temperature (black 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**) Recovery of transfused platelets after 2 hours, in fresh (pre-storage) (F) and stored (S) samples shown as mean ± SEM **B**) Platelet recovery shown as percentage of the subject's fresh autologous radiolabeled platelets, **C)** Survival of transfused platelets of fresh (pre-storage) (F) and stored (S) samples shown as mean ± SEM, **D)** Survival shown as percentage of the subject's fresh autologous radiolabeled control platelets, **E)** Representative traces taken from radiolabeling data (multiple hit model - solid lines, and tangent line - dotted lines). Fresh (purple line with horizontal intersections), 5 day room temperature stored (black line with triangles), 5 day 4°C-stored (black line with circles), 10 day 4°C-stored (red line with diamonds), 20 day 4°C-stored (green line with squares).

*p<0.05, **p<0.01, ***p<0.001, ns= not significant, n=21 for RT and n=5-7 for all other groups.

Figure 3: In Vitro Platelet Metabolism Parameters: Glucose and lactate levels measured by blood gas reader. Room temperature (black 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). F= Fresh (pre-storage) sample, S= stored sample. **A)** Glucose levels of fresh (pre-storage) (F) and stored (S) samples shown as mean ± SEM and **B)** Glucose levels shown as percentage of corresponding fresh samples, **C)** Lactate levels of fresh (pre-storage) (F) and stored (S) samples shown as mean ± SEM and **D)** Lactate levels shown as percentage of corresponding fresh samples. *p<0.05, **p<0.01, ***p<0.001 comparing stored samples (absolute), or percentage of fresh as indicated, ns= not significant, n=5-7 for all samples

Figure 4: In Vitro Platelet Activation Parameters: Platelet αIIbβ3 integrin activation was measured by PAC-1 antibody binding and α-granule secretion by P-selectin exposure by flow cytometry. PAC-1 antibody binding was measured at baseline (B) or after stimulation with the agonist collagen (C). P-selectin was measured at baseline without agonist. 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)** PAC-1 binding levels shown as

mean ± SEM and **B)** PAC-1 binding after stimulation with collagen as percentage of fresh (pre-storage) sample stimulated with collagen **C)** Representative scatter plots of baseline (pre-storage, no agonist), fresh (pre-storage, stimulated with collagen), 5 day RT (room temperature, stimulated with collagen), 5 day, 15 day, 20 day 4°C-storage (all stimulated with collagen) **D)** Representative histograms of 5 day room temperature stored platelets stimulated with collagen (black), and 5 day 4°C-stored platelets (grey). **E)** Percentage P-selectin positive events (CD62P binding) shown as mean and ± SEM. Same groups as outlined above. (F) indicates fresh (pre-storage) sample, and (S) stored sample. **F)** P-Selectin exposure as percentage of corresponding fresh samples. *p<0.05, **p<0.01, ***p<0.001, ns= not significant, n=4-8 for all samples

Figure 5: In Vitro Platelet Apoptosis Parameters: Platelet mitochondrial membrane potential measured as JC-1 dye 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 ratio was measured by flow cytometry in baseline stored sample (B) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP)-stimulated stored sample (P) and shown as mean ± SEM, and **B)** JC-1 (FL2/FL1) stored baseline samples as percentage of the corresponding fresh baseline samples (pre-storage) shown as mean ± SEM **C)** Representative scatter plots of baseline (pre-storage), CCCP (pre-storage, stimulated with CCCP), 5 day RT (room temperature, baseline), 5 day, 15 day, 20 day 4°C-storage (all baseline) **D)** Caspase 3,7 activation measured by flow cytometry and shown as mean fluorescence intensity (MFI) ± SEM. **E)** Caspase 3,7 activation shown

as percentage of pre-storage sample. *p<0.05, **p<0.01, ***p<0.001, ns= not significant, n=4-8 for all samples

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Figure 1

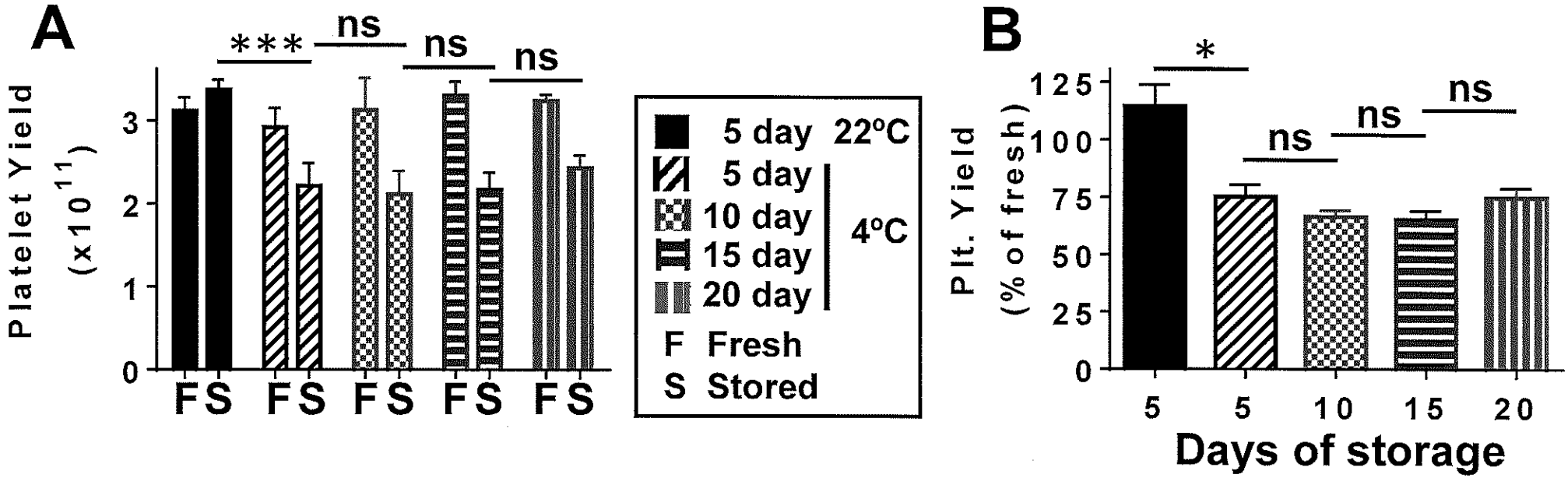
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Figure 2

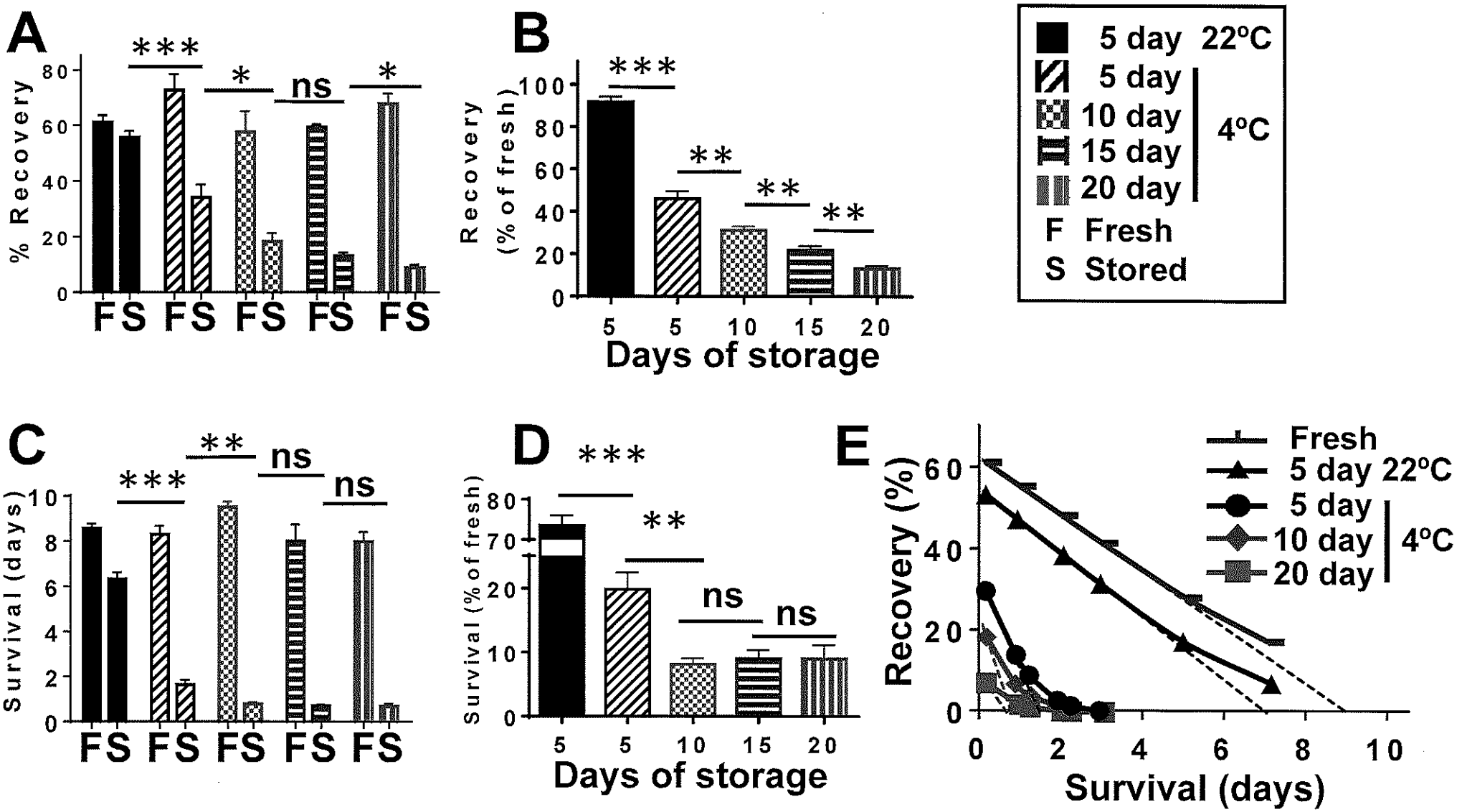
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Figure 3

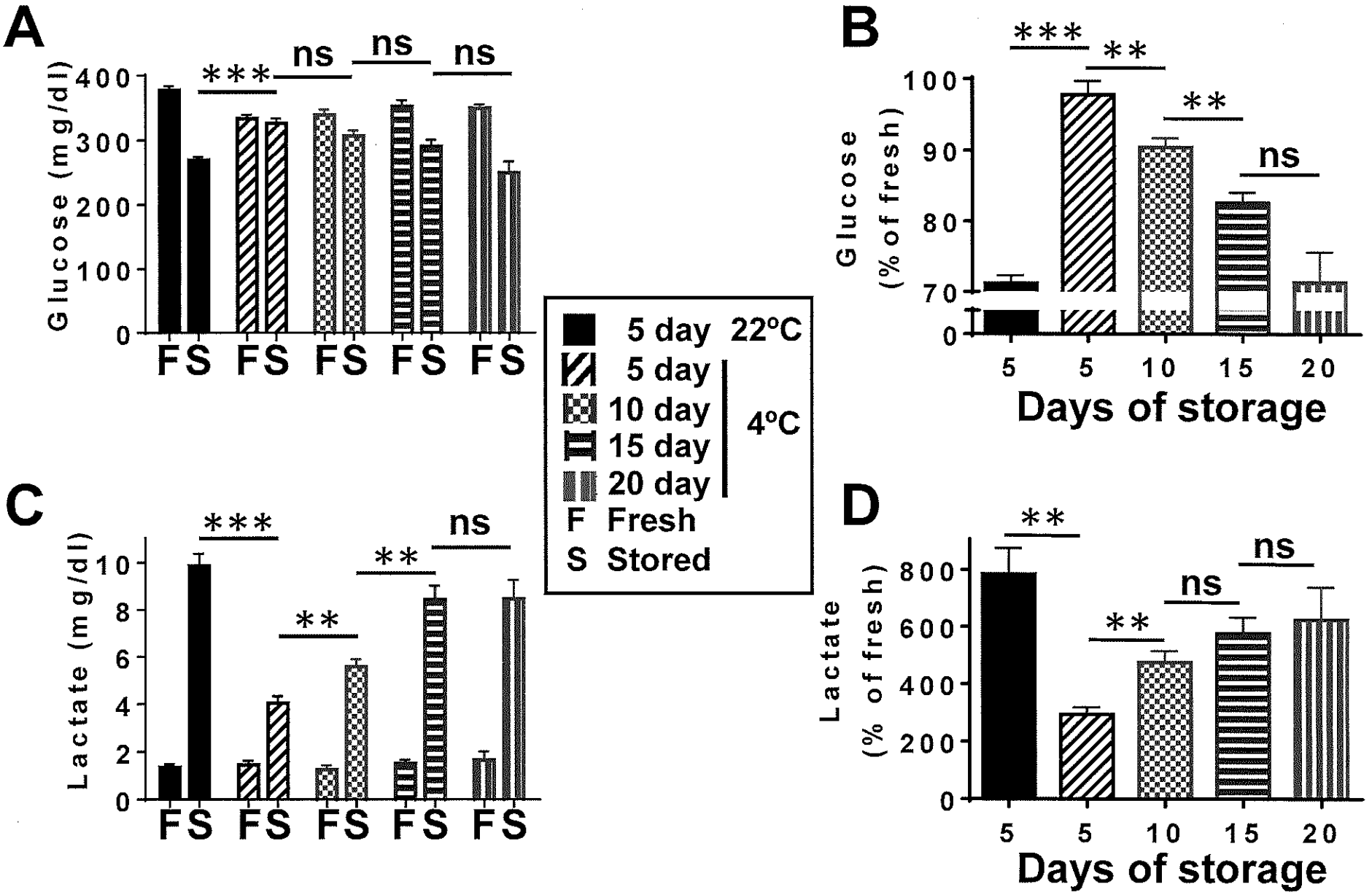
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Figure 4

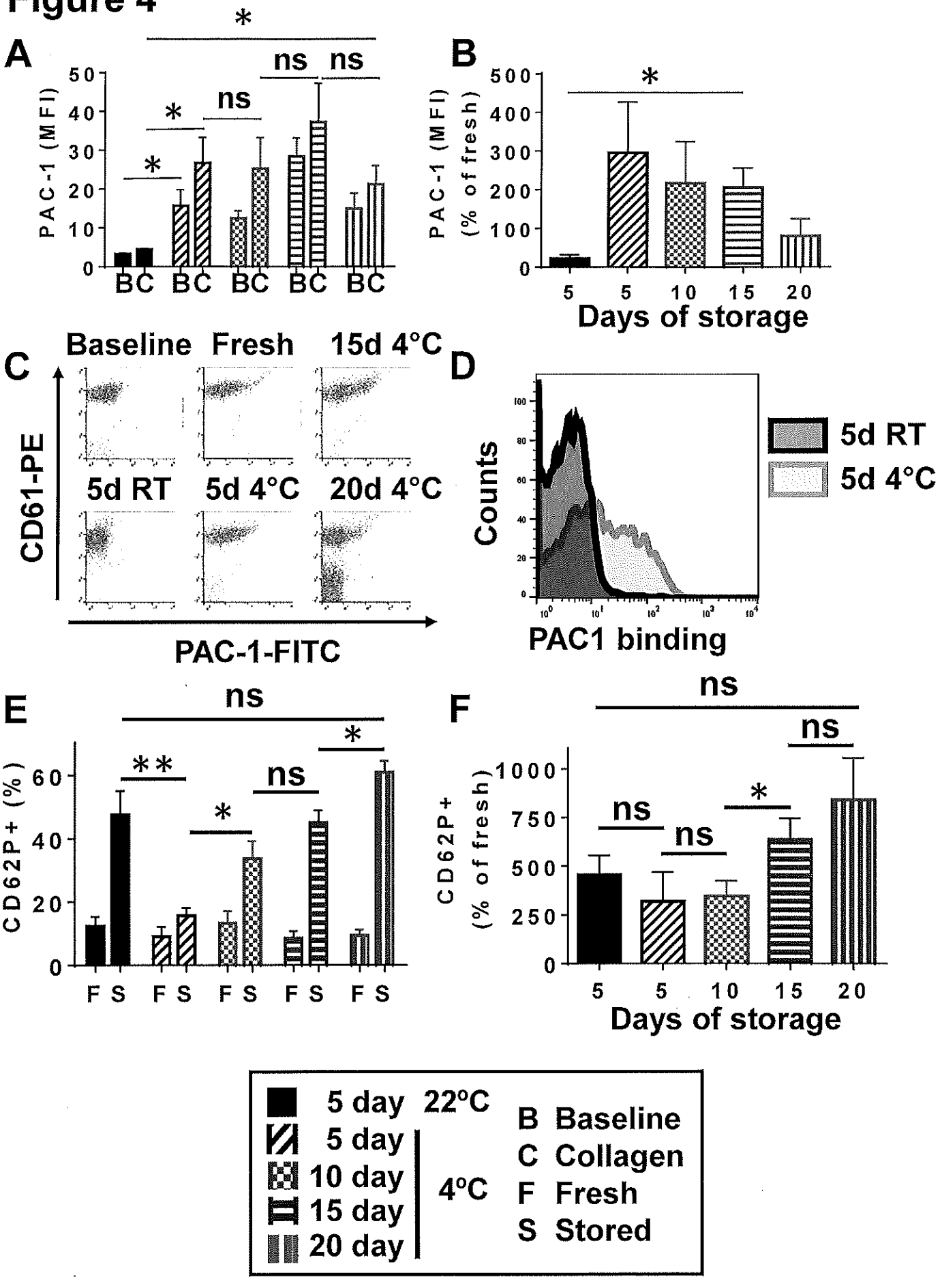
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Figure 5

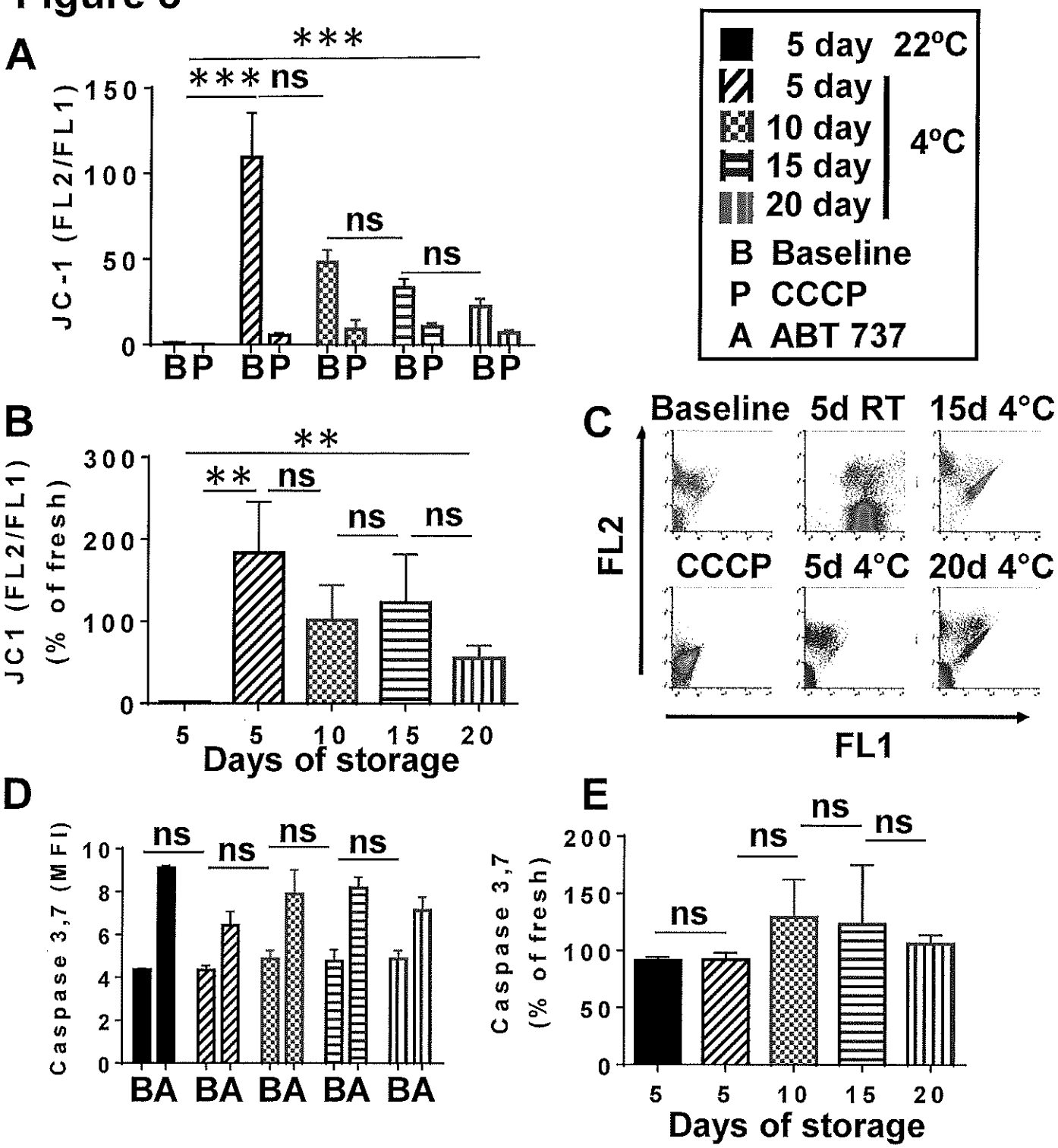
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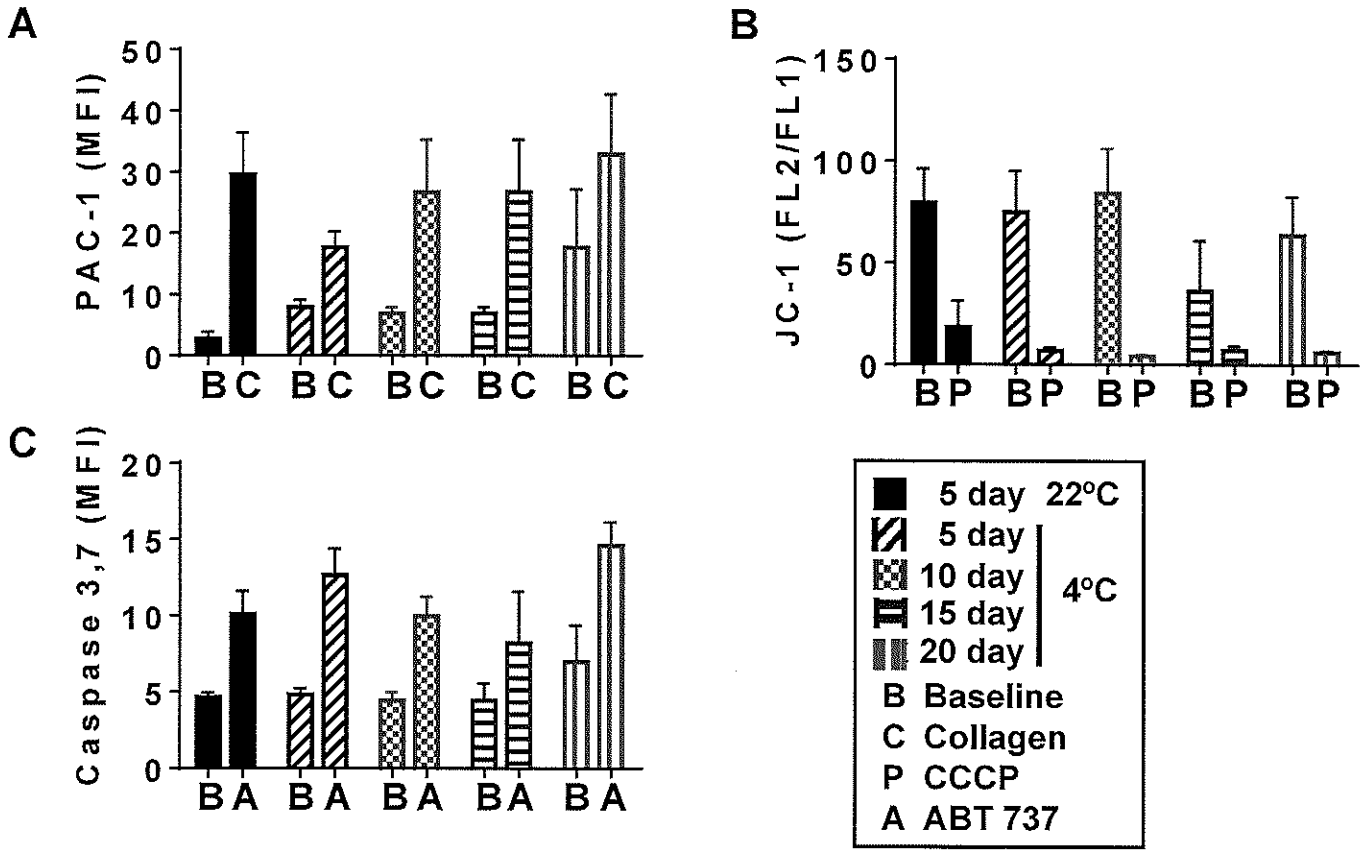
Figure S1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B A B A B A B A B A
5 day 22°C
5 day
10 day
15 day
20 day
4°C
B Baseline
C Collagen
P CCCP
A ABT 737

Figure S1: Fresh (pre-storage) platelet responses: 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)** Platelet αIIbβ3 integrin activation was measured by PAC-1 antibody binding by flow cytometry. PAC-1 antibody binding was measured at baseline (B) or after stimulation with the agonist collagen (C). PAC-1 binding levels plotted as mean ± SEM, **B)** Platelet mitochondrial membrane potential measured as ration of JC-1 dye red (FL2) to green (FL-1) ratio. JC-1 FL2/FL1 ratio was measured by flow cytometry in baseline stored sample (B) and carbonyl cyanide m-chlorophenyl hydrazone (CCCP)-stimulated stored sample (P) and plotted as mean ± SEM, **C)** Caspase 3,7 cleavage measured by flow cytometry and plotted as mean fluorescence intensity (MFI) ± SEM. Baseline (B), ABT737 (A), n=4-8 for all samples

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 4ºC. 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 reinfusion.

Results/Findings: Over storage time, platelet recoveries (% of fresh) declined significantly from 5 to 10 days (p=0.003), from 10 and 15 days (p=0.009), and 15 to 20 days (p=0.019). Platelet survivals 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 ΔHCO3, Δglucose, Δlactate, Δmicroparticles, 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 (4ºC)-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.

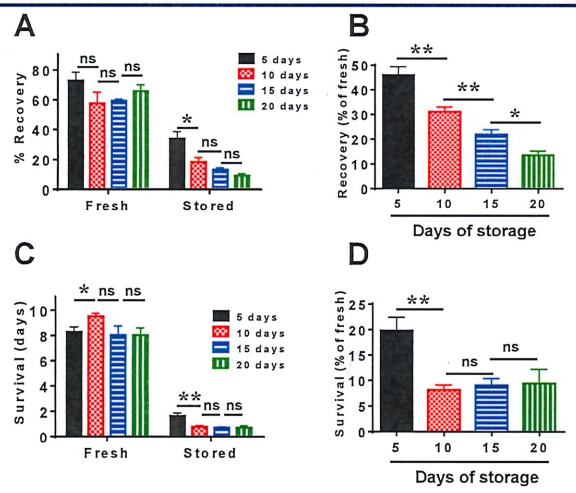

Figure 1: In vivo parameters: Healthy human subjects received either fresh or 4°C- stored autologous platelet transfusions. (A) Recovery of transfused fresh or stored platelets at 2 hour time point. (B) Recovery of stored platelets as percentage of fresh sample. (C) Survival of fresh or stored platelets in days (D) Survival of transfused platelets as percentage of fresh. *p<0.05, **p<0.01, ***p<0.001, ns= not significant.

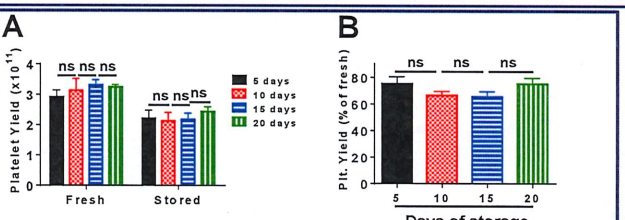

Figure 2: Platelet yields: Total platelet yield in the component was calculated by multiplying the platelet count of the sample times the volume of the component (platelet count x component volume = actual platelet yield) (A) Platelets were sampled at baseline (fresh), or after designated storage period.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

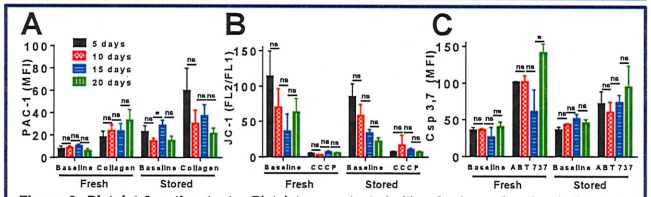

Figure 3: Platelet function tests: Platelets were tested either fresh or after the designated storage time point (A) Platelets were tested at baseline or after addition of 20µg/ml collagen. Integrin activation was tested by adding the activation-specific PAC-1 antibody. (B) Platelets were tested at baseline 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 BH3 mimetic ABT737 (positive control). A fluorescently labeled probe to caspase 3,7 was added. p<0.05, ns= not significant.

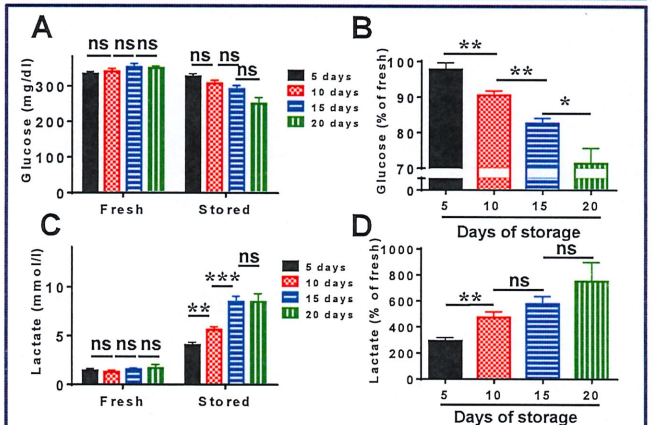

Figure 4: In vitro metabolic parameters: Units were sampled at baseline (fresh) or after designated storage period and tested for glucose or lactate by a commercially available blood gas instrument. (A) Absolute glucose values at baseline (fresh) and after designated storage period. (B) Glucose values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

The authors have no conflict of interest to disclose

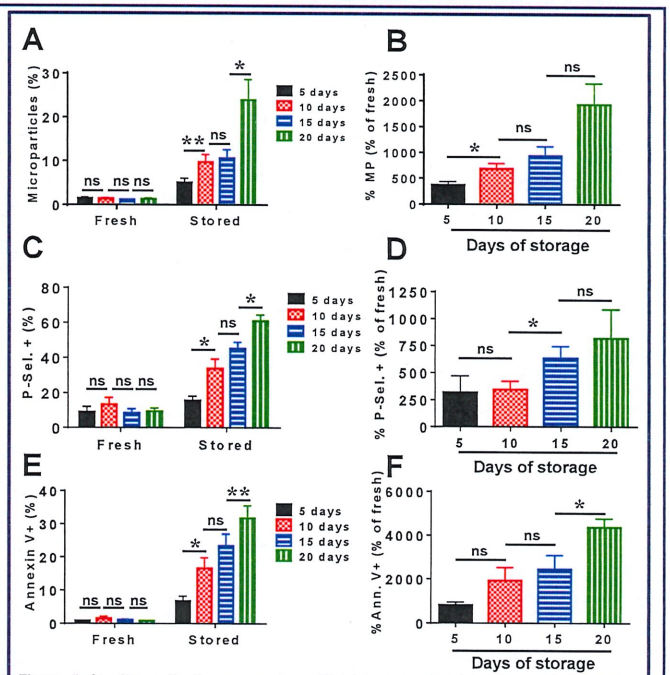

Figure 5: In vitro activation parameters: Platelets were stored in plasma at 4ºC and sampled at baseline (fresh), or after designated storage period. (A), CD61-positive microparticles measured by flow cytometry. (B), Microparticles as percentage of fresh samples. (C), P-selectin (CD62P)-positive events 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 4ºC in plasma could be used to expand the available supply of platelets to treat bleeding patients.

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 which includes funds contracted to Terumo of $1,402,000

Study/Product Aim(s)

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

Approach

This is a non-clinical, exploratory study of apheresis platelets stored in the cold (4° C). We are testing radiolabeled recovery and survival in health volunteers utilizing two sequential Indium-111 labels. The first radiolabeled infusion is performed using an aliquot from the extended stored apheresis platelet unit. The test unit is stored from 3-20 days. The second infusion is a fresh comparator obtained from and administered to the subject one week later. Various invitro tests are also performed. Study title Cold Apheresis Platelets in Plasma (CAPP).

Refrigerated platelets in plasma- stored for up to 20 days

Accomplishment: Enrollment ongoing to evaluate apheresis platelets at 4° C

Timeline and Cost

Activities CY	15	16	17	18
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				
Estimated Budget ($K)	**$1.124**	**$1.592**	**$1.745**	**$2.698**

Dated: 14NOV2019
Please note that Terumo has returned $517,837 to BWNW for project use.

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 **Isoplate/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

CAPP is an exploratory study only. For confirmation of results the FDA requires a full set of in vivo platelet recovery/survival data and complimentary in vitro platelet quality data for 22-24 subjects for the selected cold storage period.

Budget Expenditure to Date

Projected: $4,100,464 ($2,698,464 to Bloodworks + $1,402,000 to Terumo)
Actual Expenditure: Bloodworks - $2,689,464 ($00 remaining) + Terumo $1,381,300 - $863,463 spent = $517,837 which has been returned to BWNW.