Optimizing whole blood storage: hemostatic function of 35-day stored product in CPD, CP2D, and CPDA-1 anticoagulants

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BACKGROUND: Transitioning from whole blood (WB) to components developed from efforts to maximize donor yield. Components are advantageous for specific derangements, but treating hemorrhage with components requires significantly more volume to provide similar effects to WB. Because storage lesion and waste remain problematic, this study examined hemostatic function of refrigerated WB stored for 35 days in anticoagulants citrate–phosphate-dextrose-adenosine (CPDA-1), citrate–phosphate-dextrose (CPD), or citrate–phosphate-double dextrose (CP2D).

METHODS: Refrigerated WB units from healthy donors were sampled over 35 days. Global hemostatic parameters were measured by thromboelastometry, thrombogram, platelet aggregometry, and platelet adhesion to collagen under shear conditions. The effects of transfusion filtration and mixing 35-day stored product with fresh WB were evaluated.

RESULTS: Countable platelets declined as aggregation clusters appeared in microscopy. While gross platelet agonist-induced aggregation declined over time, normalization revealed aggregation responses in remaining platelets. Peak thrombin generation increased over time. Clot strength diminished over storage in tissue factor–activated samples (normalized by filtration of aggregates). Functional fibrinogen responses remained consistent throughout. Filtration was necessary to maintain consistent platelet adhesion to collagen beyond collection day. Few differences were observed between anticoagulants, and stored/fresh mixing studies normalized coagulation parameters.

CONCLUSIONS: WB is easier to collect, store, and transfuse. WB provides platelets, an oft-neglected, critical resuscitation component, but their individual numbers decline as aggregates appear, resulting in diminished coagulation response. WB has better performance in these assays when examined at earlier time points, but expirations designated to specific anticoagulants appear arbitrary for hemostatic functionality, as little changes beyond 21 days regardless of anticoagulant.

The use of whole blood (WB) for treatment and prevention of hemorrhagic shock has its roots in the battlefields of World War I,[1] when citrated WB transfusions proved to be an effective strategy for treatment of the most severely injured combat casualties.[2,3] WB use by the military in the early 20th century established the beginnings of the "walking blood bank" concept through widespread usage of fresh whole blood (FWB) collected during combat. WB use by the military has continued throughout every major conflict since World War I,[4] but since Vietnam the use of WB has declined in favor of component therapy.[5]

The impetus for transitioning to blood component therapy developed from efforts to maximize the therapeutic yield

ABBREVIATIONS: ASPI = assay for determination of platelet function triggered by arachidonic acid; CP2D = citrate–phosphate-double dextrose; CPD = citrate–phosphate-dextrose; CPDA-1 = citrate–phosphate-dextrose-adenosine; CT = clotting time; EXTEM = tissue factor pathway activation thromboelastometry; FFP = fresh frozen plasma; FIBTEM = functional fibrinogen thromboelastometry; FWB = fresh whole blood; INTEM = contact pathway activation thromboelastometry; MCF = maximum clot firmness; PPP = platelet-poor plasma; PRP = platelet-rich plasma; ROTEM = rotational thromboelastometry; TRAP = thrombin receptor-activating peptide; WB = whole blood.

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of each blood donor. By separating WB to components, multiple patients could benefit; for example, red blood cell (RBC) concentrates could be given for anemia or platelets for thrombocytopenia. These individual components can be stored under different conditions to improve their quality. Despite a lack of comprehensive clinical trial data comparing outcomes to WB transfusion, component therapy became the most widely used method for all transfusion services, including patients with traumatic hemorrhage in both civilian and military settings, as it remains to this day. Strategies have been developed to adapt component transfusion therapy to the needs of bleeding patients using RBCs, fresh frozen plasma (FFP), and platelets given in a 1:1:1 ratio[6] as an alternative to the often unavailable WB product. It has been noted that this ratio is often difficult to achieve outside of trauma centers—platelets, with their short shelf life of 5 days, are frequently unavailable or saved for the most serious cases. However, studies have shown that in some cases treating traumatic hemorrhage with blood components leads to a resuscitation milieu requiring a larger volume to achieve the same hemostatic effect when compared to WB.[7,8] Recent studies indicate that this is largely due to the poor aggregation response of stored, aged platelets, although revised storage modalities have been explored to improve platelets' functionality (as measured by aggregation and clot formation) over longer durations.[9–12] However, all blood components suffer from a "storage lesion," resulting in an overall reduced resuscitation capacity and a need to transfuse more product to achieve desired effects as products age.[13]

Platelets play a critical role in maintaining hemostasis and have convincingly been shown to contribute to better outcomes and lower mortality when used with other transfused blood products.[14–16] While current US Food and Drug Administration blood banking guidelines promote platelet storage at room temperature (resulting in a platelet product with a severely reduced aggregometry response within a few days due to the platelet storage lesion), our group has shown that refrigeration of platelets at 4 °C results in preservation of metabolic activity and procoagulant function as observed through improvements in aggregation response, clot strength, and adhesion under flow conditions.[11,17,18] Storage of platelets within their WB environment at 4 °C could combine a more functional platelet product with easier administration, enhanced availability, and increased storage capacity, improving the ability to provide hemostatic resuscitation in the prehospital setting, during early in-hospital resuscitation, and in austere combat environments.

While component therapy has been the more favored approach, recent conflicts have renewed interest in WB use on the battlefield.[19,20] However, a comprehensive study of hemostatic properties of cold-stored WB over time in different anticoagulants has not been performed. Citrate–phosphate-dextrose (CPD) became the standard anticoagulant (for both WB and components) in the late 1950s; CPD-stored blood was granted a shelf life of 21 days (2–4 °C). By 1978, CPD supplemented with adenine (CPDA-1) was in use, increasing the available storage time of WB and RBCs to 35 days. Extensions in shelf life were attributed to adenine's contribution to the increased synthesis of adenosine triphosphate.[21] Citrate–phosphate-double dextrose (CP2D) has been less commonly used as an anticoagulant for blood and blood products since the 1970s, with the higher concentration of dextrose and thus higher osmolarity than CPD intended to support RBCs.[22] However, CP2D-anticoagulated WB has the same 21-day shelf life as CPD.

While CPD is still the primary choice for WB, the 35-day shelf life of CPDA-1 is appealing for logistical reasons and waste reduction—albeit with a greater likelihood of diminished hemostatic capacity due to developing storage lesion at later time points. This work examines the hemostatic performance of WB stored at 4 °C over 35 days in vitro, with a comparison of WB units anticoagulated with CPD, CP2D, and CPDA-1.

METHODS

Collection of FWB

FWB was collected from healthy donors according to an approved institutional standard operating procedure in 450-mL CPD bags (BB*AGT456A2, Terumo BCT; n = 7, all male), 450-mL CP2D bags (121–63, Haemonetics; n = 4, all male), or 500-mL CPDA-1 bags (4R3327E, Fenwal; n = 10, 9 male), all with the same expected ratio of anticoagulant to blood (1:7). Units were stored without agitation in a walk-in refrigerator set to 4 °C with constant monitoring. An additional sample of blood was collected in blood collection tubes (K2-EDTA vacutainers, Becton Dickinson) from each donor for baseline measurements of complete blood count (CBC).

Sample preparation

On each sampling day (0, 3, 7, 14, 21, 28, and 35), bags were gently mixed by hand, and approximately 15 mL of blood was slowly removed in a sterile environment by Luer lock syringe. Of this, 2 mL was centrifuged at 200 × *g* for 10 minutes to create a platelet-rich plasma (PRP) layer separated from RBCs, and 2 mL was centrifuged at 2000 × *g* for 20 minutes (twice) to obtain a platelet-poor plasma (PPP).

WB CBC was measured at each time point in an automated hematology analyzer (ABX Micros 60, Horiba). Essential blood chemistry was measured by a blood analyzer (i-STAT, CG4+ cartridge; Abbott Laboratories).

Coagulation function

Coagulation function of stored WB samples was evaluated by rotational thromboelastometry (ROTEM delta; Instrumentation Laboratory, Bedford, MA) using three tests: tissue factor pathway activation (EXTEM), contact pathway activation (INTEM), and functional fibrinogen (FIBTEM) tests

incorporating cytochalasin D to inhibit platelet function; 300-μL samples were mixed with sample reagents according to manufacturer's instructions and run in duplicate tests.

Thrombin generation

Thrombin generation was measured in PPP samples (using PPP-low reagent, 1 pM tissue factor and 4 μM phospholipid; Diagnostica Stago) and PRP samples (using PRP reagent, 1 pM tissue factor only; Diagnostica Stago) on each testing day using the calibrated automated thrombogram assay by mixing 80 μL of sample with 20 μL of reagent, incubating for 10 minutes at 37°C, and mixing by autoinjector with 20 μL of FluCa buffer (Z-Gly-Gly-Arg-AMC fluorogenic substrate and calcium chloride; Diagnostica Stago). Thrombograms were collected with a computer-controlled microplate fluorometer and luminometer (Fluoroskan Ascent FL, ThermoFisher Scientific) with computer software (Thrombinoscope, Thrombinoscope BV). Samples were run in triplicate with an additional triplicate set run in parallel using the thrombin calibrator instead of reagent to account for inner filter effect and substrate consumption rate.

Platelet aggregation

Aggregation of platelets in WB was evaluated using a multiplate impedance aggregometer (DiaPharma). Briefly, 300 μL of stored WB was mixed with 300 μL of 0.9% NaCl (with 3 mM $CaCl_2$ if required for a particular agonist) and incubated for 3 minutes at 37°C before the addition of 20 μL of platelet agonist: collagen, ADP, thrombin receptor-activating peptide (TRAP)-6, arachidonic acid (ASPI), and ristocetin (using 50 μL instead of 20 μL) were all applied as agonists. Samples were monitored for 6 minutes with maximum aggregation used as the endpoint.

Adhesion of platelets to collagen under flow conditions

Platelet adhesion function was measured using a shear flow assay system (BioFlux 1000, Fluxion Biosciences). Channels of a BioFlux 48-well plate were coated with reagent grade type I collagen (25 μL of a 100 μg/mL stock solution; Helena Laboratories) for 1 hour, followed by a 15-minute wash with 0.5% bovine serum albumin (Sigma-Aldrich); unadhered collagen was flushed out with excess phosphate-buffered saline.

Stored WB samples were mixed with 1 μM of calcein AM (ThermoFisher Scientific) and allowed to incubate in the dark at 37°C for 30 minutes. Blood was added to the inlet well and exposed to pneumatic pressure adjusted by the BioFlux control software to generate a shear rate of 920 s^{-1} in approximation of arterial flow, 100 s^{-1} for a model of venous flow, or 4000 s^{-1} to simulate a pathological scenario. Fluorescent microscopy (490/525 em/ex) images of platelets adhered to the surface were collected every 30 seconds for a period of 10 minutes using computer software (MetaMorph, Molecular Devices).

Standard transfusion filtration

Because there was some evidence in initial studies that showed formation of visible aggregates in BioFlux assays over time, additional WB units (n = 4 each of CPD, CP2D, and CPDA-1 anticoagulated; all male donors) were collected for follow-up studies on stored blood after passing through a standard 200-μm transfusion filter (10010985; BD Carefusion). At each time point, a fresh transfusion filter was sterilely welded to the blood bag, and 12 mL of blood was collected under sterile conditions into a 15-mL conical tube via gravity flow. The timing of each collection was monitored by stopwatch (Fig. S1). Filtered samples were analyzed on CBC, ROTEM, and BioFlux as described above.

Fresh and stored blood mixing assays

At the end of storage (Day 35), mixing studies were performed in ROTEM (EXTEM, INTEM, and FIBTEM) and in multiplate (collagen, ADP, TRAP-6, ASPI, and ristocetin agonists) by combining 35-day stored WB with FWB (type-matched donors collected in citrate vacutainers) at ratios of 1:2, 1:3, 1:4, and 1:5 (fresh-to-stored) to determine if a threshold of FWB was required to improve coagulation function in a transfusable product bundle.

Statistical analysis

Data collection, aggregation, and statistical analyses were performed with computer software (Excel 2010, Microsoft Corporation; and Prism 7.01, GraphPad Software, Inc.). Two-way analysis of variance tests determined intergroup differences for studies comparing CPD, CP2D, and CPDA-1 at each time point, comparing time points versus baseline measures, filtered versus unfiltered samples where appropriate, and the ratios of fresh-to-stored blood at end of storage.

RESULTS

Complete blood count and chemistry

Standard CBC and blood chemistry results (Table 1) showed a general decline in white blood cells (WBCs) and platelets over storage duration. The drop in WBCs (compared to baseline) only reached statistical significance on Day 35 with CPD and on Day 14 with CP2D, whereas platelets were significantly decreased by Day 3 (CP2D) or Day 7 (CPD and CPDA-1). Mean platelet volume, RBC count, hematocrit, and hemoglobin did not significantly decline over time in any anticoagulant (Table S1). Additionally, only one unit was shown to have statistically nonzero hemolysis over time, and the maximum measured hemolysis of that unit was only 0.51%.

There were sporadic differences observed between CBC measurements at the same time points in different anticoagulants (Table 1), but these did not appear to be indicative of a conclusive trend (e.g., RBC count was significantly different

TABLE 1. Changes to whole blood cellular and chemistry characteristics during storage over 35 days

Day		CPDA-1	CPD	CP2D	CPDA-1	CPD	CP2D
			pH			**Lactate**	
0		7.24 ± 0.18	7.19 ± 0.09	7.23 ± 0.02	2.26 ± 1.79	1.57 ± 0.45	2.26 ± 0.51
3		7.17 ± 0.11	7.07 ± 0.06	7.21 ± 0.07	4.94 ± 0.98*	5.33 ± 0.20*	7.05 ± 0.63*
7		7.07 ± 0.15*	6.93 ± 0.12*	6.98 ± 0.04*	7.97 ± 1.23*	8.90 ± 1.79*	9.99 ± 0.99*
14		6.86 ± 0.05*	6.81 ± 0.06*	6.96 ± 0.05*	11.84 ± 0.95*	12.70 ± 1.31*	15.48 ± 1.35*
21		6.74 ± 0.04*	6.74 ± 0.07*	6.83 ± 0.07*	15.07 ± 1.24*	16.28 ± 2.31*	18.57 ± 0.75*
28		6.69 ± 0.08*	6.69 ± 0.11*	6.67 ± 0.04*	17.88 ± 1.37*	18.07 ± 1.78*	OOR
35		6.68 ± 0.08*	6.65 ± 0.06*	6.63 ± 0.03*	19.33 ± 1.01*	18.84 ± 2.01*	OOR
			WBC			**PLT**	
0	U	4.13 ± 0.84	6.90 ± 0.67	8.14 ± 2.73	139.75 ± 40.53	148.00 ± 40.41	148.50 ± 35.57
	F	4.23 ± 0.63	4.91 ± 1.63	8.31 ± 2.88	159.00 ± 30.53	104.75 ± 55.15	154.88 ± 42.48
3	U	4.10 ± 1.41	7.03 ± 1.50	8.20 ± 4.14	112.50 ± 52.98	93.25 ± 35.57	77.75 ± 23.17*
	F	5.01 ± 1.07†	5.76 ± 1.09	8.60 ± 3.72	113.75 ± 45.86	75.38 ± 37.41	79.75 ± 27.77*
7	U	3.35 ± 0.79	5.85 ± 1.05	6.05 ± 2.67	80.50 ± 28.38*	68.50 ± 33.61*	71.63 ± 29.38*
	F	4.58 ± 1.20	5.08 ± 1.63	3.80 ± 1.27	93.13 ± 35.04*	79.38 ± 30.35	80.13 ± 30.40*
14	U	3.14 ± 0.56	4.96 ± 1.29	5.20 ± 1.94	72.88 ± 14.91*	71.79 ± 29.44*	88.50 ± 33.45*
	F	4.35 ± 1.14†	3.63 ± 1.85	5.24 ± 2.02*	104.13 ± 19.54	87.13 ± 26.73	85.88 ± 32.20*
21	U	2.88 ± 0.52	4.15 ± 1.38	3.80 ± 1.29*	74.25 ± 11.78*	73.50 ± 23.60*	79.38 ± 29.35*
	F	3.90 ± 0.96	3.05 ± 0.00	3.95 ± 1.18*	114.63 ± 19.24	115.50 ± 0.00	83.25 ± 24.60*
28	U	2.78 ± 0.46	4.17 ± 1.24	3.10 ± 1.35*	83.63 ± 29.21	84.13 ± 16.82*	87.63 ± 33.40*
	F	3.36 ± 0.82†	3.54 ± 1.46	3.80 ± 0.48*	113.13 ± 25.87	82.63 ± 22.31	90.50 ± 23.23*
35	U	2.66 ± 0.33	3.49 ± 1.07*	3.05 ± 0.73*	82.13 ± 25.42	76.50 ± 23.97*	96.63 ± 30.26
	F	2.54 ± 1.80	3.30 ± 1.98	3.31 ± 0.87*	118.88 ± 31.07	88.13 ± 21.87	90.00 ± 37.30*

Data shown as mean ± standard deviation.
F = filtered; OOR = out-of-range (lactate measurement is limited on the maximum end at 20 mM); PLT = Platelets ($\times 10^3/\mu l$); U = unfiltered; WBC = white blood cells ($\times 10^3/\mu l$).
* $p < 0.05$ versus same sample on Day 0
† $p < 0.05$ versus unfiltered sample at same time point.

between CPD and CP2D on Day 3 but not at any other time point).

In a follow-up study, CBC was also conducted to determine if cell populations were disturbed by standard transfusion filtration. Filtration was performed with only four units of each anticoagulant, but there were no significant changes to blood cell components.

In all three anticoagulants, pH declined significantly over storage duration, likely due to an observed 10-fold increase in lactate (and complementary reduction in bicarbonate; Table S1). pH was not statistically different between blood samples in different anticoagulants at each given time point. Lactate was above the limit of detection (20 mM) in some samples by Day 28 and all samples on Day 35, and CP2D-anticoagulated blood had a lactate that was statistically greater than both CPD and CPDA-1 blood by Day 14. Bicarbonate was similarly lower in CP2D blood versus CPD and CPDA-1 blood by Day 14, and base deficit mirrored these trends over storage duration. Within the storage bag, PO_2 remained broadly stable and PCO_2 generally increased (Fig. S2).

Thrombin generation

With the calibrated automated thrombogram assay of thrombin generation in PRP and PPP collected from the WB units, a general trend toward increased thrombin generation was observed in all samples over time (Fig. 1). In PRP, lag time to initiation of thrombin generation decreased over time in all three anticoagulants, while in PPP lag time was maintained over the duration of storage in all three anticoagulants, although the CP2D units had a higher mean lag time versus CPDA-1 or CPD for Days 0 and 7 ($p < 0.001$). Endogenous thrombin potential, a measurement reflecting prothrombin concentration and total coagulation enzymatic availability, was retained across storage. However, peak thrombin, a more direct measurement of thrombin generation, showed significant increases at longer storage times in both PRP and PPP. It is worth noting that the samples were somewhat activated on Day 0 (likely due to shear experienced by drawing through the needle), and thus a drop in peak thrombin was observed between Days 0 and 3 for PPP (statistically insignificant for CPDA-1 and CP2D, $p < 0.05$ for CPD). Thrombograms used to calculate these parameters are shown in Fig. S3.

Thromboelastometry

Coagulation function was examined by ROTEM at each time point. With tissue factor pathway activation through EXTEM reagents (Fig. 2), clotting time (CT) increased gradually over storage in all anticoagulants, although these increases were not statistically significant from baseline values, nor were any differences between anticoagulants significant. However, after filtration, the CP2D samples on Day 35 had a large increase in CT on Day 35 (42% increase vs. Day 28; $p < 0.0001$).

EXTEM angle (correlated with rate of clot formation) declined over storage in both CPDA-1 ($p = 0.019$ for Day 7 vs. baseline) and CPD ($p = 0.039$ for Day 28 vs. baseline), and although there was a decline over time in CP2D, it was

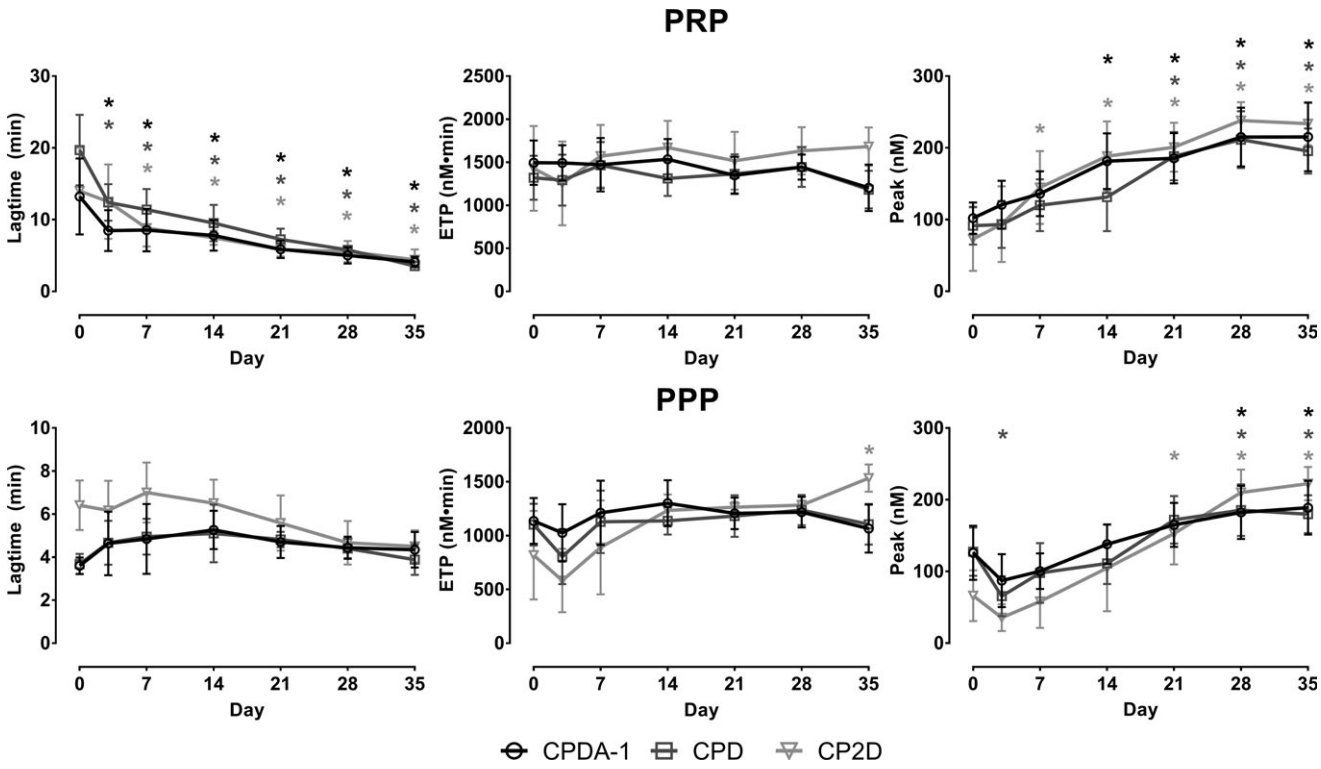

Fig. 1. In PRP samples, lag time to thrombin generation decreases over time, while PPP samples were not statistically different at the end of storage. Endogenous thrombin potential is maintained throughout storage in both PRP and PPP samples, with the only deviation seen in CP2D at day 35. Peak thrombin increases throughout storage in both PRP and PPP. Data shown as mean with standard deviations. * $p < 0.05$ versus same sample on Day 0.

not significant by Day 35 ($p = 0.35$ vs. baseline). Angle differences between anticoagulants were not significant. After filtration, only CPDA-1 had a significant change by Day 28 ($p = 0.036$ vs. baseline).

EXTEM maximum clot firmness (MCF, a measurement related to clot strength) also declined across storage, with CPDA-1 samples reaching significant differences versus baseline by Day 3 ($p = 0.0006$), CPD by Day 14 ($p = 0.032$), and CP2D by Day 21 ($p = 0.028$). CP2D did not experience the same degree of decrease between Days 0 and 3, and as such had a larger MCF than CPD and CPDA-1 (significant only on Days 3 and 28). After using the transfusion filter, changes to MCF were normalized, with differences versus Day 0 occurring only on Day 28 (CPDA-1; $p = 0.044$) or Day 35 (CPD, $p = 0.006$; CP2D, $p = 0.017$).

FIBTEM reagents allow for exploration of deficiencies in the plasma coagulation factors irrespective of platelet contribution (Fig. S4). Mean CT values increased over storage duration in all anticoagulants (although significantly so only in CP2D). Angle and MCF remained constant across 35 days of storage. The use of the transfusion filter did not change these trends. The constancy of these three parameters indicates that the time-dependent changes observed in the various EXTEM ROTEM assays are primarily due to altered platelet function over storage.

The INTEM assay of contact pathway activation was only conducted on unfiltered samples (Fig. S5), showing similar trends to what was seen in EXTEM. CT increased over time, significantly so in CPDA-1 by Day 14 ($p = 0.004$) and by Day 21 in CPD ($p = 0.025$) and CP2D ($p = .012$). There were no significant differences between anticoagulants, but there was a noticeable decrease versus baseline in both CPDA-1 ($p = 0.021$ on Day 3) and CPD ($p = 0.043$ on Day 7) samples. INTEM MCF declined across the storage duration in all anticoagulants (CPDA-1 on Day 3, $p = 0.01$; CPD on Day 21, $p = 0.0026$; CP2D on Day 21, $p = 0.019$).

Platelet agonist-induced aggregation

Impedance aggregometry measurements in the multiplate using five aggregation-inducing agonists showed similar effects regardless of agonist or anticoagulant: Day 0 responses to all agonists were strong in all anticoagulants, and all declined rapidly by Day 3. Figure 3 shows platelet aggregation responses to collagen; ADP, TRAP, ASPI, and ristocetin agonist effects are shown in Fig. S6. Based on the observation that the platelet count was rapidly declining (see Table 1), the aggregation response was also normalized to platelet count, which demonstrated that the remaining platelets continued to have moderate aggregation responses

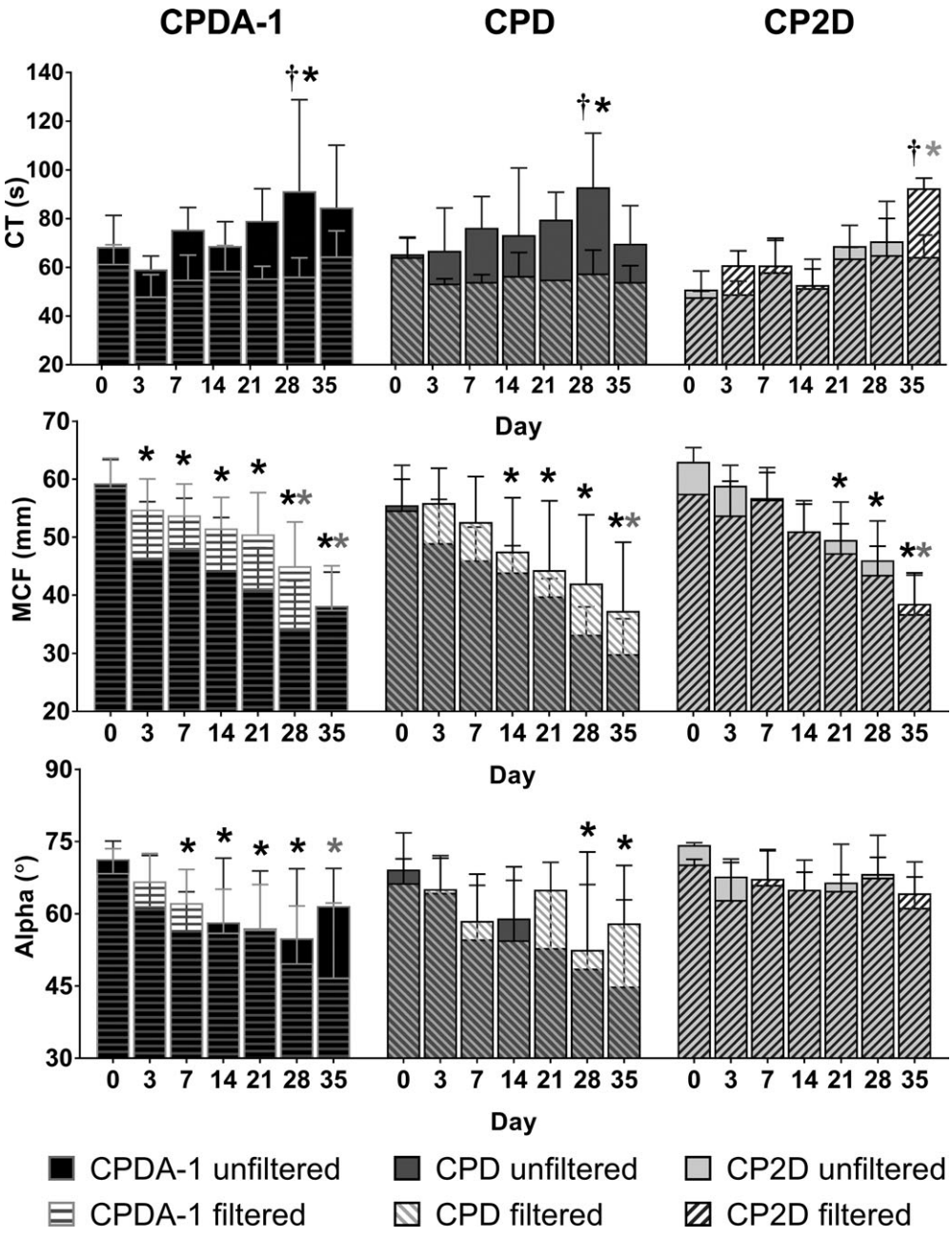

Fig. 2. ROTEM (EXTEM activated) assays of coagulation in whole blood stored over 35 days show trends in increasing CT and decreasing MCF and alpha over storage, changes which are mitigated post-filtration. CT, coagulation time (s); MCF, maximum clot firmness (mm); Alpha, α-angle (°). * $p < 0.05$ versus same sample on Day 0 (black for unfiltered sample, gray for filtered sample); † $p < 0.05$ for the comparison of unfiltered versus filtered samples on the same day.

over the first 14 days, especially in the CPD samples. More specifically, in CPD WB, collagen-stimulated aggregation of platelets declined by 47.9% between Days 0 and 3, but when normalized for platelet count this decrease was only 16.9%.

Platelet adhesion to collagen under flow

When fluorescently labeled platelets were exposed to arterial shear (920 s^{-1}) for a period of 10 minutes, there was significant observable adhesion of platelets to a collagen layer on Day 0, which noticeably transformed into masses of aggregates by Day 3 and beyond (representative images in CPDA-1 anticoagulated samples at each time point shown in Fig. 4). These aggregates were formed prior to adhering to the substrate, although they would grow over time and frequently release from the surface to continue down the channel. This prompted the usage of the transfusion filter set in later samples, and filtration of blood delayed the appearance

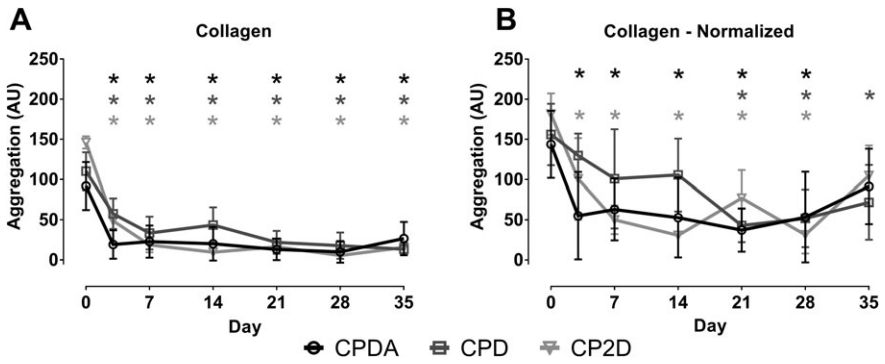

Fig. 3. (A) Collagen-stimulated platelet aggregation in the multiplate shows a rapid decline in function over time. (B) This functional decrease corresponds to the decreased platelet count (Table 1), and when samples were normalized versus count, functionality was elevated. * $p < 0.05$ versus same sample on Day 0. See Fig. S4 for additional agonist responses.

of these aggregate masses to Day 14. No noticeable differences were observed versus other anticoagulants.

Additionally, tests with a pathologically high shear (4000 s^{-1}) were conducted (Fig. S7), and very few platelets or aggregates adhered to the collagen beyond Day 0 of storage under this high-flow condition. Tests with low venous shear (100 s^{-1}) showed moderate adhesion over the first 14 days, followed by the appearance of large aggregates (Fig. S8). However, this result may be skewed, as collagen alone is not the most appropriate substrate for low-flow adhesion.[23]

Coagulation function of 35-day stored mixed with fresh blood

Mixing studies of expiring WB with FWB demonstrated an improvement in functional coagulation responses (Figs. 5A-C). In CP2D, a mixture of one part FWB with five parts 35-day stored WB was sufficient to normalize MCF in ROTEM EXTEM assays; statistically significant improvements of EXTEM MCF in 35-day stored CPD WB were observed with a 1:4 ratio of fresh-to-stored blood. EXTEM CT values were normalized at the 1:5 ratio, even for CPDA-1, which had the highest mean CT values of the stored products. EXTEM angles showed similar trends in improvement with low ratio mixtures. INTEM also followed the EXTEM parameters' trends of improvement by mixing with FWB (Fig. S9). FIBTEM CT was improved by mixing FWB with the stored WB, but MCF and angle were already maintained over storage and did not improve.

Platelet aggregation function improvement in mixed samples was negligible when measured in multiplate (see Fig. 5D for collagen agonist response; other agonists are shown in Fig. S10), but these samples were not normalized to platelet count and do not reflect the impact of mixing on fresh, functional platelets.

DISCUSSION

Component therapy has been the standard of care for blood product storage and transfusion, and a 1:1:1 ratio of RBCs, FFP, and platelets has been proposed as a guiding principle for hemostatic resuscitation since as early as 2003.[24] However, two key factors make component therapy less than ideal. First, platelets have been limited to a maximum of 5 days' storage, and while standard room temperature–stored platelets achieve sufficient recovery and survival metrics, they are deficient in coagulation function compared to their freshly collected, healthy counterparts. While RBCs and FFP have relatively long shelf lives (42 days and ≥ 1 year, respectively), platelets are often omitted from resuscitation except in well-supplied trauma centers that can afford to justify their usage from an economic, need-based, and/or waste-avoidance standpoint. Second, component separation requires processing time and equipment, relegating its practice to blood banks and specialized locations, with advanced notice required. Component therapy in austere environments is thus limited to processed-and-shipped material, further reducing functionally useable shelf life with transit time.

Cold storage of WB provides a compromise: It is easier to collect and prepare and requires only one storage modality (4 °C) versus three in classical component therapy (4 °C for RBCs, 22°C for platelets, −20°C for FFP). However, the shelf life of WB (21–35 days) is shorter than that of RBCs or FFP. But the ease of WB transfusion should not be underestimated as a benefit—transfusion of a typical massive transfusion pack of six RBC units, six plasma units, and one apheresis platelet unit requires spiking 13 bags versus eight for an equivalent volume of WB (approximately 4 L). The complexity of component therapy can limit its full implementation in staff-constrained environments.

Because fibrinogen is one of the first coagulation cascade components to be depleted in trauma,[25] the retention of clot strength as measured by the FIBTEM assay in WB throughout storage is encouraging. The importance of fibrinogen replacement in hemorrhage has been established,[26,27] although additional sources such as fibrinogen concentrates or cryoprecipitate may be necessary in conjunction with WB to restore sufficient levels.[28,29]

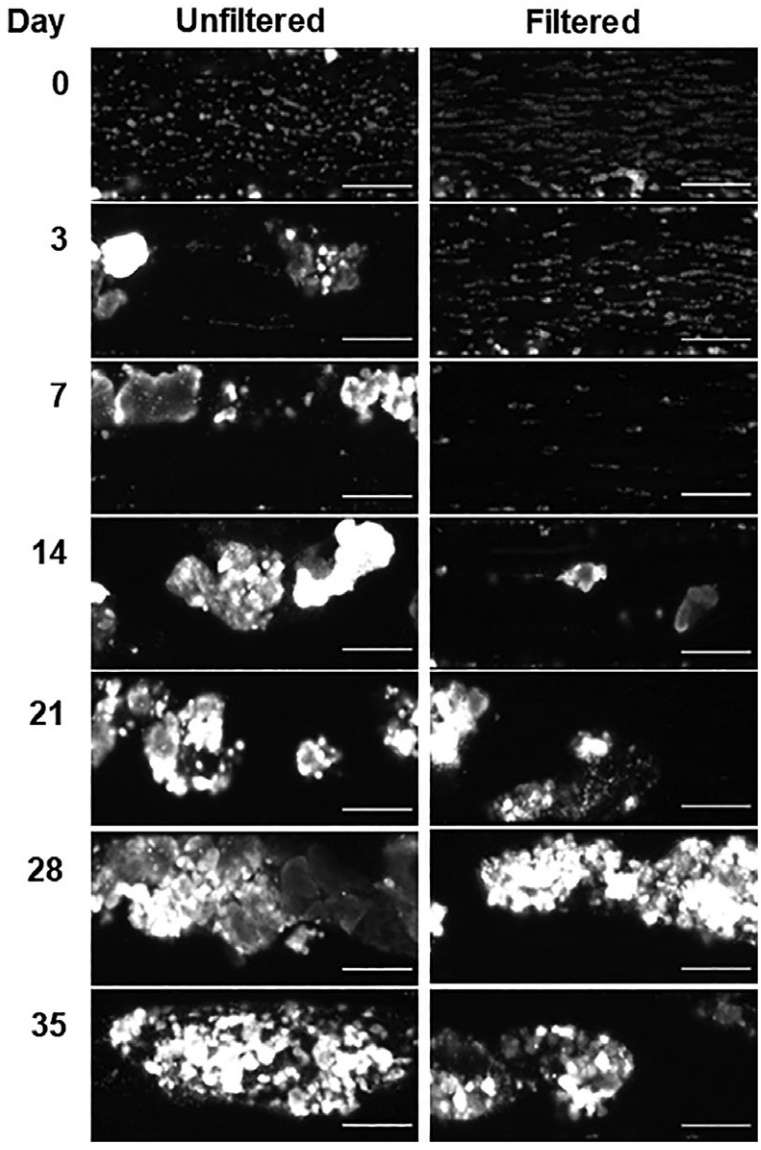

Fig. 4. Representative images of platelet adhesion to a collagen-coated surface under an arterial shear rate (920 s^{-1}) show that CPDA-1 anticoagulated blood platelets (left column) adhere in small clusters on Day 0, but larger aggregate structures are observed on following days. After standard transfusion filtration (right column), platelet adhesion occurs without large aggregate formation through Day 7. Scale bars are 200 μm.

As shown in these studies, the hemostatic capability of the platelets contained in the stored WB products declined in a manner consistent with cold-stored platelet concentrates (though this is mitigated in those stored in platelet additive solutions).[9,30] There was a reduction in platelet count accompanied by a global decline in platelet aggregation functional response. These changes to platelet number and aggregation response aligned with the appearance of the aggregated masses in the adhesion assays, suggesting that the disappearance of countable platelets was due to their participation in those aggregates. As the use of the standard transfusion filter removed these aggregates (at least for the first week after storage) but caused no change in clot strength as measured by ROTEM, it can be inferred that those aggregates are not contributing to this aspect of clot function. Unpublished studies in our lab have indicated that the visible network the aggregates are composed of consists largely of fibrin or fibrinogen, although this has not yet been positively identified; interaction of platelets with fibrinogen would result in activation and exhaustion, explaining the decline in platelet count and aggregation response.[17,30] As aggregometry was not conducted on the filtered samples, it is unknown whether the postfiltration platelets would show mean improved aggregation response compared to the population at large. However, while many platelets were "lost" in storage as observed in unfiltered blood samples, those remaining in the countable

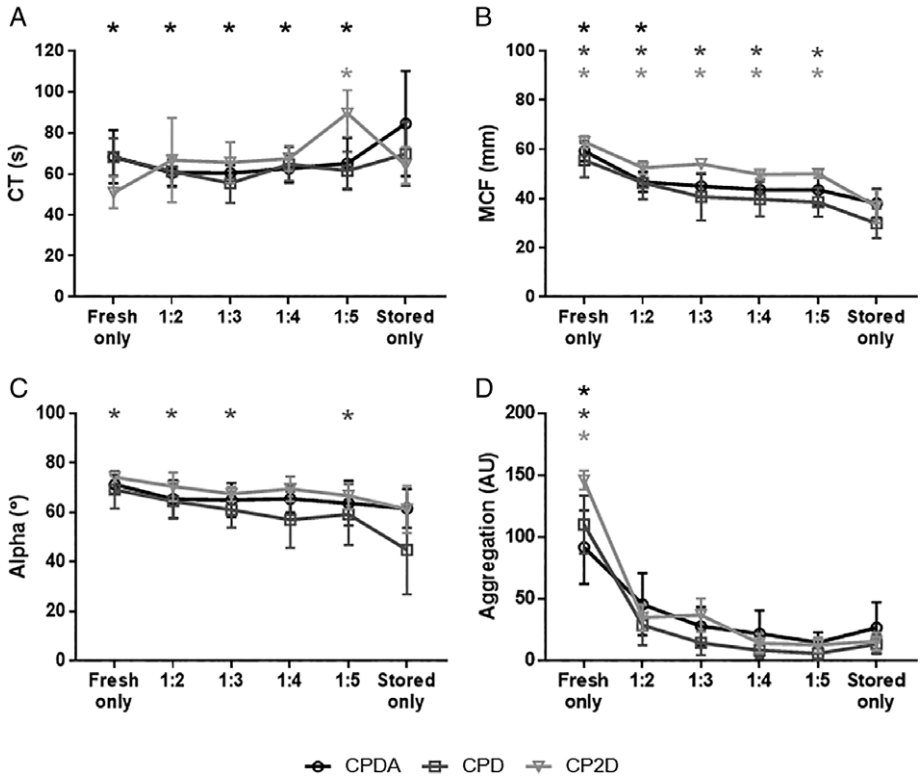

Fig. 5. FWB mixed with 35-day stored WB at the given ratios shows improvements to (A) EXTEM CT (in CPDA-1) and (B) EXTEM MCF (for all anticoagulants) with (C) slight improvement for CP2D EXTEM alpha versus 35-day stored blood. (D) Collagen-induced aggregation in 35-day stored samples is only slightly improved (not normalized to platelet count). * $p < 0.05$ versus "stored only" samples.

population maintained some aggregation and, at the very least, contributed their phospholipid surfaces as focal points for coagulation enzyme complex formation.

Platelet adhesion was also retained in some form throughout the 35-day storage period. Typically, quantifiable data is gathered from images collected in the BioFlux 1000 device (e.g., fluorescently labeled platelet intensity and area coverage), but the aggregates made this data too erratic and restricted the reporting to qualitative images. Differences in platelet adhesion between shear rates should be explored in greater detail in the future, especially using in vivo models to determine if variable wounding patterns will result in variable responses to transfusion therapy. In this study, arterial shear was shown to produce small nodes of adhesion on the collagen surface that were observed throughout the study, but these were frequently masked by aggregates in unfiltered samples. Even after filtration, only the first 7 days most closely represented what was seen in a fresh blood product's adhesion capacity. The reappearance of aggregates in filtered samples on Day 14 and beyond was possibly due to conglomeration of smaller aggregates (<200 μm) after filtering, but this was not confirmed. Regardless, the platelets contributed to clot strength throughout storage and produced much stronger clots than just RBCs and plasma, as seen by comparing the EXTEM and FIBTEM assays.

Despite the time-dependent deteriorations in platelet function, stored WB has continued to show its benefits to the bleeding patient.[31,32] Therefore, the results of these in vitro assays should not be taken as a warning against using stored WB but instead as an invitation to consider storing WB for even longer periods of time. Since very few of the measured parameters show a significant change beyond the current CPD shelf life of 21 days, further studies should be conducted to evaluate the efficacy of WB in restoring hemostasis even after 35 days of storage.

The resurgence of FWB from a "walking blood bank" as an acceptable (and oftentimes preferable) resuscitation solution in trauma, particularly in military settings, has offered additional lifesaving options, particularly compared to use of crystalloid and colloid volume replacers/expanders, which restore perfusion but drive progression of coagulopathies.[33,34] However, FWB has its own set of limitations, not the least of which is availability—particularly in cases requiring massive transfusion, which would require the rapid activation of several donors. Further, FWB cannot be fully tested for transfusion-transmitted disease prior to use. These studies suggest an alternative paradigm that may improve WB resuscitation strategies, particularly in austere settings: If a supply of cold-stored WB was maintained (using the same storage capabilities that are currently used for RBCs), even after 4 to 5 weeks of storage this blood could be used to supplement a

smaller volume of FWB, minimizing the reduced coagulation effects shown in this study. However, as mentioned previously, it should be noted that the results of these fresh and stored blood-mixing studies were not corrected for platelet count.

Finally, there appears to be little difference between CPDA-1, CPD, and CP2D anticoagulants with respect to these measured functional parameters, despite their differently mandated expirations. Because there were fewer CP2D units (n = 4) versus CPDA-1 (n = 10) and CPD (n = 7), observed differences and similarities should be reviewed in expanded studies before commitment to a specific anticoagulant. It is clear that a fresher product provides greater hemostatic functionality per these assays, particularly over the first 14 days, but these studies show no reason for CPD or CP2D to be restricted to a shorter shelf life than CPDA-1. Of course, blood has many other functions besides hemostasis (e.g., oxygen-carrying capacity), and these tests were not capable of identifying derangements to those functions that might occur as a result of extended storage.

Future work should be conducted to validate these results in vivo: models using stored blood (up to 35 days) and mixtures of fresh and stored blood will confirm or reject the prospect of considering later expiration dates and a transition from component therapy as the primary method of resuscitation.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Additional chemistry and CBC values in stored whole blood.

Fig. S1. Time required for 12 mL of stored whole blood to pass through the 200-μm filter increases over the first 7 days and then plateaus. This corresponds to the appearance of aggregates (visible under microscopy) that are seemingly restricting flow through the filter. Data shown are means with standard deviation as error bars. * $p < 0.05$ versus same sample on Day 0.

Fig. S2. PCO_2 and PO_2 fluctuate over the course of storage. Data shown are means with standard deviation as error bars. * $p < 0.05$ versus same sample on Day 0.

Fig. S3. Representative thrombograms generated at each time point for PRP and PPP collected from the different anticoagulant-stored WB samples reflect increasing thrombin generation over time.

Fig. S4. ROTEM FIBTEM (exploring only the plasma and fibrinogen contributions to clot formation) measurements in stored whole blood. Only CP2D showed significant changes over time in CT. Data shown are means with standard deviations for error bars. * $p < 0.05$ versus same sample on day 0. † $p < 0.05$ versus unfiltered sample at same time point.

Fig. S5. ROTEM INTEM (contact activation pathway) measurements in stored WB show a decrease in coagulation function over time, with increasing clotting time, decreasing maximum clot firmness, and decreasing alpha angle (rate of clotting). Data shown are means with standard deviations for error bars. * $p < .05$ versus same sample on Day 0.

Fig. S6. Additional multiplate assays of aggregation following stimulation by agonists ADP, TRAP-6, ASPI, and ristocetin demonstrate significant changes as early as Day 3 in platelets from WB, which are mitigated when normalized by platelet count (see Table 1). * $p < 0.05$ versus same sample on Day 0.

Fig. S7. With pathologically high shearing (4000 s^{-1}), platelet adhesion to a collagen-coated surface is limited beyond Day 0 in the BioFlux 1000 microfluidics platform. These representative CPDA-1 anticoagulated blood samples underwent filtration through the standard transfusion filter. Scale bars are 200 μm.

Fig. S8. With low (venous) shearing (100 s^{-1}), limited adhesion of platelets was observed over the first 14 days in the BioFlux 1000 platform. Representative filtered CPDA-1 anticoagulated WB samples show the formation of large aggregates on Day 21. Scale bars are 200 μm.

Fig. S9. Day 35-stored WB mixed with FWB shows similar trends with the INTEM assay as what was seen in EXTEM assays (see Fig. 2); FIBTEM MCF and angle were unaffected by lengthy storage, and mixing with fresh blood did not improve these parameters, but CT was shorter for CPDA-1 (with 1:5 fresh-to-stored ratio) and CPD (with 1:3 ratio) samples. * $p < 0.05$ versus 35-day stored samples ("stored only").

Fig. S10. Multiplate-measured platelet aggregation (with ADP, TRAP, ASPI, and ristocetin agonists) was not significantly improved in 35-day stored WB samples by mixing with FWB. These samples were not normalized versus platelet count. * $p < 0.05$ versus 35-day stored samples ("stored only").