

Primary hemostatic capacity of whole blood: a comprehensive analysis of pathogen reduction and refrigeration effects over time

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BACKGROUND: Whole blood (WB) has been used in combat since World War I as it is readily available and replaces every element of shed blood. Component therapy has become standard; however, recent military successes with WB resuscitation have revived the debate regarding wider WB use. Characterization of optimal WB storage is needed. We hypothesized that refrigeration preserves WB function and that a pathogen reduction technology (PRT) based on riboflavin and ultraviolet light has no deleterious effect over 21 days of storage.

STUDY DESIGN AND METHODS: WB units were stored for 21 days either at 4°C or 22°C. Half of each temperature group underwent PRT, yielding four final treatment groups (n = 8 each): CON 4 (WB at 4°C); CON 22 (WB at 22°C); PRT 4 (PRT WB at 4°C); and PRT 22 (PRT WB at 22°C). Testing was at baseline, Days 1-7, 10, 14, and 21. Assays included coagulation factors; platelet activation, aggregation, and adhesion; and thromboelastography (TEG).

RESULTS: Prothrombin time (PT) and partial thromboplastin time increased over time; refrigeration attenuated the effects on PT ($p \leq 0.009$). Aggregation decreased over time ($p \leq 0.001$); losses were attenuated by refrigeration ($p \leq 0.001$). Refrigeration preserved TEG parameters ($p \leq 0.001$) and PRT 4 samples remained within normal limits throughout the study. Refrigeration in combination with PRT inhibited fibrinolysis ($p \leq 0.001$) and microparticle formation ($p \leq 0.031$). Cold storage increased shear-induced platelet aggregation and ristocetin-induced platelet agglutination ($p \geq 0.032$), as well as GPIb-expressing platelets ($p \leq 0.009$).

CONCLUSION: The in vitro hemostatic function of WB is largely unaffected by PRT treatment and better preserved by cold storage over 21 days. Refrigerated PRT WB may be suitable for trauma resuscitation. Clinical studies are warranted.

INTRODUCTION

Transfusion medicine was born on the battlefields of World War I with the lifesaving infusion of citrated whole blood (WB) into severely injured combat casualties. WB remained the product of choice for preventing and treating hemorrhagic shock through World War II, the Korean War, and the Vietnam War. WB was used because it was readily available from in-theater donors—the so-called “walking blood bank”—and because it replaced all components of shed blood required for oxygen transport and

ABBREVIATIONS: aPTT = activated partial thromboplastin time; ASPI = arachidonic acid agonist; COL = collagen; GPIb α = glycoprotein Ib alpha; K = clot formation; LY30 = clot lysis at 30 minutes; LY60 = clot lysis at 60 minutes; MA = maximum amplitude (clot strength); PC = platelet concentrates; PMT = photomultiplier tube; PPP = platelet-poor plasma; PRP = platelet-rich plasma; PRT = pathogen reduction technology; PT = prothrombin time; R = clotting time; RH = high ristocetin concentration; RIPA = ristocetin-induced platelet agglutination; RL = low ristocetin concentration; RT = room temperature; SIPA = shear-induced platelet aggregation; TEG = thromboelastography; TRAP = thrombin receptor-activating peptide-6; TTG = total thrombin generation; WB = whole blood; α -angle = alpha angle (angle of clot formation).

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Supported by: the MPMC, Terumo BCT Biotechnologies.
doi: 10.1111/trf.12048

TRANSFUSION 2013;53:137S-149S.

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 01 JAN 2013		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Primary hemostatic capacity of whole blood: a comprehensive analysis of pathogen reduction and refrigeration effects over time				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Pidcoke H. F., McFaul S. J., Ramasubramanian A. K., Parida B. K., Mora A. G., Fedyk C. G., ValdezâDelgado K. K., Montgomery R. K., Reddoch K. M., Rodriguez A. C.,				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 13	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

hemostasis.¹ Low hemagglutinin-titer Type O WB, stored in citrate-phosphate-dextrose anticoagulant (CPD) and refrigerated, was used as a universal resuscitation fluid by US Forces throughout the Vietnam conflict. This universal WB product, as well as type-specific WB, was collected from donor centers in the United States and Japan, shipped to Vietnam, and used over the course of a 21-day storage period.² Current US military guidance for using WB in the treatment of life-threatening bleeding requires ABO blood type specificity to the recipient because of concerns of hemolytic reaction. Other investigators address this controversial policy in detail within this supplement.³⁻⁵

The wars in Iraq and Afghanistan, the largest military engagements since the Vietnam War, have been fought in the age of component therapy and an era of heightened awareness of the risks of transfusion-transmitted disease (TTD). The overwhelming majority of transfusions to combat casualties in the recent conflicts have been component based,⁶ although the apparent success of fixed-ratio transfusion to approximate WB (1:1:1 platelet [PLT] : fresh frozen plasma [FFP] : red blood cell [RBC]) and the use of fresh WB suggest that a larger role for WB could reasonably be incorporated into future trauma resuscitation strategies.⁷⁻¹² A number of challenges remain to the wider use of WB. These include implementing pathogen risk mitigation, determining the role and timing of PLT-sparing leukoreduction, and optimal storage conditions.¹³

In the US Military, WB is used for emergency transfusions within 24 hours of collection, before results of complete, post hoc pathogen testing are available. A pathogen reduction technology (PRT), which uses riboflavin and ultraviolet (UV) light to damage the nucleic acids of white blood cells (WBCs), parasites, bacteria, and viruses, is being considered for TTD risk mitigation. The effects of this technology and subsequent storage conditions on the hemostatic properties of WB are poorly understood but must be characterized if PRT-treated WB is to be used to treat hemorrhage in severely wounded service members.

Hemostasis is a complex process involving the interaction of PLTs with RBCs and plasma proteins and is locally regulated by both biochemical factors such as PLT secretory mediators and biophysical properties such as local blood flow. We hypothesized that pathogen inactivation with riboflavin and UV light has no deleterious effect on the

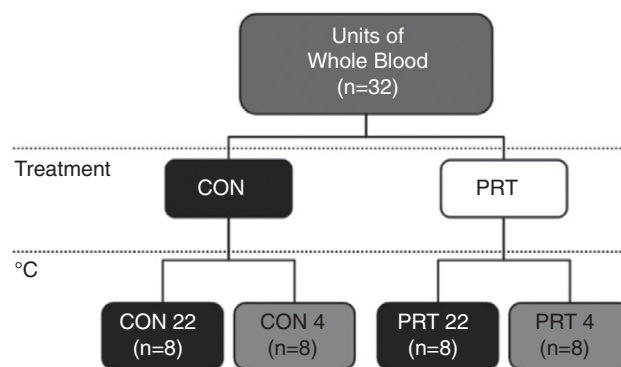


Fig. 1. Study design. WB units ($n = 32$) were divided into control (CON: no pathogen reduction performed) and treatment (PRT: pathogen reduction performed) and then stored for 21 days under two temperature conditions, resulting in four treatment groups: CON 4, untreated WB stored at 4°C ($n = 8$); CON 22, untreated WB stored at 22°C ($n = 8$); PRT 4, pathogen-reduced WB stored at 4°C ($n = 8$); and PRT 22, pathogen-reduced WB stored at 22°C ($n = 8$). WB was tested at baseline, after PRT, and on Days 1-7, 14, and 21.

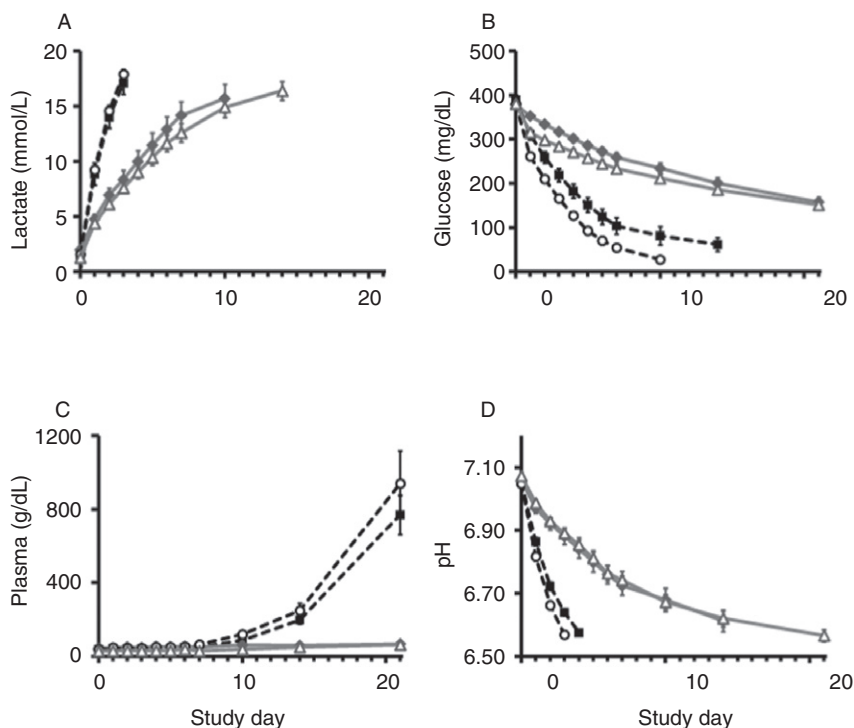


Fig. 2. Sample quality over time. Multiple measures assessed sample quality: (A) lactate, (B) glucose, (C) hemolysis, and (D) pH changed significantly over time; however, alterations were attenuated by refrigeration (A-D: $p \leq 0.001$). Glucose differences due to PRT were small but significant (B: $p \geq 0.008$). The other parameters were not significantly altered by PRT (A, C-D: $p \geq 0.270$). —●— = CON 22; - -●- = CON 4; —▲— = PRT 22; - -▲- = PRT 4.

TABLE 1. Complete blood count

Study day	CON 4	CON 22	PRT 4	PRT 22	CON 4	CON 22	PRT 4	PRT 22
WBC ($\times 10^3/\mu\text{L}$)					Granulocytes ($\times 10^3/\mu\text{L}$)			
0	5.0 $\pm$ 0.5	5.4 $\pm$ 0.5	4.9 $\pm$ 0.3	6.6 $\pm$ 0.9	2.9 $\pm$ 0.3	3.2 $\pm$ 0.4	3.0 $\pm$ 0.2	4.3 $\pm$ 0.8
1	5.8 $\pm$ 0.4	5.6 $\pm$ 0.5	4.8 $\pm$ 0.2	6.2 $\pm$ 0.9	3.2 $\pm$ 0.3	3.1 $\pm$ 0.4	2.8 $\pm$ 0.2	2.5 $\pm$ 0.4
2	5.5 $\pm$ 0.4	5.4 $\pm$ 0.5	4.8 $\pm$ 0.3	6.1 $\pm$ 0.8	2.8 $\pm$ 0.3	2.6 $\pm$ 0.4	2.8 $\pm$ 0.2	0.9 $\pm$ 0.2
3	5.8 $\pm$ 0.5	5.4 $\pm$ 0.4	4.9 $\pm$ 0.3	6.1 $\pm$ 0.9	2.9 $\pm$ 0.5	2.1 $\pm$ 0.3	2.4 $\pm$ 0.3	0.4 $\pm$ 0.1
4	5.3 $\pm$ 0.5	5.3 $\pm$ 0.4	4.6 $\pm$ 0.2	5.7 $\pm$ 1.1	2.1 $\pm$ 0.4	1.6 $\pm$ 0.2	1.9 $\pm$ 0.3	0.3 $\pm$ 0.0
5	5.5 $\pm$ 0.5	5.3 $\pm$ 0.4	5.2 $\pm$ 0.4	5.9 $\pm$ 0.8	1.6 $\pm$ 0.3	1.4 $\pm$ 0.2	2.0 $\pm$ 0.5	0.6 $\pm$ 0.4
6	5.5 $\pm$ 0.5	5.5 $\pm$ 0.5	5.0 $\pm$ 0.3	5.9 $\pm$ 0.8	1.1 $\pm$ 0.2	1.1 $\pm$ 0.2	1.4 $\pm$ 0.4	0.2 $\pm$ 0.1
7	4.9 $\pm$ 0.6	5.3 $\pm$ 0.6	4.2 $\pm$ 0.6	5.2 $\pm$ 0.4	0.9 $\pm$ 0.2	0.8 $\pm$ 0.2	1.2 $\pm$ 0.3	0.2 $\pm$ 0.0
10	5.2 $\pm$ 0.4	5.5 $\pm$ 0.6	5.0 $\pm$ 0.3	5.8 $\pm$ 0.8	0.5 $\pm$ 0.1	0.9 $\pm$ 0.5	0.7 $\pm$ 0.2	0.4 $\pm$ 0.1
14	4.9 $\pm$ 0.4	5.2 $\pm$ 0.5	4.5 $\pm$ 0.3	5.7 $\pm$ 0.8	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1	0.3 $\pm$ 0.1	0.9 $\pm$ 0.2
21	4.9 $\pm$ 0.4	5.3 $\pm$ 0.5	4.4 $\pm$ 0.3	5.7 $\pm$ 0.8	0.3 $\pm$ 0.0	0.7 $\pm$ 0.2	0.3 $\pm$ 0.0	1.5 $\pm$ 0.2
RBC ($\times 10^3/\mu\text{L}$)					Hb (g/dL)			
0	4.1 $\pm$ 0.2	4.1 $\pm$ 0.2	4.1 $\pm$ 0.1	4.2 $\pm$ 0.1	13 $\pm$ 0	13 $\pm$ 1	13 $\pm$ 0	13 $\pm$ 1
1	4.2 $\pm$ 0.2	4.0 $\pm$ 0.2	3.7 $\pm$ 0.2	4.0 $\pm$ 0.4	13 $\pm$ 1	12 $\pm$ 1	12 $\pm$ 0	13 $\pm$ 1
2	3.9 $\pm$ 0.2	4.1 $\pm$ 0.1	3.8 $\pm$ 0.1	3.9 $\pm$ 0.1	12 $\pm$ 1	13 $\pm$ 1	12 $\pm$ 0	12 $\pm$ 0
3	4.1 $\pm$ 0.1	3.9 $\pm$ 0.2	3.8 $\pm$ 0.2	4.0 $\pm$ 0.1	13 $\pm$ 0	12 $\pm$ 1	12 $\pm$ 1	12 $\pm$ 1
4	3.9 $\pm$ 0.2	4.1 $\pm$ 0.2	3.6 $\pm$ 0.1	4.1 $\pm$ 0.3	12 $\pm$ 1	13 $\pm$ 1	12 $\pm$ 0	13 $\pm$ 1
5	4.0 $\pm$ 0.2	4.0 $\pm$ 0.2	3.6 $\pm$ 0.2	3.9 $\pm$ 0.4	13 $\pm$ 1	12 $\pm$ 1	12 $\pm$ 1	12 $\pm$ 1
6	4.1 $\pm$ 0.2	4.1 $\pm$ 0.2	3.9 $\pm$ 0.1	3.9 $\pm$ 0.1	13 $\pm$ 0	13 $\pm$ 1	12 $\pm$ 0	12 $\pm$ 1
7	4.1 $\pm$ 0.2	4.1 $\pm$ 0.2	3.6 $\pm$ 0.2	3.9 $\pm$ 0.2	13 $\pm$ 0	13 $\pm$ 1	12 $\pm$ 1	12 $\pm$ 1
10	3.9 $\pm$ 0.2	4.1 $\pm$ 0.2	4.0 $\pm$ 0.2	3.8 $\pm$ 0.2	12 $\pm$ 1	13 $\pm$ 1	13 $\pm$ 1	12 $\pm$ 1
14	3.8 $\pm$ 0.3	4.0 $\pm$ 0.2	3.7 $\pm$ 0.1	3.8 $\pm$ 0.2	12 $\pm$ 1	12 $\pm$ 1	12 $\pm$ 0	12 $\pm$ 1
21	4.0 $\pm$ 0.1	4.2 $\pm$ 0.2	3.8 $\pm$ 0.1	3.9 $\pm$ 0.1	13 $\pm$ 0	13 $\pm$ 1	12 $\pm$ 0	12 $\pm$ 0
HCT (%)					PLT ($\times 10^3/\mu\text{L}$)			
0	37 $\pm$ 1	37 $\pm$ 2	37 $\pm$ 1	38 $\pm$ 1	156 $\pm$ 16	171 $\pm$ 17	150 $\pm$ 16	183 $\pm$ 25
1	38 $\pm$ 2	36 $\pm$ 2	33 $\pm$ 1	36 $\pm$ 3	142 $\pm$ 21	191 $\pm$ 11	140 $\pm$ 9	164 $\pm$ 18
2	35 $\pm$ 2	37 $\pm$ 1	34 $\pm$ 1	35 $\pm$ 1	160 $\pm$ 17	189 $\pm$ 15	132 $\pm$ 10	174 $\pm$ 13
3	37 $\pm$ 1	36 $\pm$ 2	34 $\pm$ 2	36 $\pm$ 1	165 $\pm$ 15	195 $\pm$ 16	122 $\pm$ 13	170 $\pm$ 13
4	35 $\pm$ 1	38 $\pm$ 2	33 $\pm$ 1	37 $\pm$ 3	146 $\pm$ 18	189 $\pm$ 13	108 $\pm$ 14	163 $\pm$ 16
5	37 $\pm$ 2	37 $\pm$ 2	33 $\pm$ 2	36 $\pm$ 3	151 $\pm$ 16	192 $\pm$ 15	118 $\pm$ 9	166 $\pm$ 16
6	37 $\pm$ 1	38 $\pm$ 2	36 $\pm$ 1	36 $\pm$ 1	146 $\pm$ 14	187 $\pm$ 14	108 $\pm$ 11	165 $\pm$ 10
7	37 $\pm$ 1	38 $\pm$ 2	33 $\pm$ 2	36 $\pm$ 2	145 $\pm$ 13	190 $\pm$ 21	107 $\pm$ 10	164 $\pm$ 16
10	35 $\pm$ 2	38 $\pm$ 2	37 $\pm$ 2	36 $\pm$ 2	138 $\pm$ 11	177 $\pm$ 16	98 $\pm$ 9	162 $\pm$ 16
14	35 $\pm$ 2	36 $\pm$ 2	34 $\pm$ 1	36 $\pm$ 2	128 $\pm$ 10	178 $\pm$ 17	88 $\pm$ 7	171 $\pm$ 16
21	37 $\pm$ 1	38 $\pm$ 2	35 $\pm$ 0	39 $\pm$ 1	110 $\pm$ 7	166 $\pm$ 19	94 $\pm$ 10	173 $\pm$ 17

PLT count decreased with both refrigeration and PRT ($p \leq 0.007$). Temperature storage differences in WBCs among treatment groups were significant but not clinically relevant ($p = 0.037$; Table 1); PRT effects did not differ between groups ($p = 0.275$). Granulocytes decreased over time, a process accelerated by RT storage and PRT ($p \leq 0.047$; Table 1). HCT, Hb, and RBCs did not change relative to baseline or differ between groups ($p \geq 0.817$).

hemostatic function of WB stored either at 4°C or room temperature (RT; 22°C) over several days. To this end, we assessed hemostatic function at multiple levels, including changes in levels of coagulation factors in plasma, PLT activation status, PLT response to chemical agonists, PLT adhesion receptor levels, PLT function under static and flow conditions, and overall clot properties. Our comprehensive analyses demonstrate that hemostatic function in vitro is mostly unaffected by PRT treatment and is better preserved at 4°C versus RT storage.

METHODS

Institutional review board approval was obtained for blood collection from 32 healthy adult donors, and standard blood donation guidelines were followed. Thirty-two units (450 ± 50 mL) of WB were placed in commercially available bags containing 63 mL of CPD anticoagulant (Teru-

flex, Terumo Medical Corporation, Somerset, NJ). PRT was performed on half of the units collected (16 of 32; see Fig. 1). Each unit was drained into the citrated polyvinyl chloride illumination bag (attached to a storage bag; set manufactured by Terumo BCT, Lakewood, CO), and riboflavin solution was added (35 mL of 500- μM riboflavin in 0.9% NaCl; Terumo BCT), roughly a 5%-10% dilution compared with control bags. The illumination/storage set was weighed, the hematocrit (HCT) of the contents was determined, and the set was placed in the illuminator and exposed to UV light (265-400 nm of phosphor; Mirasol System, Terumo BCT) for a dose of 80 J/mL_{RBC}. The majority (99%) of the total energy reaching the product through the plastic is in the UV-B (280-315 nm) and UV-A (315-400 nm) spectral regions. After treatment, the contents of the illumination bag were drained into the attached storage bag, and the illumination bag was removed. All WB units were stored for 21 days under two temperature

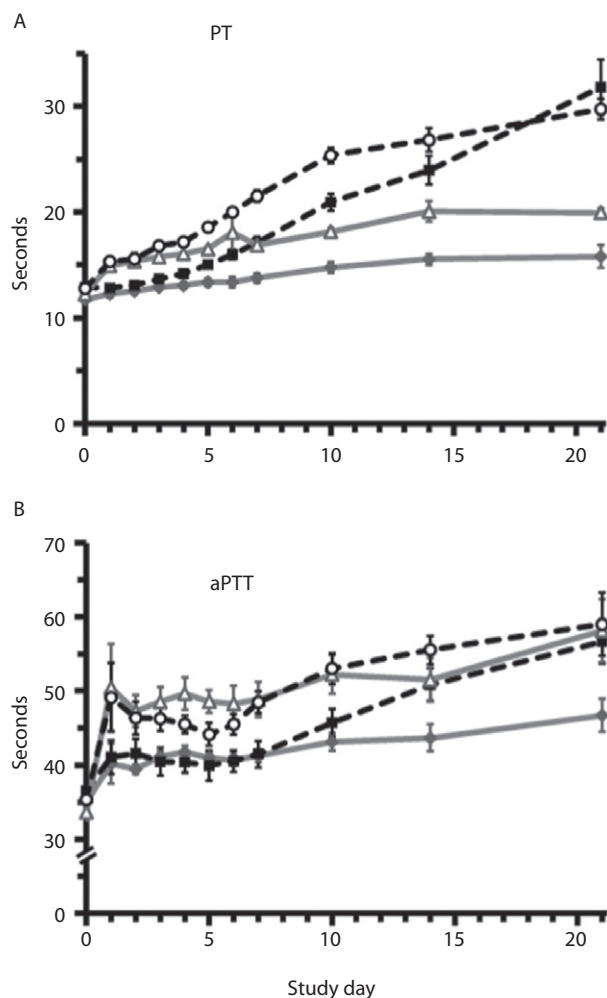


Fig. 3. PT and aPTT. PT and aPTT increased over time; however, refrigeration attenuated the effect on PT (A: $p \leq 0.009$). Mean PT levels were within reference ranges through Day 7 in the CON 4 group compared with Day 4 for CON 22. PRT prolonged both PT and PTT (A-B: $p \leq 0.009$), and mean values were above reference ranges by posttreatment Day 1. --- = CON 22; — = CON 4; --- = PRT 22; — = PRT 4.

conditions, resulting in four treatment groups (Fig. 1): CON 4, untreated WB stored at 4°C ($n = 8$); CON 22, untreated WB stored at 22°C ($n = 8$); PRT 4, pathogen-reduced WB stored at 4°C ($n = 8$); and PRT 22, pathogen-reduced WB stored at 22°C ($n = 8$). WB was tested at baseline and on Days 1-7, 10, 14, and 21. Between sampling, WB units were protected from light and UV radiation within a metal-lined storage container.

At each testing point, a Luer lock access port was attached with the use of a sterile technique and a 14-mL aliquot of WB collected. Tests performed included: complete blood count (Coulter Ac-T diff2 Analyzer, Beckman Coulter, Inc., Brea, CA); prothrombin time (PT) and activated partial thromboplastin time (aPTT; STA-4 Ana-

lyzer, Diagnostica Stago, Inc., Parsippany, NJ); and plasma-free hemoglobin (Hb; Plasma/Low Hb Photometer, HemoCue, Ängelholm, Sweden) for determination of sample hemolysis, as a measure of WB integrity. Thromboelastography (TEG; Haemonetics Corporation, Braintree, MA) with kaolin activation measured WB hemostatic characteristics. Parameters recorded were clotting time (R), clot formation (K), alpha angle (α -angle), maximum amplitude (MA), clot lysis at 30 and 60 minutes (LY30 and LY60, respectively), and total thrombin generation (TTG). TTG was estimated by taking the first derivative of the TEG tracing as previously described.¹⁴

PLT function was assessed with multiple electrode impedance aggregometry (Multiplate 5.0 Analyzer, Dynabyte Medical, Munich, Germany), and samples were stimulated with adenosine diphosphate (ADP), collagen (COL), thrombin receptor-activating peptide-6 (TRAP), arachidonic acid agonist (ASPI), and two concentrations of ristocetin (ristocetin low [RL]: 0.2 mg/mL; and ristocetin high [RH]: 0.77 mg/mL, respectively). Multiple electrode aggregometry data were reported in arbitrary units defined as area under the curve. Shear effects and von Willebrand factor (vWF)-mediated PLT adhesion and aggregation were measured with a cone-and-plate analyzer (DiaMed Impact-R, Bio-Rad Laboratories, Hercules, CA). Adhesion was measured as percent surface coverage (%SC), and aggregation as aggregate size (AS; in square micrometer). A commercial analyzer (STA-R Evolution Hemostasis System, Diagnostica Stago, Inc.) measured d-dimer, antithrombin III (ATIII), fibrinogen (FIB), factor V (FV), factor VIII (FVIII), protein C, and vWF. Results for PRT-treated samples were corrected for the dilution due to the addition of riboflavin. Soluble CD40 ligand (sCD40L) released by PLTs into stored blood was measured with an enzyme-linked immunosorbent assay kit (human sCD40L Platinum [extra sensitive], eBioscience, Inc., San Diego, CA).

Microparticles (MPs) in platelet-poor plasma (PPP), prepared by centrifugation ($3000 \times g$) for 10 minutes, were quantified and characterized as to their cellular origin, antigen expression, and size distribution by flow cytometry (BD FACS Canto II, BD Biosciences, San Jose, CA) with a forward scatter (FSC) photomultiplier tube (PMT) resolution of 200-nm events. Bead mixtures (201, 390, 505, 794, and 990 nm; Bangs Laboratories, Fishers, IN) were used to generate a standard curve against the median FSC PMT data from which MP sizes were determined. An MP gate was set between 990 and 201 nm. Hank's balanced salt solution (HBSS) was filtered through a sterile 0.1- μ m membrane, and sheath fluid was filtered through two inline 0.1- μ m membranes.

For identification of cellular origin and antigen expression, an antibody panel was prepared with fluorescein isothiocyanate conjugated lactadherin (phosphatidylserine [PS] expression; Haematologic Technologies,

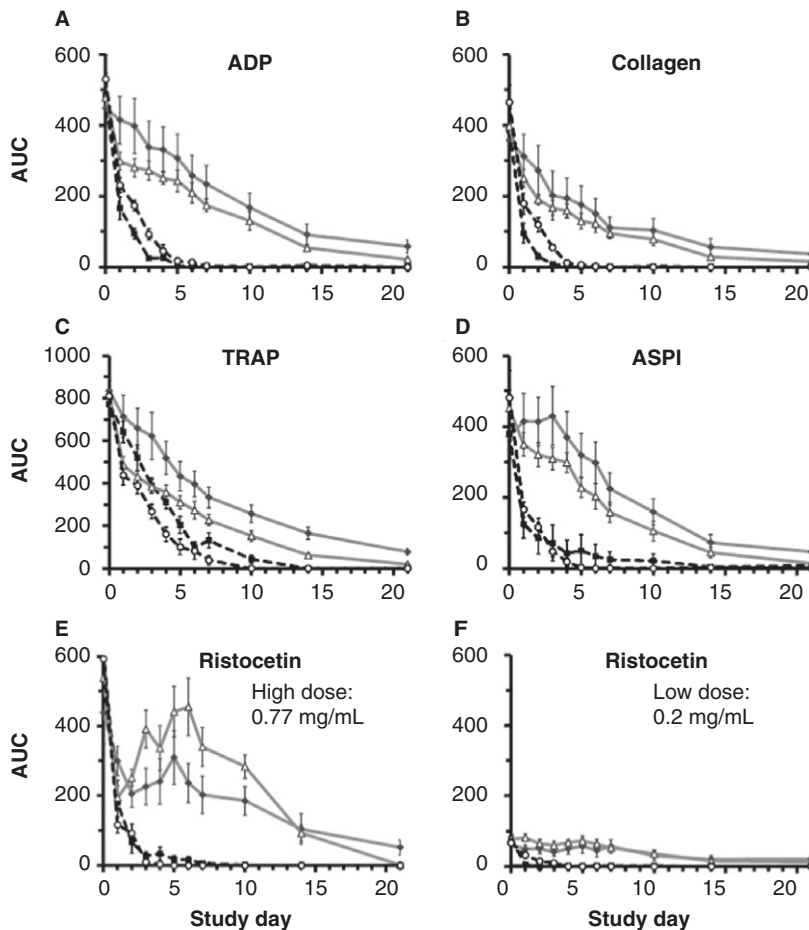


Fig. 4. Multiple electrode PLT aggregometry. A repeated measures analysis demonstrated that agonist-stimulated aggregation decreased over time in all four treatment groups (A-F: $p \leq 0.001$); however, losses were attenuated in samples stored at 4°C compared with those stored at 22°C (A-F: $p \leq 0.001$). PRT-treated samples demonstrated lower COL-, TRAP-, and RL-stimulated aggregation compared with controls (B, C, F: $p \leq 0.033$), but differences were not significant in ADP-, ASPI-, and RH-stimulated samples (A, D, E: $p \geq 0.242$). Aggregation immediately after PRT, with the exception of ADP and TRAP, were within one standard deviation of healthy subject ranges at our center. —●— = CON 22; —■— = CON 4; ····△···· = PRT 22; —◆— = PRT 4.

Inc., Essex Junction, VT); PE-Cy7 conjugated anti-human CD45 (WBCs; eBioscience); PE conjugated anti-human CD142 (tissue factor [TF]; BD Biosciences), PE-Cy-5 conjugated anti-human CD62P (P-Selectin [P-Sel], PLTs, BD Biosciences), APC conjugated anti-human CD235a (glycophorin A, RBC; BD Biosciences), and APC-H7 conjugated anti-human CD41a (glycoprotein IIb [GPIIb], PLTs; BD Biosciences) with the use of the recommended volumes from the manufacturers.

Appropriate isotype control for each antibody was combined in a separate tube. Five microliters of PPP was added to the antibody and isotype panels, incubated for 30 minutes on ice, and 900 μ L of filtered HBSS was added to each tube. The sample was analyzed by flow cytometry, and electronic noise was excluded from the MP gate by setting

the appropriate instrument threshold. FSC PMT and side scatter data were acquired with the use of logarithmic settings. The concentration of MPs was measured with the use of BD TruCount absolute counting tubes (BD Biosciences) with a known number of beads. For absolute MP counts, 5 μ L of PPP and 995 μ L of HBSS were added to each TruCount tube, and data collected until 1000 beads were counted. Data analysis was performed with the use of BD FACS Diva software (BD Biosciences).

A second set of experiments was undertaken to further characterize the effects of PLT storage on shear-induced platelet aggregation (SIPA) following a protocol described by Montgomery and colleagues¹⁵ Platelet-rich plasma (PRP) was obtained via a standard protocol and stored without agitation at 4°C or 22°C. Samples were tested at baseline and on Days 2 and 7. Briefly, 80 μ L of washed PLTs was resuspended to a final concentration of $1-5 \times 10^7$ /mL in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/Tyrode's buffer with Ca^{2+} , Mg^{2+} , 2 mg/mL FIB (EMD Chemicals, Billerica, MA), and 5 μ g/mL vWF (Haematologic Technologies), and exposed to shear forces of 2500 or 10,000 s^{-1} for 120 s at 37°C in a computer-controlled 0.5° cone-and-plate rheometer (Anton-Paar, Graz, Austria). Ten-microliter samples were immediately fixed in 1% formaldehyde, and PLT aggregation was measured by flow cytometry.¹⁵ Glycoprotein Ib alpha (GPIb α) receptor expression levels were

estimated by the binding of the fluorescently conjugated anti-GPIb α monoclonal antibody AK2 (Abcam, Boston, MA) to stored PLTs. Ristocetin-induced platelet agglutination (RIPA) was performed based on our standard protocol with the use of washed PLTs with 0.75-mg/mL ristocetin and 5- μ g/mL vWF.¹⁵

Statistical analysis was performed with SAS 9.2 software (SAS Institute, Cary, NC). Data were reported as mean values $\pm$ standard error of the mean, unless noted otherwise. Differences between treatment groups were evaluated with repeated measures analysis of variance (RM-ANOVA) with analysis of individual effects (time, PRT treatment, and temperature storage) to determine significance, which was set at $p < 0.05$. Reported p values reflect RM-ANOVA with analysis of individual effects, unless

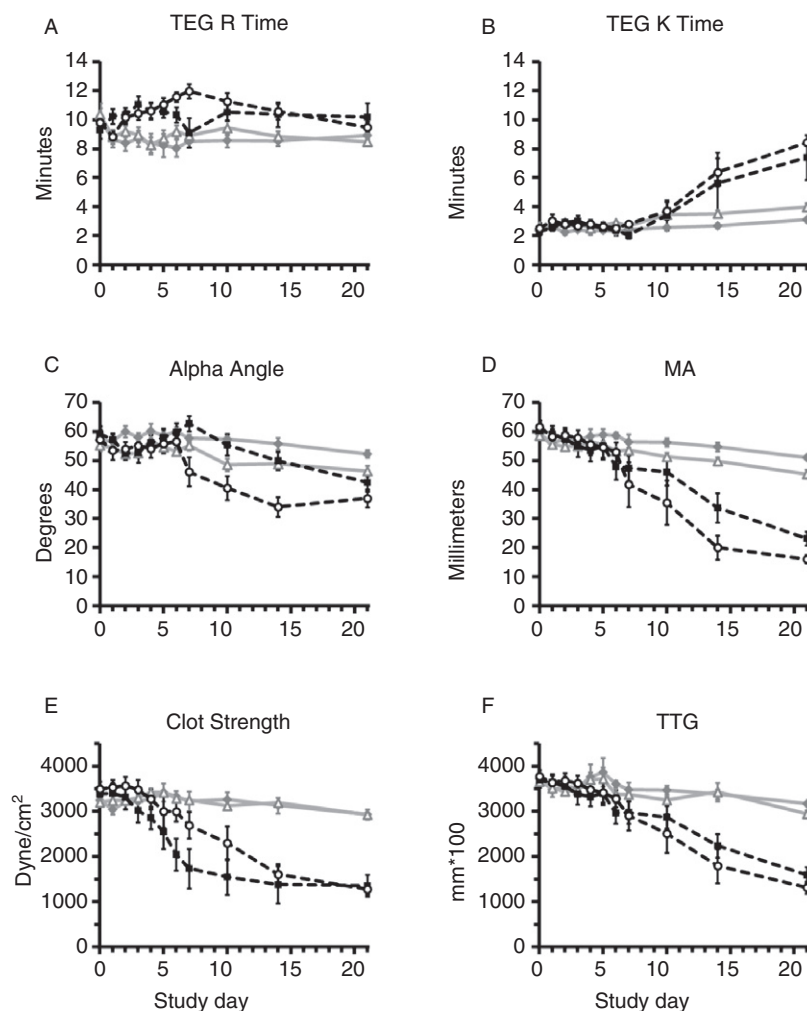


Fig. 5. TEG. Storage at 4°C preserved TEG R time, K, α -angle, MA, clot strength, and TTG relative to samples stored at RT (A-F: $p \leq 0.001$). Differences in TEG clot strength and TTG were not significant after PRT treatment compared with controls by RM-ANOVA (E-F: $p \geq 0.237$). R, K, α -angle, and MA were altered by PRT treatment (A-D: $p \leq 0.045$), but results for samples in cold storage, including those that underwent PRT, remained within normal subject limits throughout the study. $\cdots\circ\cdots$ = CON 22; $\cdots\bullet\cdots$ = CON 4; $\cdots\circ\cdots$ = PRT 22; $\cdots\triangle\cdots$ = PRT 4.

explicitly stated otherwise. When a single p value was given without reference to individual effects, the p value applies to all three, and the mathematical symbol “ $\leq$ ” or “ $\geq$ ” was used. Post hoc analysis for within-time-point comparisons was made via the Holm-Sidak method. Interactions were evaluated by one-way or two-way ANOVA, as appropriate.

RESULTS

Multiple measures assessed sample quality over time. Lactate, a by-product of anaerobic respiration, increased significantly with storage, but refrigeration slowed accu-

mulation ($p \leq 0.001$; Fig. 2A). Glucose, analyzed as a measure of metabolic activity, decreased significantly; however, losses were attenuated by refrigeration ($p \leq 0.001$; Fig. 2B). Hemolysis, a reflection of RBC integrity, was higher in samples stored at RT, particularly after Day 7 ($p = 0.014$; Fig. 2C), and remained essentially unchanged in refrigerated samples (Fig. 2C). Storage at 4°C slowed the decrease in pH over time ($p \leq 0.001$; Fig. 2D). Differences due to PRT were only significant for glucose measurements (glucose: $p \leq 0.008$, Fig. 2B; lactate, hemolysis, and pH: $p \geq 0.270$, Fig. 2A, C-D).

PLT count decreased with both refrigeration and PRT ($p \leq 0.007$, Table 1). Temperature storage differences in WBCs among treatment groups were significant but not clinically relevant ($p = 0.037$, Table 1). Granulocytes decreased over time, a process accelerated by RT storage and PRT ($p \leq 0.047$, Table 1). HCT, Hb, and RBCs did not change relative to baseline or differ between groups ($p \geq 0.817$, Table 1). Soluble CD40L, a potent inducer of inflammatory responses and an indication of decreased PLT viability when elevated,¹⁶ was not significantly altered by storage temperature, PRT, or storage duration ($p \geq 0.231$), and mean values remained within normal subject levels in all groups (minimum: 2.3 ± 1.0 ng/mL; maximum: 5.7 ± 1.0 ng/mL; eBio-science product insert normal subject range: nondetectable, 7.5 ng/mL).

Coagulation results differed between the type of test performed and between subject groups. PT and partial thromboplastin time (PTT) increased over time, but refrigeration attenuated the effect on PT ($p \leq 0.009$; Fig. 3A). Mean PT levels were within the reference ranges determined by our clinical laboratory (PT: 12.1–14.7 sec) through Day 7 in the CON 4 group compared with Day 4 for CON 22. PRT prolonged both PT and PTT ($p \leq 0.009$; Fig. 3A-B), and mean values were above reference ranges by Posttreatment Day 1.

A repeated measures analysis demonstrated that agonist-stimulated impedance aggregation decreased over time in all four treatment groups ($p \leq 0.001$; Fig. 4A-F); however, losses were attenuated in samples stored at 4°C compared with those stored at 22°C ($p \leq 0.001$; Fig. 4A-F). PRT-treated samples demonstrated lower

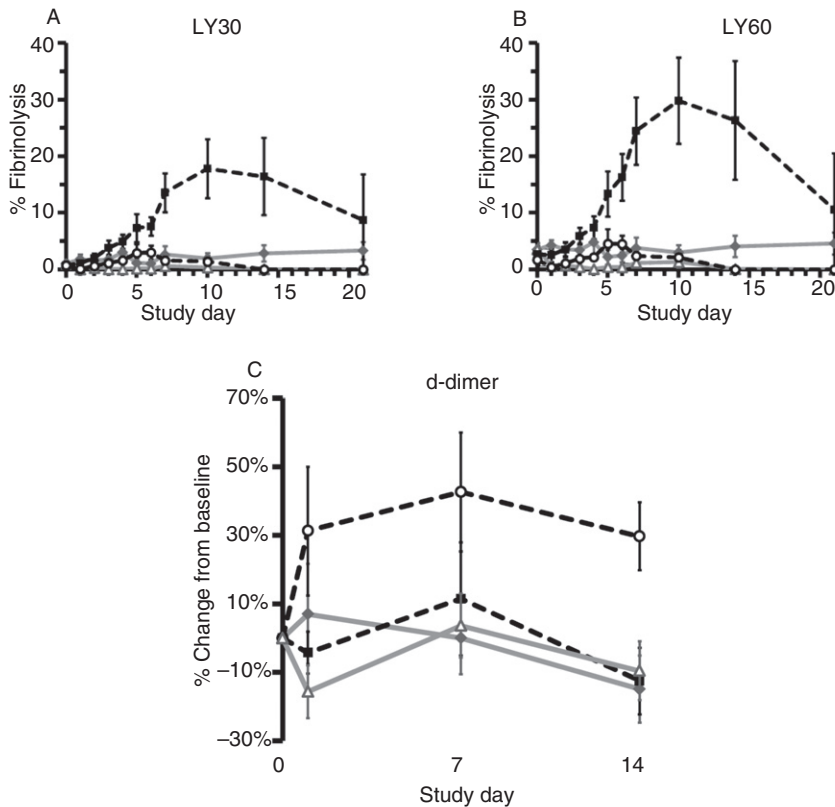


Fig. 6. Fibrinolysis: (A) LY30; (B) LY60; and (C) d-dimer. Fibrinolysis as measured by TEG was inhibited by both cold storage and PRT compared with control samples stored at RT (A: LY30 $p = 0.012$; B: LY60 $p \leq 0.001$). d-dimer did not accumulate in unstimulated stored blood; the CON 22 samples did not differ significantly from other groups, and most values were approximately within $\pm 30\%$ of baseline (C: $p = 0.412$). ---○--- = CON 22; —●— = CON 4; —○— = PRT 22; —●— = PRT 4.

COL-, TRAP-, and RL-stimulated aggregation compared with controls ($p \leq 0.033$; Fig. 4B, C, F), but differences were not significant in ADP-, ASPI-, and RH-stimulated samples ($p \geq 0.242$; Fig. 4A, D-F).

Storage at 4°C preserved TEG R, K, MA, clot strength, and TTG relative to samples stored at RT ($p \leq 0.001$; Fig. 5A-F). Differences in TEG clot strength and TTG were not significant after PRT compared with controls by RM-ANOVA ($p \geq 0.237$; Fig. 5E, F). R, K, α -angle, and MA were altered by PRT treatment, but results for samples in cold storage, including those that underwent PRT, remained within normal subject limits throughout most of the study.¹⁷ Fibrinolysis as measured by TEG was inhibited by both cold storage and PRT compared with control samples stored at RT (LY30: $p = 0.012$; LY60: $p \leq 0.001$; Fig. 6A, B). There was no difference between groups in d-dimer accumulation over storage time, and most values were approximately within $\pm 30\%$ of baseline ($p = 0.412$; Fig. 6C).

The effects of PRT and temperature storage conditions on coagulation factors were variable. ABO blood type distribution was not equal between groups (CON 4: 100% type O;

CON 22: 25% type O, 37.5% type A, 25% type B, 12.5% type AB; PRT 4: 62.5% type O, 37.5% type A; and PRT 22: 50% type O, 50% type A). Mean baseline values for most plasma measurements differed between groups ($p \leq 0.05$), possibly due to the differences in distribution of ABO blood groups because of donor scheduling constraints; therefore, results are shown as percent change from baseline. Refrigeration prevented decline in FIB and ATIII levels. PRT caused an initial drop in FIB (26%-30%; PRT: $p \leq 0.001$; Fig. 7A); however, mean values remained within normal subject ranges (145-348 mg/dL) for all groups (lowest: 173 ± 6 mg/dL, Day 1, PRT 4).¹⁸ Temperature did not have a significant effect (TEMP: $p = 0.364$; Fig. 7A). PRT had no immediate effect on ATIII, and losses over time were attributable to storage at RT ($p \leq 0.001$; Fig. 7B). Differences between treated and untreated groups stored at the same temperature were not significant in pairwise comparisons of any given day ($p \geq 0.471$; Fig. 7B). Protein C levels decreased over time and due to storage at RT ($p \leq 0.001$; Fig. 7C) but not due to PRT treatment ($p = 0.529$). Refrigeration diminished losses. FV and FVIII decreased due to PRT and over time ($p \leq 0.001$; Fig. 7D, E). Storage temperature had no effect on FV ($p = 0.243$), but cold storage affected FVIII adversely ($p = 0.036$). Levels of vWF remained within $\pm 10\%$ of baseline (Fig. 7F).

Cone-and-plate aggregometry measures GPIIb-vWF PLT adhesion reported as %SC, followed by GPIIb/IIIa-FIB-mediated aggregation expressed as AS. Refrigeration significantly attenuated the decline in %SC over time ($p \leq 0.001$; Fig. 8A), whereas PRT effects were not significant ($p = 0.058$). Diminished AS, reflecting loss of GPIIb/IIIa and FIB-mediated PLT aggregation, was attenuated by refrigeration ($p \leq 0.019$; Fig. 8B); PRT-related differences were not significant ($p \geq 0.125$). Mean values were within healthy subject limits throughout the period studied. These findings were consistent with results from ristocetin-induced impedance aggregometry as described above. We observed a similar pattern in platelet concentrates (PCs) derived from PRP and stored at 4°C versus 22°C for 7 days (Fig. 9). SIPA and RIPA levels were both higher after cold storage compared with RT (SIPA Day 2: $p = 0.032$; RIPA Day 7: $p = 0.002$; Fig. 9A, B). Cold storage increased both the percentage of GPIIb-expressing PLTs and the expression level, as measured by AK2, by Day 7 compared with RT storage ($p \leq 0.009$; Fig. 9C, D).

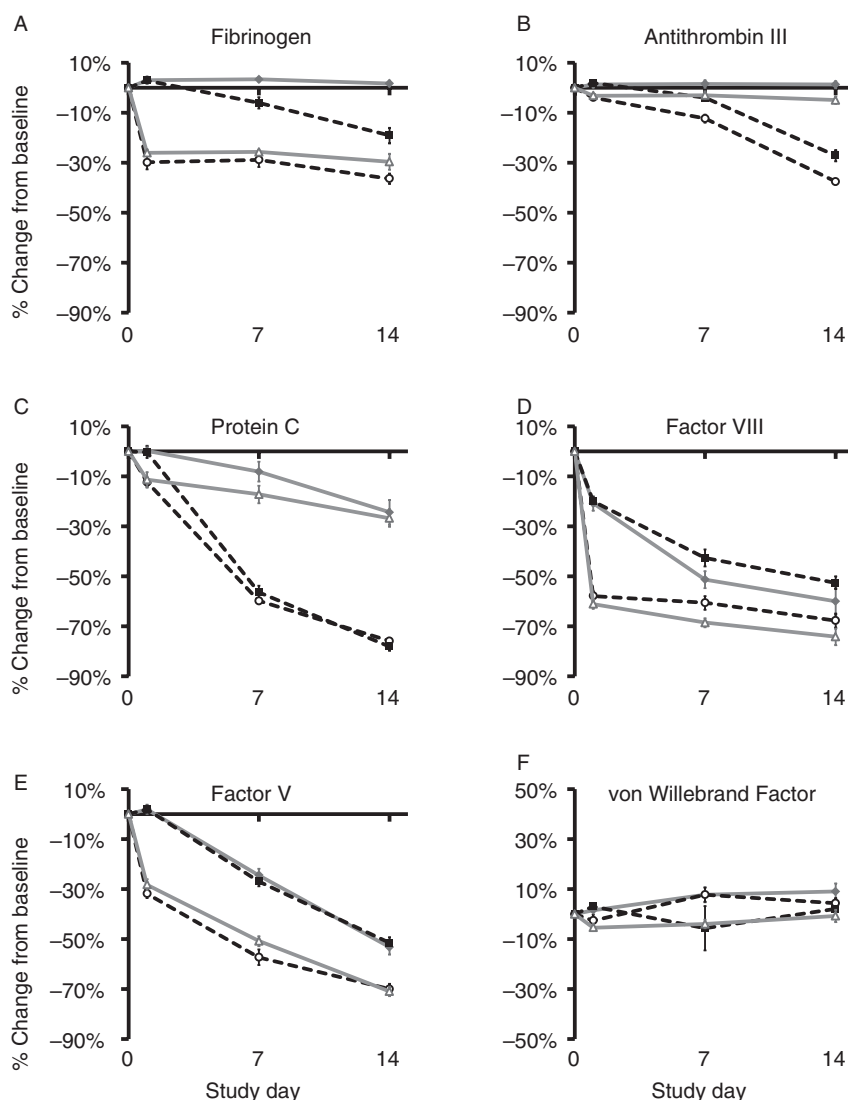


Fig. 7. Plasma analysis. The effects of PRT treatment and temperature storage conditions on coagulation factors were variable. PRT caused an initial drop in FIB, which decreased by 26%-30% but temperature did not have a significant effect (A: PRT $p \leq 0.001$; TEMP $p = 0.364$). PRT had no immediate effect on ATIII, and losses were attributable to storage at RT (B: PRT $p \geq 0.471$ by pairwise comparison; TEMP $p \leq 0.001$). Protein C levels decreased over time, but refrigeration diminished losses, and PRT treatment had no effect (C: TEMP $p \leq 0.001$; PRT 0.529). FV and FVIII decreased due to PRT and over time (D-E: PRT $p \leq 0.001$; DAY $p \leq 0.001$). Storage temperature had no effect on FV ($p = 0.243$). PRT did not change vWF, and levels remained within 10% of baseline (F: TEMP $p = 0.007$; PRT & DAY $p \leq 0.091$). --- = CON 22; — = CON 4; - - - = PRT 22; — = PRT 4.

MP subpopulations increased over time in most treatment groups ($p \geq 0.05$, $n = 6-8$ per treatment group; Fig. 10). The combination of PRT and 4°C was associated with the lowest MP levels; otherwise, temperature and PRT effects varied among MP subpopulations (Fig. 10). Size distributions fell into two ranges: 201-330 and 330-1000 nm. Abundance ratios were approximately 90% (201-330 nm) and 10% (330-1000 nm), respectively, throughout the

study. The PS-expressing subpopulation was the most abundant MPs found (up to 31% of events; Fig. 10A). As a group, PS MPs were not significantly altered by either storage temperature or pathogen reduction, but up to 68% of this population was positive for other markers, the overwhelming majority of which were PLTs in origin (50% of PS MP). RBC-, WBC-, and PLT-derived MP distributions responded to refrigeration and PRT treatment in divergent ways (Fig. 10B-E). Cold storage significantly reduced RBC-derived MP accumulation ($p \leq 0.001$; Fig. 10B). A similar effect was seen in WBC, PLT, and TF-expressing PLT MP but only when combined with PRT ($p \leq 0.031$; Fig. 10C-E). Interestingly, increases in PLT MP were also minimized by RT storage in the absence of PRT. PLT MPs were further examined for P-Sel membrane expression; temperature and PRT effects were not significant in this population ($p = 0.154$; Fig. 10F).

DISCUSSION

The experience of military and civilian surgeons since World War I has demonstrated that treatment of severe hemorrhage requires blood replacement. Among civilian and military trauma surgeons, component therapy to treat massive hemorrhage has recently moved toward reapproximating WB with RBCs, FFP, and PLTs after reports of improved survival.^{6,9,10,19-24} This approach is complicated by the logistical challenges of 1) managing RBC inventory age; 2) balancing thawed plasma and FFP inventories to provide plasma in a timely manner; and 3) ensuring sufficient PLT products, which are limited by a 5-day shelf life and the risks associated with RT storage. Bacterial contamination of PLTs remains a common problem, despite scientific investigations spanning decades and involving considerable financial investments; more problematic still is the human cost in severe septic events and fatalities.^{25,26} To minimize sepsis risk, PLTs are quarantined for 24 hours while awaiting culture results, which effectively limits the shelf life to 4 days. PLTs are stored at RT to maximize PLT circulation time, as refrigeration leads to PLT clearance within 48 hours of transfusion.²⁷ Unfortunately, this practice comes

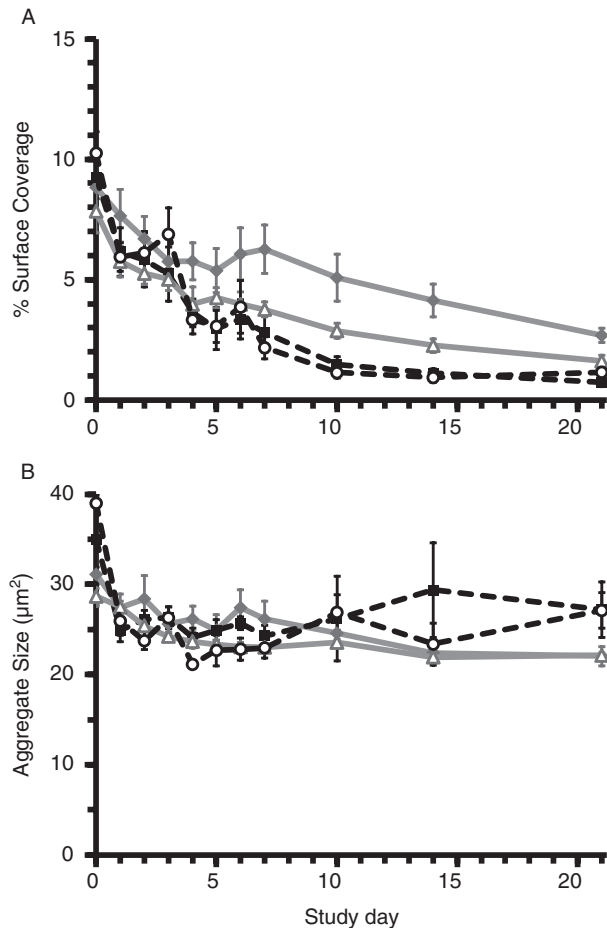


Fig. 8. Cone-and-plate aggregometry. (A) Comparison of the %SC as a measure of vWF and GPIb-mediated PLT adhesion in the four storage conditions demonstrated that refrigeration significantly attenuated the loss of function observed over time (%SC: TEMP and DAY $p \leq 0.001$). Differences in the PRT samples were not significant (%SC: PRT $p = 0.058$). CON 4 values were within healthy subject limits through Day 14 compared with 3–4 days in WB stored at 22°C. (B) Change in AS, a measure of GPIIb/IIIa and FIB-mediated PLT aggregation, was attenuated by refrigeration (AS: TEMP and DAY $p \leq 0.019$). Treatment-related differences between groups were not significant ($p \geq 0.125$), and mean values were within healthy subject limits throughout the period studied. $\cdots\circ\cdots$ = CON 22; $\cdots\triangle\cdots$ = CON 4; $\cdots\circ\cdots$ = PRT 22; $\cdots\triangle\cdots$ = PRT 4.

at the cost of a high risk of contamination and may have clinical consequences of increased morbidity.²⁸

Currently, WB is used by the military as a rescue product for severe hemorrhage when component therapy is unavailable or ineffective.^{6,7,10–13} Storage is limited to 24 hours at RT. This is due to concern for PLT viability and because, as an emergency product, it does not undergo comprehensive in-theater infectious disease testing. Both military and civilian clinicians have reported promising results in patients who were treated with WB, possibly

related to the avoidance of unbalanced resuscitation strategies characterized by early RBC and late hemostatic plasma and PLT transfusions.^{9,10,12,19,29} The use of WB for wider applications has an inherent teleological rationale, as it replaces what is lost during hemorrhage, but the important considerations that must be addressed before such a strategy is broadly applied include pathogen mitigation, storage temperature and time, and maintenance of hemostatic capacity.

PLTs are integral to the hemostatic potential of WB, and recent reviews of the military experience demonstrate that they have a closer association with beneficial outcomes than other blood components.⁶ Expansion of WB use would necessitate storage for more than 24 hours, and cold storage would be beneficial in inhibiting bacterial growth. However, refrigeration causes accelerated PLT elimination due to two mechanisms: clustering and desialylation of the GPIb α receptor with subsequent clearance via Mac-1 integrin binding by hepatic and splenic macrophages, and GPIb α receptor binding to Ashwell–Morell receptors on hepatocytes.^{30,31} PLT activation and accelerated clearance is also seen in vivo after exposure to cold temperatures;³⁰ these mechanisms have been the rationale for eliminating the use of cold PLTs after the phenomenon was first observed more than five decades ago.^{30,32} Severe traumatic hemorrhage is a highly acute problem limited by total blood volume and flow rate; however, the question of whether a highly active but transient PLT population might be useful for massive bleeding was once debated but not pursued for reasons that remain unclear.³³

The practical use of WB would require storage beyond 24 hours and thus presents a conundrum: while refrigeration promotes the accelerated clearance of PLTs, storage at RT poses the risk of bacterial contamination as well as decreased blood cell viability due to metabolic stress.^{25,26,34} Methods to nonspecifically reduce pathogens are currently under investigation and may hold the key to a safe, cost-effective, and hemostatic WB product,^{13,35–44} either with or without refrigeration. Here, we have comprehensively evaluated the in vitro effects of PRT treatment, refrigeration, and RT storage on coagulation and hemostasis over a 21-day period.

The data from these experiments indicate that loss of PLT function over time can be attenuated by refrigeration. A limitation of this study is that we did not try to balance ABO blood type between groups. As a result, all the blood in the CON 4 group was type O, which is associated with lower levels of vWF and FVIII.^{45,46} Because this group displayed the best aggregation and coagulation function over time, it is unlikely that differences in the experimental data associated with cold storage were attributable to unequal ABO distribution.

Pathogen reduction causes an initial drop in several measures of coagulation; however, PRT WB does not

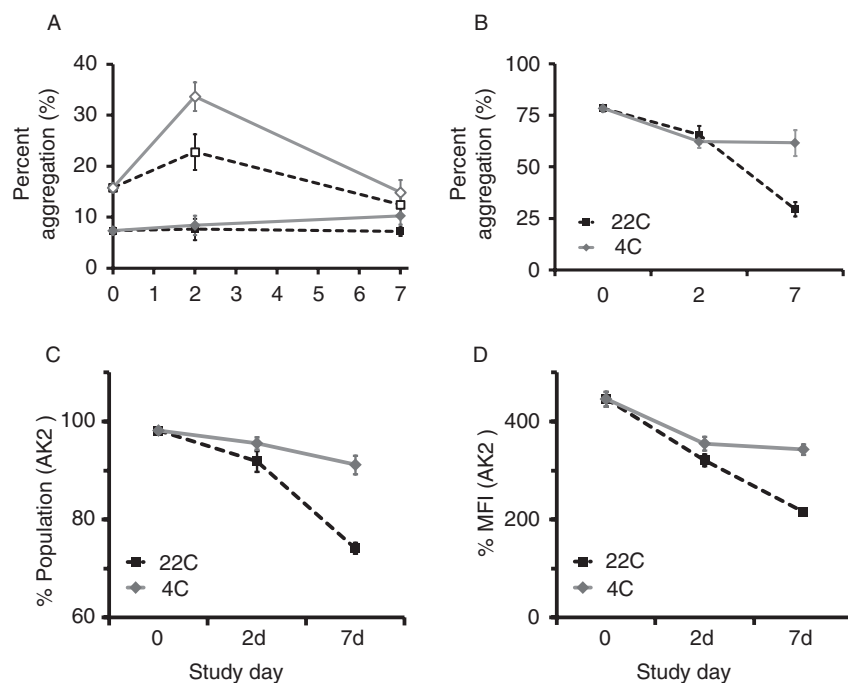


Fig. 9. (A) SIPA, (B) RIPA, and (C-D) measures of GPIIb/IIIa expression. PC stored at 4°C versus 22°C for 7 days demonstrated higher SIPA and RIPA results after cold storage compared with RT (SIPA Day 2: $p = 0.032$; RIPA Day 7: $p = 0.002$). GPIIb-expressing PLTs, as measured by AK2, were more abundant by Day 7 in refrigerated PLTs (C: $p \leq 0.001$), and mean fluorescence intensity was significantly higher at Day 7 (D: $p = 0.004$). $\bullet$ — $\bullet$ = 22°C 2500 s⁻¹; $\blacktriangle$ — $\blacktriangle$ = 4°C 2500 s⁻¹; $\bullet$ — $\bullet$ = 22°C 10,000 s⁻¹; $\blacktriangle$ — $\blacktriangle$ = 4°C 10,000 s⁻¹.

subsequently differ from control in many assays. Flow cytometry demonstrated that pathogen reduction in combination with cold storage prevents or reduces MP formation from several cell types and confirmed that antibody detection of GPIIb/IIIa was preserved with refrigeration. Release of the PLT-derived inflammatory mediator sCD40L was minimal and not significantly increased from baseline in all conditions tested. PLT viability after pathogen reduction has been shown to correlate with a low glucose consumption rate,^{36,47} and we found that both PRT and refrigeration delayed reductions in glucose plasma levels, evidence of slowed metabolism in the cellular constituents of WB. Hemolysis, a measure of RBC lysis, was minimal in all groups through Day 7 and minimized after that by storage at 4°C with or without PRT treatment.

PT and aPTT are widely used measures of coagulation function that have been criticized as poor predictors of bleeding. These two parameters were greatly affected by time and PRT treatment, but PT prolongation was largely prevented for a week by refrigeration. As expected, the prolongations in PT and PTT were associated with decreases in the activities of a number of coagulation factors, in particular FV and FVIII. Similarly, agonist-stimulated PLT aggregation, as measured by impedance

aggregometry, was decreased by time and RT storage, but unlike PT and PTT, PRT-related decreases were less marked and not significant in the case of ADP, ASPI, and RH. A better measure of PLT aggregation under flow, namely, shear-mediated PLT aggregation, was relatively preserved at 4°C, as measured by cone-and-plate aggregometry in both WB and PC, suggesting that PLT hemostatic function is more dependent on temperature than the storage solution. Taken together, these data suggest that refrigerated PRT WB could support primary hemostasis and retain utility during extended storage.

In contrast to the results of the reductionist methods of blood component analysis described above, most TEG parameters for cold-stored WB remained largely within normal limits over the 21-day WB storage period.¹⁷ These data indicate that PLT and coagulation function in cold-stored WB may be sufficient to provide hemostatic resuscitation, although this needs to be validated with in vivo studies. The TEG finding of diminished clot lysis associated with pathogen reduction and refrigeration is intriguing and suggests

that PRT WB may complement the beneficial effects of the antifibrinolytic, tranexamic acid in trauma resuscitation, particularly in the most coagulopathic patients who demonstrate hyperfibrinolysis.

The clinical significance of these findings has yet to be established. These data indicate that PRT-treated, refrigerated WB can maintain hemostatic function when stored for several weeks, making it a potentially useful adjunct in austere environments when components from US blood banks cannot be obtained. If PRT WB provides adequate PLT function and coagulation factor activity to support hemostasis in the setting of severe bleeding, physicians could prepare it in anticipation of mass casualties with refrigerated, treated blood donated by soldiers in a forward blood bank. Our TEG results suggest that refrigerated PRT WB may be sufficient to restore hemostasis, even at 21 days of storage; however, this remains to be studied in in vivo models. Further refinements to PRT WB such as inhibition of glycosyltransferases could prevent or delay PLT clearance.^{32,48-51} In summary, these data demonstrate that refrigerated PRT WB retains substantial in vitro hemostatic activity over 21 days of storage, suggesting the viability of an expanded role for WB in hemorrhage resuscitation. Clinical studies of its safety and efficacy are warranted.

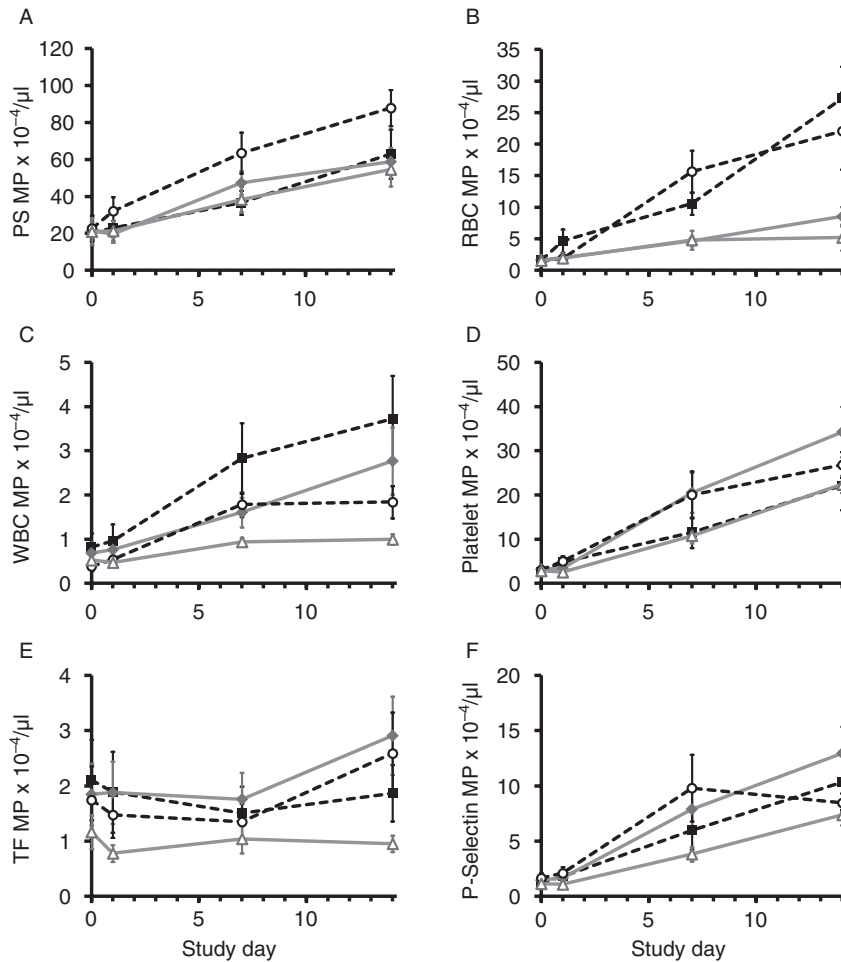


Fig. 10. MP analysis. MP distribution was variably affected by both temperature and PRT, whereas MP subpopulations increased over time ($p \geq 0.05$). (A) PS-expressing MPs were in greatest abundance and increased 2.5- to 4-fold by Day 14 (all groups, $p \leq 0.012$). Differences due to temperature storage and PRT were not significant ($p \geq 0.05$). (B) RBC MP were three to four times higher in samples stored at 22°C compared with 4°C ($p \leq 0.001$), whereas PRT did not cause significant effects ($p = 0.148$). (C) PRT and refrigeration in combination significantly reduced WBC MP accumulation ($p \leq 0.011$); values in the PRT 4 group did not differ from baseline over time ($p = 0.063$). (D) PRT and refrigeration in combination significantly reduced PLT MP accumulation ($p \leq 0.015$). Interestingly, increases in PLT MP were also minimized by RT storage in the absence of PRT. (E) PRT and refrigeration in combination significantly reduced TF MP accumulation ($p \leq 0.031$). (F) Changes due to PRT and refrigeration were not significant in P-Sel MP formation ($p = 0.154$). $\cdots\bigcirc\cdots$ = CON 22; $\cdots\bullet\cdots$ = CON 4; $\cdots\triangle\cdots$ = PRT 22; $\cdots\blacktriangle\cdots$ = PRT 4.

CONFLICT OF INTEREST

This study was conducted under a protocol reviewed and approved by the US Army Medical Research and Materiel Command Institutional Review Board and in accordance with the approved protocol.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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