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Hemostatic function of apheresis platelets stored at 4 °C and 22 °C

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Abstract

Introduction—Platelet refrigeration decreases the risk of bacterial contamination and may preserve function better than standard-of-care room temperature storage. Benefits could include lower transfusion-related complications, decreased costs, improved hemostasis in acutely bleeding patients, and extended shelf-life. In this study, we compared the effects of 22°C and 4°C storage on the functional and activation status of apheresis platelets (APs).

Methods—APs (n = 5 per group) were stored for 5 days at 22°C with agitation (RT) versus at 4°C with agitation (4C+AG) and without (4C). Measurements included platelet counts, mean platelet volume, blood gas analytes, aggregation response, thromboelastography, TxB₂ and sCD40L release, activation markers and microparticle formation.

Results—Sample pH levels were within acceptable limits for storage products (pH 6.2-7.4). Platelet glucose metabolism (P < 0.05), aggregation response (ADP: RT 0; 4C+AG 5.0 ± 0.8; 4C 5.6 ± 0.9; P < 0.05), and clot strength (MA: RT 58 ± 2; 4C+AG 63 ± 2; 4C 67 ± 2; P < 0.05) were better preserved at 4°C compared to RT storage. Refrigerated samples were more activated compared to RT (P < 0.05), although TxB₂ (P < 0.05) and sCD40L release (P < 0.05) were higher at RT. Agitation did not improve the quality of 4°C-stored samples.

Conclusion—AP stored at 4°C maintain more viable metabolic characteristics, are hemostatically more effective, and release fewer pro-inflammatory mediators than AP stored at RT over 5 days. Given the superior bacteriologic safety of refrigerated products, these data suggest that cold-stored platelets may improve outcomes for acutely bleeding patients.

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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.

Keywords

Hemostasis; coagulation; aggregation; clot strength; cold storage; activation

Introduction

Current blood-banking recommendations are that platelets be stored in incubators at 22°Celsius (°C), with gentle agitation for no longer than 5 days (1). This limited shelf-life is necessary due to the risk of bacterial contamination that leads to life-threatening transfusion-related infections (2-4). These storage practices also result in a platelet storage lesion that is associated with a decline in platelet hemostatic function (5).

Cold storage at 4°C could prolong shelf-life by diminishing the risk of bacterial sepsis, decreasing platelet metabolism, and maintaining functionality. Transportation of 4°C platelets would be convenient because the infrastructure for other refrigerated blood products, such as red blood cells (RBCs), is already in place (6). Cold platelets may be a better hemostatic product. Several *in vitro* studies from the 1970s showed that refrigeration of platelets results in better metabolic and functional responses such as minimal lactate accumulation, better aggregation response, and adhesion to subendothelium (7-9). *In vivo* human studies have shown that 4°C platelets function better than room temperature (RT) platelets in reducing the bleeding times in thrombocytopenic patients, aspirin-treated volunteers, and in aplastic thrombocytopenic patients shortly after transfusion (10-12).

Despite promising *in vitro* and *in vivo* studies in controlling acute bleeding, the practice of cold storage for transfusion was abandoned during the 1970s due to the belief that clinically effective platelets should be both hemostatically functional and survive in circulation for several days as indicated for prophylactic transfusion. It was shown by Murphy and Gardner that platelets stored for up to 18 hours in cold (2-4°C) show decreased recovery and survival upon transfusion compared to their RT (22-24°C) counterparts, i.e., the lifespan ($t_{1/2}$) of cold- and RT-stored platelets are 1.3 and 3.9 days, respectively (13). Consequently, when transfused to thrombocytopenic patients, cold stored platelets are as effective as RT-stored platelets at 24 h post-transfusion though not at 72 h suggesting cold storage may be acceptable for therapeutic transfusion (i.e., therapy for active hemorrhage) (14). In recent years, Hoffmeister and colleagues have shown that platelet clearance and function may be entirely separate attributes, and that appropriate modification (such as glycosylation) may prolong platelet survival in circulation (15-17). Concurrent reports from several groups have confirmed that platelets stored at 4°C are “healthier” than platelets stored at room temperature (18). Together, these studies have prompted the reconsideration of cold stored platelets as a transfusion product to treat bleeding patients (18-20). We have recently shown that cold storage of platelet rich plasma (PRP) enhances platelet activation and aggregation under shear, and cold storage of platelets in whole blood improves their performance in a panel of functional assays compared to RT-stored platelets (21-22).

In this study, we characterized the effects of storage at both 22°C and 4°C for up to 5 days with apheresis platelets (AP) using functional and activation assays. It is particularly important to evaluate the effect of storage on AP since apheresis is the most common

method of platelet isolation with 80% use in the United States and 50% in Europe, and it has been shown that the method of preparation of platelet concentrate (i.e., buffy coat, apheresis or PRP) is a determinant of platelet storage lesion (23-27). We hypothesized that apheresis platelets stored at 4°C would demonstrate more viable metabolic characteristics, perform better in functional tests, form stronger clots, and release fewer inflammatory mediators compared to platelets stored at 22°C.

Materials and Methods

Reagents and Suppliers

Calcium and kaolin used in thromboelastography were obtained from Haemonetics Corp (Braintree, MA). Multiplate agonists were obtained from Diapharma (West Chester, OH). FITC-conjugated bovine lactadherin was purchased from Haematologic Technologies, Inc (Essex Junction, VT). Gibco 1× Hanks Balanced Salt Solution (HBSS) without calcium and magnesium was obtained from Invitrogen Life Technologies (Carlsbad, CA). The following anti-human monoclonal mouse antibodies were purchased from BD Biosciences (San Jose, CA): APC-conjugated anti-CD62P (P-selectin), clone AK-4; PE conjugated anti-CD 154 (CD40 ligand or CD40L), clone TRAP1. PerCP conjugated mouse monoclonal anti-CD42b (glycoprotein Ib receptor, clone HIP-1 was purchased from BioLegend (San Diego, CA). For absolute particle counts TruCount tubes from BD Biosciences were utilized. Thromboxane B₂ EIA kits were purchased from Cayman Chemicals (Ann Arbor, MI) and Human soluble CD40L (sCD40L) Extra Sensitive Platinum enzyme-linked immunosorbent assay (ELISA) kits were purchased from eBioscience (Vienna, Austria). CG4⁺ and CHEM8⁺ cartridges were purchased from Abbott Laboratories (Abbott Park, IL).

Platelet Storage and Handling

Single AP units were collected in acid-citrate-dextrose (ACD)-plasma from healthy donors ($n=5$) using a Trima Accel Automated Blood Collection System, Terumo BCT (Lakewood, CO) 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. 10 ml aliquots were obtained from the donor bag and sterilely transferred into 15-ml BCSI mini-bags (Seattle, WA) for storage, with one mini-bag used for each time point and condition. The mini-bags were stored for 5 days in one of the following conditions: RT in a Food and Drug Administration (FDA)-approved platelet incubator with agitation (RT); in a walk-in refrigerator at 4°C with agitation (4C+AG) or without agitation (4C). Platelet function, characteristics, and release markers were analyzed for all stored samples on the day of collection (Baseline or Day 1), Day 3, and Day 5. Samples stored at 4°C were allowed to come to RT for 30 minutes and gently massaged before testing commenced. AP was centrifuged for 10 minutes at 3000×g to obtain platelet-poor plasma (PPP) where applicable. For RBC reconstitution, whole blood was collected in ACD vacutainer tubes, spun at 3500 × g for 10 minutes, and the plasma and buffy coat layers were carefully aspirated from the RBCs.

Laboratory equipment and measurements

All tests were performed at the U.S. Army Institute of Surgical Research at Joint Base San Antonio/FT Sam Houston according to routine protocols. Platelet count and mean platelet volume (MPV) were determined in duplicate using a standard cell counter (Coulter Ac-T diff2 hematology system, Beckman Coulter, Brea, CA). Mean platelet component was determined using an Advia 120 Haematology System (Bayer Diagnostics, Tarrytown, NY). Blood pH, sodium (mM), potassium (mM), chloride (mM), glucose (mg/dL), pCO₂ (mmHg), pO₂ (mmHg), bicarbonate (mM), and lactate (mM) were determined using an i-STAT point-of-care device (Abbot Labs, Abbott Park, IL). Thromboelastography (TEG) was performed using a TEG5000 device (Haemoscope Corp., Niles, IL) with kaolin activation. Aggregation was conducted using a Multiplate impedance aggregometer (Verum Diagnostica, Munich, Germany) with adenosine diphosphate (ADP), collagen, and thrombin receptor activating peptide (TRAP) as agonists. Platelet markers were assessed by flow cytometry with BD FACS Canto I and microparticle counts were determined using a BD FACS Canto II, RUO flow cytometer equipped with a FSC-PMT capable of detecting particles down to 200 nm. Particles were considered microparticles if their sizes fell within the range of 200-1000 nm.

Blood gas analysis

Blood gas and chemistry analyses in fresh and stored AP were performed using CG4⁺ and CG8⁺ cartridges, respectively. PPP samples (100 µl/cartridge) were obtained to test for metabolic parameters.

Thromboelastography (TEG)

All samples were tested as previously described (28). Briefly, samples were recalcified by the addition of CaCl₂ (0.2M, 30 µL) to a TEG sample cup followed by kaolin-treated AP (330 µl). All tests were performed at 37°C in duplicate.

Impedance aggregometry

Impedance aggregometry was performed according to established methods (28). Briefly, 300 µl of AP diluted with PPP to a concentration of 250×10³ platelets/µl was incubated with 300 µl of 3mM NaCl₂/CaCl₂ solution (Verum Diagnostica). After incubation, 20 µl of agonist (6.5 µM ADP, 3.2 µg/ml Collagen, or 32 µM TRAP) was added. The tests were carried out for 6 minutes in duplicate, and area under the curve (AUC) was reported. Aggregation was also performed in the presence of RBCs with a platelet count of 300×10³/µl and 40% hematocrit for 12 minutes.

Surface receptor expression

Geometric mean fluorescence intensity (GMFI) and abundance (% positive) was determined for the following platelet markers: CD42b (glycoprotein Ib receptor or GPIb), CD62P (P-selectin), lactadherin (phosphatidylserine surface expression), and CD154 (CD40L surface expression molecule). Ten thousand (10,000) events per sample were recorded for each marker, and forward and side scatter characteristics were used to identify the platelet

population. For microparticle analysis, absolute particle counts were determined using TruCount tubes (BD Biosciences).

ELISA

Commercially available kits were used to assess sCD40L and thromboxane B₂ (TxB₂) levels released into plasma during storage. PPP was stored at -80°C until analysis by ELISA. All plasma samples were diluted before testing to ensure that the concentrations fell within the standard range prescribed by the manufacturer.

Statistics

The data collected were analyzed by one-way ANOVA for repeated measures with a post-hoc Bonferroni adjustment for pairwise comparisons. Significance from baseline (Day 1) and between groups was determined when $P < 0.05$. Data in tables are mean \pm SD, and data in graphs are mean \pm SEM, unless otherwise stated. Microsoft Excel (Microsoft Corp., Redmond, WA) was used to manage data and analysis was performed using the statistical software JMP (Version 10, SAS Institute Inc., Cary, NC).

Results

Platelet Count, MPV and MPC

We obtained platelet counts for all AP samples to assess loss of single platelets during storage in minibags as described in the Methods section (Table 1A). We observed a significant drop in the platelet count in both 4C+AG and 4C samples by Day 5 ($P < 0.05$), along with macroscopic platelet clumping, but the platelet number was unchanged in RT samples. The decrease in platelet number in 4°C samples on Day 5 compared to fresh platelets (Day 1 or baseline) was ~20%; however platelet counts were stable and the clumping effect was abolished when refrigerated platelets were stored in full-sized bags (Table 1B). MPV remained unchanged in both RT and 4°C samples suggesting no significant change in the size of single platelets. The mean platelet component (MPC) decreased significantly after 5 day RT storage ($P < 0.05$) suggesting platelet degranulation due to RT storage. Platelets stored at 4°C maintained MPC. Stored platelet samples were visually inspected for swirling according to the guidelines set by Bertolini (29). RT platelets maintained their ability to swirl at Day 5 of storage, while swirling in 4°C-stored samples was nonexistent, most likely due to the shape change that occurs during storage at 4°C (30).

Platelet metabolism

We measured the pH and blood gases in stored platelet samples using i-STAT, and the results are tabulated in Table 2. All samples maintained acceptable levels of pH, i.e., between 6.2 and 7.4 (31). We observed an increase in lactate production and glucose consumption in both RT- and 4°C-stored platelets. The lactate levels in RT-stored platelets increased by ~300% and ~600%, and glucose levels dropped by 8% and 25% by Day 3 and Day 5, respectively ($P < 0.05$). In comparison, the lactate levels in 4°C-stored platelets increased only by 150% and 225% by Day 3 and Day 5, respectively ($P < 0.05$), and the glucose levels did not change significantly. Correspondingly, more bicarbonate was consumed and carbon dioxide released in RT- than 4°C-stored platelets ($P < 0.05$). The

dissolved oxygen levels were comparable and remained high, suggesting adequate gas exchange during the storage period. The glucose consumption and lactate production rates were 3- and 2-fold higher, respectively, during RT storage than during cold storage over 5 days (Table 3). There was no difference between 4°C platelets stored under non-agitated or agitated conditions. Thus, these parameters suggest that 4°C-stored platelets show a significant decrease (more than 2-3 fold) in metabolic rate compared to RT-stored platelets over 5-day storage. A small, but significant difference in sodium and chloride levels was observed at Day 5 in RT samples, and in potassium levels in 4C and 4C+AG samples.

Platelet aggregation

Next, we tested the aggregation response of fresh and stored platelets to physiologically-relevant agonists including ADP, collagen, and TRAP (Figure 1). Cold-stored platelets aggregate significantly better than both RT-stored and fresh platelets when stimulated with ADP (Figure 1A) and collagen (Figure 1B). However, in the case of TRAP (Figure 1C), all storage products decreased compared to fresh platelets, and no differences were seen between groups. There was no difference between 4°C platelets stored under non-agitated or agitated conditions. These data show that 4°C-stored platelets are more responsive to activating stimuli than RT-stored platelets.

We also evaluated the effect of RBC on aggregation response by adding 40% RBC to platelets stored for 5 days (Figure 1D-E). The baseline AUC is higher than in Figure 1A-C because aggregation was monitored for 12 minutes instead of 6. We observed that the addition of RBC significantly increased the response of fresh platelets to ADP. The response of RT platelets to ADP and collagen stimulation again declined significantly both with and without RBCs compared to baseline, while the response of cold-stored platelets was not enhanced by the addition of RBC. Responses of Day 5 4C samples to ADP and collagen stimulation over 12 minutes were similar to baseline. Unlike the six minute data, the 4C collagen response was not enhanced at Day 5 compared to fresh. Decreases in TRAP aggregation over time were not significant in 12-minute aggregation tests for any group, and responses did not were not changed by the addition of RBC.

Clot formation and strength

We tested the kinetics of formation, strength, and stability of clots formed from fresh or stored platelets using thromboelastography. TEG measures time to initial fibrin formation (reaction time or R), time to clot formation (clot time or K), rate of clot formation (α angle), clot strength (maximum amplitude or MA), and clot lysis at 30 minutes (LY30). As can be seen in Figure 2A, the time to initial fibrin formation, R time, was significantly faster with storage at 4°C without agitation, resulting in faster clot formation at Day 3 both compared to baseline and RT storage. The clot generation time, K, for the RT samples was statistically different from baseline at Day 3, but differences were clinically unimportant (Figure 2B). The α -angle was not significantly changed by storage method or duration (Figure 2C). The clot strength, as measured by MA, decreased significantly on Day 5 for RT-stored platelets but remained unaffected in 4°C-stored platelets (Figure 2D). The clot lysis, as measured by LY30, increased significantly after 5 days for RT-stored platelets, but remained constant for 4°C-stored platelets (Figure 2E). With the exception of the Day 3 R values, agitation did not

alter any of the TEG parameters during storage at 4°C. Taken together, the TEG assay clearly demonstrates that 4°C-stored platelets retain the ability to support normal clotting, comparable to fresh platelets even after 5 days of storage, whereas RT-stored platelets form clots that are weak and susceptible to lysis. Conversely, cold storage enhanced initiation of fibrin formation.

Platelet activation marker expression

We measured the levels of receptors expressed on activated platelets including P-selectin, lactadherin binding to exposed phosphatidylserine (PS), and CD40L (Figure 3 A-C). We observed a significant increase in the percentage of platelets expressing P-selectin (CD62P) over storage duration in both RT and 4°C-stored platelets. The 4°C-stored platelets showed a greater increase in CD62P expression (Figure 3A) compared to RT-stored platelets. PS exposure, as measured by lactadherin binding (Figure 3B), also increased with storage duration at 4°C. We used lactadherin instead of annexin V since lactadherin binding does not require Ca^{+2} and has higher sensitivity (32). The CD40L expression on platelet surfaces were higher with cold storage and agitation on day 3 compared to baseline and RT conditions (Figure 3C). Changes in microparticle count and CD42b surface expression were not significant either over time or between cold and RT-stored samples (Figure 3D-E).

Release of pro-inflammatory mediators

When platelets become activated, they release their granular contents and other metabolites into plasma. We tested the levels of sCD40L and TxB₂ released by platelets into plasma during storage (Figure 4). TxB₂ is the stable end product of a metabolite of the arachidonic acid pathway, TxA₂, which intracellularly amplifies signals from other agonists such as ADP or thrombin. RT-stored platelets released 4-fold more TxB₂ than 4°C-stored platelets after 5 days of storage while the latter platelets did not release any significant quantities of TxB₂ (Figure 4A). Soluble CD40L is a protein expressed in platelets that is known to play a role in inflammation, thrombosis, and restenosis (33). CD40L translocates from inside to the surface of agonist-activated platelets, and is shed from the platelet surface as sCD40L. RT-stored samples released slightly more sCD40L into plasma during storage compared to baseline on Day 5 (Figure 4B).

Discussion

In this work, we demonstrated that apheresis platelets stored at 4°C are superior in metabolic and functional assays to platelets stored under standard blood banking conditions at RT. Furthermore, we find that agitation does not significantly influence the function of the 4°C-stored product. Our results suggest that 4°C-stored apheresis platelets would perform better to stop acute hemorrhage. Additional benefits of refrigeration would include decreased risk of bacterial contamination and simplified transport and storage because other blood products must be maintained at 4°C (2-4). Cold platelets may be a viable alternative to RT-stored platelets for therapeutic, as opposed to prophylactic, transfusion when immediate hemostasis is required.

As platelets stored in plasma metabolize glucose via the glycolytic pathway, they produce lactate and free hydrogen ions, which are buffered by bicarbonate in plasma to yield carbon dioxide and water (34). Glucose levels in 4°C-stored platelets did not change over time, consistent with decreased metabolic rates in the refrigerated samples. Similarly, changes in lactate and bicarbonate levels were also small. A similar effect was described in platelet concentrates from PRP stored at 4°C for up to 3 days (8). Stable pH levels correlate with preserved platelet viability, thus the nearly constant pH measurements in the stored 4°C samples indicate that platelet quality is better maintained during refrigeration. In contrast, the RT-stored samples demonstrated a precipitous drop in glucose levels, increased lactate levels, and a corresponding decrease in bicarbonate levels, consistent with the increased metabolic rates previously described during RT storage (31, 35). The glucose consumption and lactate production rates during RT storage were comparable to those reported by Dumont et al (31). Surprisingly, despite better metabolic indices, the platelet counts during 4°C storage were significantly lower compared to RT storage suggesting either adhesion to the bag, clumping or cell death. This loss was abolished when platelets were stored in larger, standard-sized bags, suggesting that higher surface-to-volume ratio in the smaller bags may contribute to the reduction in platelet count.

The various markers of platelet activation including CD62P expression, PS exposure, and expression of CD40L increased during 4°C storage, but only CD62P expression was greater at RT. A similar difference in CD62P expression and PS exposure between 4°C and RT storage has been reported for PRP (36). PS exposure and the release of α -granule contents during RT storage is correlated with the loss in mitochondrial membrane potential and apoptosis due to depletion of glucose and lactate accumulation that occurs from continuous metabolic activity (37-38). These markers represent different molecular events of activation: CD62P is released from platelet α -granules; PS is externalized from the inner leaflet of the platelet membrane; and CD40L is a pro-inflammatory mediator translocated to the platelet membrane. All were higher in 4°C-stored platelets compared to RT despite significantly reduced metabolic rates. In addition, RT storage, but not cold storage, results in platelet degranulation which has been correlated with activation (39). These data suggest that distinct mechanisms of activation are triggered at each storage temperature tested. While platelet activation at RT is related to aging or senescence, we speculate that cold-induced biophysical changes such as membrane rafting, GP Ib receptor clustering, and cytoskeletal rearrangement may trigger a set of downstream biochemical pathways distinct from ageing that result in platelet activation at 4°C (15, 40-43).

In contrast, we found that RT-stored platelets release more pro-inflammatory mediators such as sCD40L and TxA₂ (measured as the more stable downstream product, TxB₂), possibly due to platelet degranulation. Elevated release of sCD40L at RT has been previously described and may play a potential role in adverse transfusion reactions (44-45). TxA₂ is produced by the oxidation of arachidonic acid during platelet activation and is in itself a potent activator of platelets, thus may result in inappropriate coagulation upon transfusion (46). In essence, a decrease in soluble inflammatory mediators suggests that cold stored platelets may be safer for transfusion than RT-stored platelets and may result in fewer adverse events such as transfusion-related acute lung injury (TRALI). Low TxB₂ release from 4°C-stored platelets indicates that arachidonic acid is relatively preserved, suggesting

that the dynamic range of platelet response to physiologic agonists after transfusion is similarly preserved and may explain our aggregometry results.

Circulating platelets are in a state of low activation but high responsiveness. The stimulation of fresh platelets with suboptimal concentrations of natural agonists activates the platelets but also decreases their response to subsequent stimulation with the same or different agonists (47-49). Both RT and 4°C storage activate platelets, but this activation leads to variable responses to chemical agonists. The markedly reduced ability of RT-stored platelets to aggregate upon stimulation with ADP, collagen and TRAP is concordant with previous studies of platelets obtained via both the buffy coat and apheresis methods (50-51). This phenomenon has been attributed to senescence due to progressive loss in energy generating machinery (52). In contrast, 4°C-stored platelets aggregate as well as fresh platelets to ADP and collagen, possibly due to cold-induced platelet activation. This trend was maintained even after the addition of RBC to mimic the physiological environment. Together, our data suggests that activation due to storage at 4°C 'primes' the platelet to a state of heightened responsiveness without compromising the innate response to stimuli. Thus, it appears that the activation state attained by platelets due to 4°C storage may be different from activation states associated with either RT storage or prior activation with chemical stimuli. The similar decline in aggregation response to TRAP activation for both storage temperatures may be due to a loss of thrombin receptors (53).

The superior ability of 4°C-stored platelets to aggregate when stimulated with chemical agonists is accompanied by improved clot strength and stability. While RT storage negatively impacts clot strength and enhances fibrinolysis compared to fresh platelets, significant loss of function was not seen in 4°C-stored samples. Specifically, TEG analysis indicates that despite pre-activation, RT-stored platelets result in the formation of weaker clots that are less resistant to fibrinolysis, probably due to low thrombin generation levels (54). The maintenance of hemostatic function in 4°C-stored platelets, as estimated by TEG, is in line with previous reports (20). Although TEG measures global coagulation, the contribution of platelets is reflected predominantly in MA. In this study, the MA values confirm that platelet integrity and function are well preserved during cold storage without significantly changing AP clot strength.

We stored platelets at 4°C both with and without the method of agitation currently employed by blood banks during standard RT storage. Our assays did not indicate that agitation during cold storage has a significant role in maintaining platelet health and function. Conversely, RT-stored platelets must be agitated either continuously or intermittently every 24 hours to prevent poor gas exchange, acidosis (pH<6), loss of normal morphology, and low platelet recovery (55-57). Thus cold storage may reduce storage costs and minimize space by eliminating the need for platelet agitators during storage and transportation to remote locations. Furthermore, the cost of 4°C stored platelets could be further lowered if reduced need for bacterial testing is proven.

In summary, our data demonstrates that cold-stored platelets have better functional competence and fewer inflammatory mediators than RT-stored platelets. Despite a few promising human trials in the 1970's, platelet refrigeration has been abandoned because of

shorter circulation time post transfusion. Platelet utilization is shifting due to clinical practice guidelines that recommend 1:1:1 transfusion ratios for massive transfusion trauma protocols. The medical environment in which prophylactic transfusions predominated is giving way to one in which active bleeding is frequently the target of therapy. When treating severe hemorrhage, the superior hemostatic effectiveness of cold platelets may actually make them the better choice. Refrigerated platelets were the standard of care for more than a decade. Our results, along with a growing body of evidence, suggest the need to seriously consider the re-instatement of cold-stored platelets as a widely-available therapeutic product.

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List of Abbreviations

RT	room temperature
4C	storage at 4 °Celsius
4C+AG	storage at 4 °Celsius with gentle agitation
AP	apheresis platelets
ADP	adenosine diphosphate
TRAP	thrombin receptor activating peptide
CD62P	P-selectin
CD40L	CD40 ligand
sCD40L	soluble CD40 ligand
CD154	CD40L surface expression molecule
CD42b	glycoprotein Ib receptor
GPIb	glycoprotein Ib
TxB₂	Thromboxane B ₂
RBC	red blood cell
HBSS	Hanks Balanced Salt Solution
ELISA	enzyme-linked immunosorbent assay
ACD	acid-citrate-dextrose
PPP	platelet-poor plasma
FDA	Food and Drug Administration
MPV	mean platelet volume
MPC	mean platelet component
TEG	Thromboelastography

GMFI	Geometric mean fluorescence intensity
AUC	area under the curve
R	reaction time (time to initial fibrin formation)
K	time to clot formation
α angle	alpha angle (rate of clot formation)
MA	maximum amplitude (clot strength)
LY30	clot lysis at 30 minutes
TRALI	transfusion-related acute lung injury

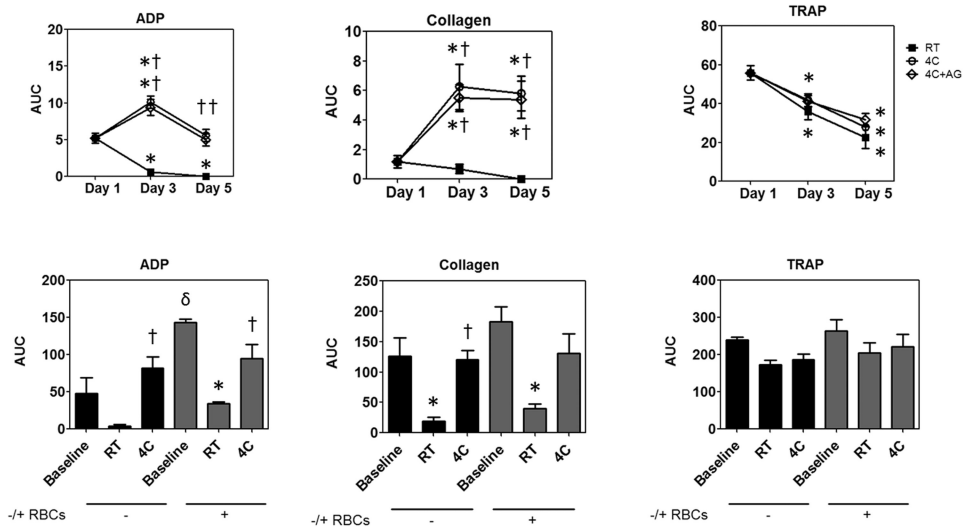


Figure 1. Aggregation of platelets without (A, B, C) and with RBCs (D, E, F) when stimulated with (A, D) ADP, (B, E) Collagen, and (C, F) TRAP. Bar graphs D, E, and F are comparisons between Baseline versus Day 5 RT and 4C AP. Treatment conditions are represented as follows: RT = ■; 4C = ○; 4C+AG = ◇. Area under the curve (AUC) are represented as mean ± SEM. Differences compared to baseline (*), compared to RT (†), and between samples without RBCs versus with (δ) are shown if results from both the one-way ANOVA for repeated measures and the post hoc Bonferroni test comparisons are significant ($P < 0.05$). (Note: - RBC AUC values are higher in graphs D-F compared to A-C because aggregation was measured over 12 minutes instead of 6.)

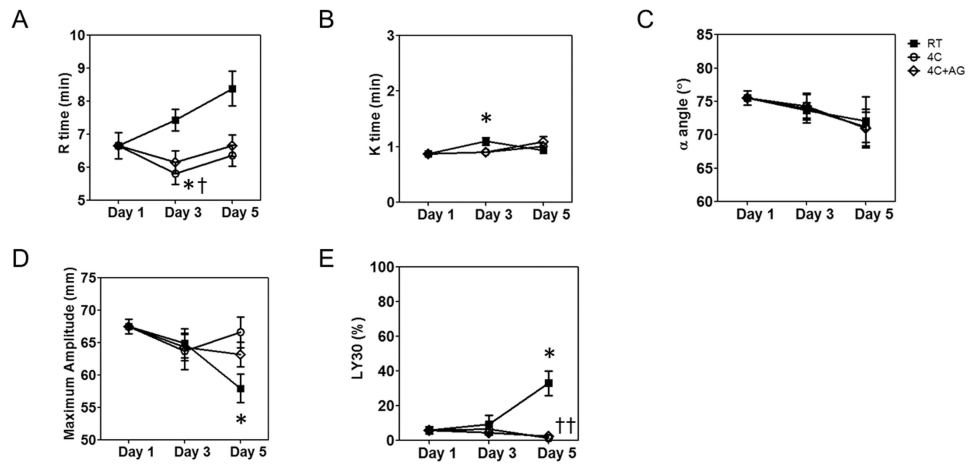


Figure 2.

Measurement of clot properties by thromboelastography. (A) R time; (B) K time; (C) α angle; (D) Maximum amplitude. Treatment conditions are represented as follows: RT = ■; 4C = ○; 4C+AG = ◇. Data are represented as mean \pm SEM. Differences from Baseline (*) and between treatment groups (†) are shown if results from both the one-way ANOVA for repeated measures and the post-hoc Bonferroni test comparisons are significant ($P < 0.05$).

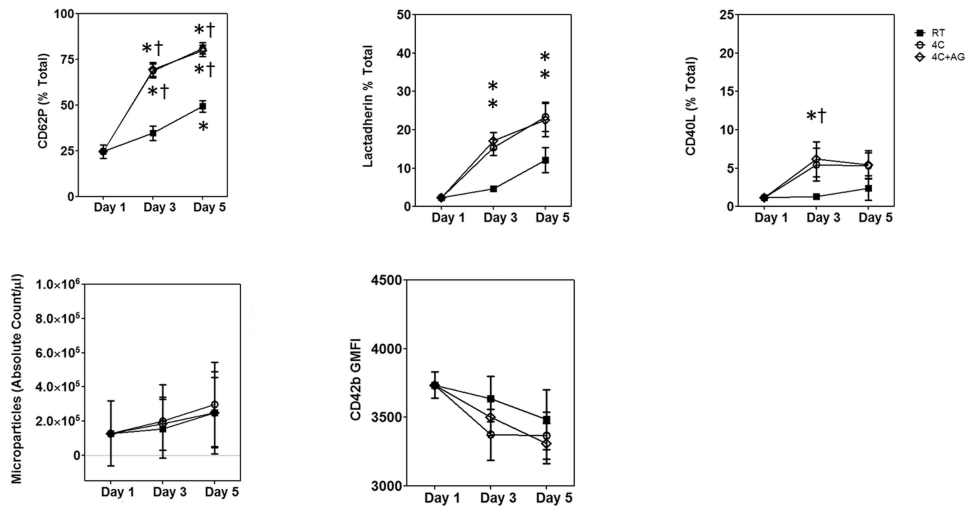


Figure 3. Estimation of surface receptor levels by flow cytometry. (A) P-Selectin; (B) lactadherin binding (PS exposure); (C) CD40L expression; (D) Microparticle release; and (E) GP Iba. Treatment conditions are represented as follows: RT = ■; 4C = ○; 4C+AG = ◇. The expression levels are represented as mean±SEM. Differences from Baseline (*) and between treatment groups (†) are shown if results from both the one-way ANOVA for repeated measures and the post-hoc Bonferroni test comparisons are significant ($P < 0.05$).

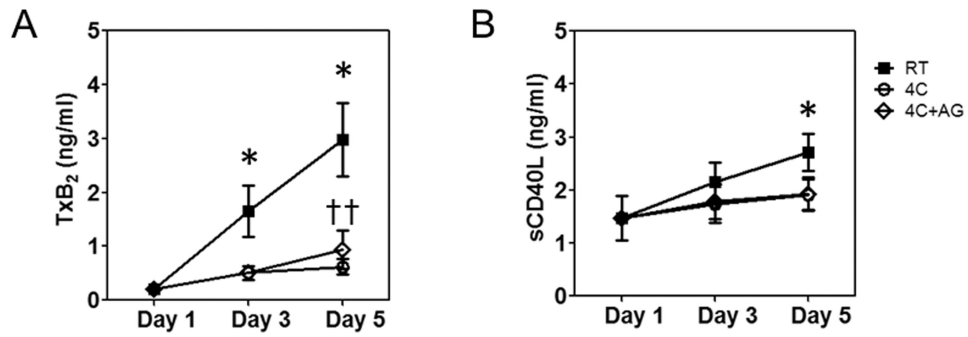


Figure 4. ELISA quantification of soluble factors released by platelets. (A) TxB₂, and (B) sCD40L. Treatment conditions are represented as follows: RT = ■; 4C = ◐; 4C+AG = ◑. The concentrations are represented as mean ± SEM. Differences from Baseline (*) and between treatment groups (†) are shown if results from both the one-way ANOVA for repeated measures and the post-hoc Bonferroni test comparisons are significant ($P < 0.05$).

Table 1

A. Platelet count $\times 10^3$, mean platelet volume (MPV), and mean platelet component (MPV) during storage in minibags (research use only). **B.** Platelet count $\times 10^3$ of apheresis platelets stored in full-sized FDA-approved platelet storage bags (n = 3).

A	Baseline		RT		4C		4C+AG	
	Day 1	Day 3	Day 5	Day 3	Day 5	Day 3	Day 5	
Count ($\times 10^3$ plts/ μ l)	1084 \pm 47	1019 \pm 66	924 \pm 149	815 \pm 152	727 \pm 153*	854 \pm 103	687 \pm 147*	
MPV (fl)	7.0 \pm 0.2	6.9 \pm 0.1	7.0 \pm 0.2	6.9 \pm 0.4	6.9 \pm 0.3	7.0 \pm 0.4	6.8 \pm 0.3	
MPC (g/dL)	25 \pm 1.8	-	20 \pm 1.7*	-	26 \pm 2.3	-	-	

Values (mean \pm SD) significant from Baseline represented by * ($P < 0.05$)

B	Baseline		4C	
	Day 1	Day 5	Day 5	Day 5
Count ($\times 10^3$ plts/ μ l)	1192 \pm 445	1187 \pm 51	1123 \pm 40	

Values (mean \pm SD) were not significantly different ($P = 0.05$)

Table 2

Metabolic and electrolyte levels in plasma during storage.

	RT			4C			4C+AG		
	Day 1	Day 3	Day 5	Day 3	Day 5	Day 3	Day 5	Day 3	Day 5
pH	7.24±.07	7.45±.16	7.28±.08	7.45±.21	7.44±.13	7.48±.25	7.44±.15		
Lactate (mg/dL)	1.77±0.6	6.85±0.8*	12.87±2.1*	4.51±0.6*	5.67±0.3 [‡]	4.83±0.7*	6.05±0.4 [‡]		
Glucose (mg/dL)	321.5±8.1	294.0±22.1*	238.3±42.5*	310.2±14.2*	307.5±25.0 [‡]	312.4±28.1*	303.5±24.6 [‡]		
Bicarbonate (mM)	18.04±1.6	10.06±2.9*	6.27±0.9*	14.00±1.7* ^δ	12.78±0.8 [‡]	14.01±2.7* ^δ	12.38±1.0 [‡]		
pCO ₂ (mmHg)	38.5±4.7	13.8±1.5*	13.1±.25*	24.1±4.2* ^δ	19.5±4.4 [‡]	22.6±5.6* ^δ	18.7±4.7 [‡]		
pO ₂ (mmHg)	91.8±9.7	113±17.8	132±41.2	120.4±32.7	139.8±26.8 [‡]	116.6±31.0	128.0±29.3 [‡]		
Sodium (mM)	137.5±1	139.5±3.7	140.25±1.3*	137.4±1.7	136.8±96 [‡]	137.8±1.6	136.5±1.3 [‡]		
Potassium (mM)	3.24±.18	3.34±.23	3.45±.31	3.52±.19*	3.60±.25*	3.52±.25*	3.55±.27 [‡]		
Chloride (mM)	97.6±.89	101.3±4.9	104.8±1.3*	99.2±2.8	99.7±1.2 [‡]	99.0±2.2	98.8±1.7 [‡]		

* Values (mean ± SD) significant from Baseline represented by ($P < 0.05$)

^δ Values significant from Day 3 RT represented by ($P < 0.05$)

[‡] Values significant from Day 5 RT represented by ($P < 0.05$)

Table 3

Glucose consumption and lactate production rates calculated from the measurements in Table 1.

	RT		4C		4C+AG	
	Day 3	Day 5	Day 3	Day 5	Day 3	Day 5
Glucose consumption from Baseline ($\mu\text{mol}/10^{12}$ platelets/hr)	30.6 \pm 7.9	75.6 \pm 25.0	17.1 \pm 11.3	25.3 \pm 3.6*	14.8 \pm 7.1*	29.4 \pm 9.8*
Lactate production from Baseline ($\mu\text{mol}/10^{12}$ platelets/hr)	71.8 \pm 7.2	97.6 \pm 23.0	36.1 \pm 5.1*	40.0 \pm 8.6*	40.3 \pm 4.9*	44.6 \pm 8.5*

* Values (mean \pm SD) significant from corresponding RT samples represented by ($P < 0.05$)