Effect of leukoreduction and pathogen reduction on the hemostatic function of whole blood

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BACKGROUND: There is renewed interest in the use of whole blood (WB) for resuscitation of patients in hemorrhagic shock. Leukoreduction with platelet-sparing filters and pathogen reduction may be used to improve the safety profile of WB, yet the effects of leukoreduction and pathogen reduction on WB hemostatic function are not well characterized.

STUDY DESIGN AND METHODS: Blood from 32 healthy group O donors was divided into treatment groups (n = 8 for each group): untreated, pathogen reduced (PR^+), leukoreduced using an in-line filter (LR^+), or PR^+LR^+. Units were stored without agitation for 21 days between 1° and 6°C, with sampling on days 0 (pre- and post-treatments), 1, 3, 5, 10, 15, and 21 for hemostatic function as assessed by thromboelastometry, thrombin generation, platelet activation factors, and platelet impedance aggregometry.

RESULTS: From day 3 (D3) to D15 of storage, platelet count was reduced in PR^+/LR^+ units compared to PR^-/LR^- units. From D10 to D21 of storage, maximum clot firmness (MCF) was reduced in PR^+/LR^+ units compared to PR^-/LR^- units. From D3 to D21 of storage, platelet aggregation was reduced in PR^+/LR^+ units compared to PR^-/LR^- units. Total thrombin generation was similar in all groups from D0 to D21.

CONCLUSIONS: The combination of LR with a platelet-sparing filter and PR significantly reduces hemostatic function compared to either treatment alone or untreated WB. The clinical consequences of LR and PR of WB in patients with severe bleeding should be examined in trials before both are used in combination in patients.

There are roughly 30,000 preventable civilian deaths from hemorrhagic shock after traumatic injury per year in the United States (US).[1] Currently, most civilian trauma patients in the US will be resuscitated using component therapy. However, there has been increasing use of cold-stored, low-titer anti-A and anti-B, group O whole blood (LTOWB) to resuscitate patients with hemorrhagic shock.[2] The rationale for the preference for LTOWB compared to components includes the fact that LTOWB is a more concentrated product compared to reconstituted whole blood (WB) using components (due to increased additive solutions in each component).[3] LTOWB also contains cold platelets (PLTs) that are more hemostatically active compared to the use of PLTs stored at room temperature when components are used.[4,5] The risk of bacterial contamination of room temperature PLTs is also greatly reduced with the use of cold-stored WB. There are also less donor exposures with LTOWB compared to the use of individual blood components. LTOWB simplifies the logistics of resuscitation as it provides a balanced resuscitation fluid in one bag instead of three. This logistic benefit

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can translate into more rapid administration of each product, which may improve outcomes for patients with life-threatening bleeding.[6] This advantage is magnified for patients with hemorrhagic shock that are treated in the prehospital phase of resuscitation.

Among the unanswered questions surrounding the use of WB is whether or not it should be leukoreduced (LR) with a platelet-sparing filter. LR is required in many but not all countries for red blood cell and WB products. LR minimizes the risk of febrile reaction, cytomegalovirus (CMV) transmission, and alloimmunization, although it has not been demonstrated to improve clinical outcomes in trauma patients.[7] In addition, LR with a platelet-sparing filter can be detrimental to the platelet aggregation response of WB, and does add an additional cost to the product.[8] Thus it remains unclear if the benefits of LR of WB are offset by a potential detriment to platelet aggregation.

The risk of transfusion-transmitted diseases (TTDs) has led to the development of pathogen-reduction (PR) technologies (PRTs). Mirasol is one method of PRT that uses ultraviolet light and riboflavin to inactivate pathogens and white blood cells (WBCs).[9] It is licensed for PLTs and plasma in some countries, and it has been documented in a randomized controlled trial (RCT) to reduce the risk of transfusion-transmitted malaria.[10] For countries with mandatory LR that want to incorporate PR of WB, it is essential to determine the effect of both processing methods in combination on the hemostatic function of WB. In addition, the American Association of Blood Banks (AABB) Standards for Blood Banks and Transfusion Services permit the use of cold-stored WB in patients of unknown ABO group,[11] and its use in trauma resuscitation is increasing in the US and internationally. It is therefore of the utmost importance to examine the functional effects of alternative methods of processing WB. The effect of concomitant LR and PR on WB has not been studied; therefore, this study evaluated changes in the hemostatic function of cold-stored WB following either LR or PR, or both.

MATERIALS AND METHODS

Whole blood collection and storage

WB was collected at a local blood center (licensed by the US Food and Drug Administration) from healthy volunteers, and the protocol to do so was approved by the Washington University School of Medicine (WUSM) institutional review board. In brief, 500 milliliter (mL) of WB was collected from each of 32 donors into the Imuflex WB-SP blood bag system (#BB*LGQ506A6), which contains citrate–phosphate-dextrose (CPD) anticoagulant. WB was then transported by car (< 1 hour [h] at room temperature [RT] in a temperature-controlled medical container) to our laboratory at WUSM for further treatment and storage. Units were allocated into four different treatment groups: PR^-/LR^-, n = 8; PR^+/LR^-, n = 8; PR^-/LR^+, n = 8; and PR^+/LR^+, n = 8. Both immediately before and after treatments, 10 mL samples were aseptically drawn from each unit. The units in the control group (PR^-/LR^-) were stored on the benchtop at RT until treatment of the other units was completed. After treatment for the other three groups, and within 3 hours of collection, all 32 units were then stored between 1° and 6°C for 21 days without agitation, as previous reports have demonstrated hemostatic function of WB is not affected by agitation.[8,12]

Leukoreduction and pathogen reduction

Leukoreduction was performed using the in-line filtration system in the Imuflex WP-SB blood bag system (see Fig. S1, available as supporting information in the online version of this paper). WB from either an un-manipulated unit or an LR unit was PR using the Mirasol system. Both treatment time and the weight of the PR bag were recorded for each sample. The PR units were rested for 15 minutes (min), then a 10 mL sample was aseptically removed, and the units placed between 1° and 6°C until the next sampling time point.

Unit sampling and testing

Over the 21-day period of cold storage, units were sampled at seven different time points (D0, D1, D3, D5, D10, D15, D21). For D0 sampling, 10 mL samples were taken from units both prior to and after either PR^+ or LR^+ treatment. For each subsequent sampling, units were kept at RT for 20 minutes before sampling. Units were sampled aseptically using a collection port and a 10 mL syringe. From each 10 mL sample from a given unit: 1.5 mL was used for impedance aggregometry studies (Multiplate), 1.8 mL was used for complete blood counts (CBCs), 1.5 mL was sent for testing in the St. Louis Children's Hospital Clinical Laboratory (activated partial thromboplastin time [aPTT], fibrinogen, blood gas and critical care analyte analysis), 0.325 mL was used for rotational thromboelastometry (ROTEM) studies, and the remaining volume was used to isolate platelet poor plasma (PPP; spun at 2500*g* for 10 minutes at RT, transferred supernatant to a new tube, spun at 10000 *g* for 10 min at RT, transferred supernatant in 0.5 mL aliquots to new tubes) which was immediately stored at −80°C for batched thrombin generation studies using a calibrated automated thrombinoscope (CAT). After 10 mL samples were isolated, sampling ports were crimped, and units were placed at 4°C until the next sampling time.

Laboratory measurements

Laboratory measurements for CBC, coagulation tests (aPTT, international normalized ratio [INR], fibrinogen), blood gas, and critical care analytes were performed according to standard procedures at the St. Louis Children's Hospital Clinical Laboratory.

Impedance aggregometry

Multiplate technology (DiaPharma Group, Inc.) was used to measure platelet aggregation in response to the following

agonists: adenosine-5′-diphosphate (ADP, #08115761001, DiaPharma), arachidonic acid (ASPI, #08115826001, DiaPharma), thrombin receptor activating peptide-6 (TRAP, #08116679001, DiaPharma), and collagen (COL, #08115842001, DiaPharma). Agonists were reconstituted and the tests were performed according to the manufacturer's protocol. Data were reported as area under the aggregation curve (AUC), as well as aggregation (height of the curve), and velocity (maximum slope of the curve).

Rotational Thromboelastometry

The ROTEM *delta* Analyzer (TEM International GmbH) was used to measure clot formation in response to extrinsic pathway activation. ExTem reagent (#503-05-US, TEM Systems Inc.) was used for activation and StarTem reagent (#503-10-US, TEM Systems Inc.) was used for recalcification. All samples were assessed according to the manufacturer's instructions, and assays were run for 60 minutes. Clotting time (CT, s), clot formation time (CFT, s), alpha angle (α, °), maximum clot firmness (MCF, mm), clot firmness at 10 and 20 minutes respectively (A10, mm; A20, mm), and maximum lysis (ML, %) were recorded.

Thrombin generation

The CAT device (Diagnostica Stago Inc.) was used to measure thrombin generation in response to activation by tissue factor (TF). Assays were performed according to the manufacturer's protocol. In brief, 80 μL of thawed PPP was added to 20 μL of reconstituted PPP-low reagent (TF, 1 pM) to induce thrombin generation. Absorbance was measured at 460 nm every 20 seconds for a total of 60 minutes. Endogenous thrombin potential (ETP, nM*min), maximal thrombin generation (Peak, nM), and time to peak (Time to Peak, min) were reported.

Soluble Factor Levels

Levels of platelet factor 4 (PF4) and soluble CD40 ligand (sCD40L) in PPP were assessed by enzyme-linked immunosorbent assay (ELISA). For PF4 detection, the Human PF4 ELISA Kit (CXCL4) SimpleStep (#ab189573, Abcam) was used according to the manufacturer's instructions. For sCD40L detection, the Human sCD40L Platinum ELISA Kit (#BMS239, eBioscience – now Thermo Fisher) was used according to the manufacturer's protocol.

Statistical analysis

Data were analyzed using GraphPad Prism software (version 7.02 for Windows, GraphPad Software) as well as R 3.4.3 (2017-11-30; R Foundation for Statistical Computing, Vienna, Austria). Data are reported as median interquartile range (IQR) throughout the manuscript. Data in figures are represented with box and whisker plots indicating the median and maximum and minimum values. Comparison of a given variable at a specific time point between treatment groups was done by one-way analysis of variance (ANOVA) (Kruskal-Wallis test), followed by multiple comparison using the Bonferroni method. A p-value of less than 0.05 was considered significant. Comparison of a given variable in a specific group between each time point was performed using a t-test (Mann–Whitney test). Comparison of kinetics of one variable over time within a single treatment group was performed by one-way ANOVA (Kruskal-Wallis test). Table S1 lists p-values for one-way ANOVA tests that demonstrated significance: inter-group analyses at a given time point (D0 Pre, D0 Post, D1, D3, D5, D10, D15 D21) are on the left, and intra-group analyses over the duration of the study (PR^-/LR^-, PR^+/LR^-, PR^-/LR^+, PR^+/LR^+) are on the right. Table S2 lists p-values for intra-group Wilcoxon rank sum paired analyses between D0 Pre- and D0 Post-values.

RESULTS

Leukoreduction, not pathogen reduction, lowers white blood cell counts

Over the period of 21 days of storage, we found that neither LR nor PR changed the number of red blood cells (RBCs) in the units (see Fig. S2, available as supporting information in the online version of this paper). As expected, LR immediately depleted the number of detectable WBCs in PR^-/LR^+ and PR^+/LR^+ units, while PR^+/LR^- units had a slight decrease in WBC concentration by D21 when compared to D0 pre-treatment (Fig. 1A; statistical analyses in Tables S1 and S2). By D3 of storage, platelet numbers were different between treatment groups, and PR treatment reduced platelet counts when compared to PR^-/LR^- and PR^-/LR^+ units (PR^-/LR^- 202.5 (134–263), PR^+/LR^- 146.5 (103–188), PR^-/LR^+ 188.5 (144–305), PR^+/LR^+ 118 (93–193), one-way ANOVA $p = 0.0035$) (Fig. 1B). Of note, PR lead to significant decreases in platelet counts compared to PR^-/LR^- and PR^-/LR^+ units throughout 15 days of storage (Fig. 1B), with the same trend occurring until D21 (yet not reaching significance). Thus, while LR reduced WBC, PR had a more pronounced effect on platelet numbers.

Pathogen reduction impairs some measures of coagulation

The prothrombin time (PT) of PR^-/LR^- treated WB gradually increased over the 21-day storage period (13.1 [12–15.5] sec to 19.25 [16.5–20.4] sec; $p < 0.001$, Table S1). The same trend was observed in the INR for all four treatment groups, as might be expected due to the inherent use of the PT value in INR calculations (see Fig. S3A, available as supporting information in the online version of this paper). The same trend was again observed in aPTT measurements, with PR causing an increase in aPTT by D21 of storage when compared to PR^-/LR^- and PR^-/LR^+ units (see Fig. S3B, available as supporting information in the online version of this paper). Lastly, while PR treatment led to a gradual increase in INR and aPTT over the 21-day storage period, fibrinogen

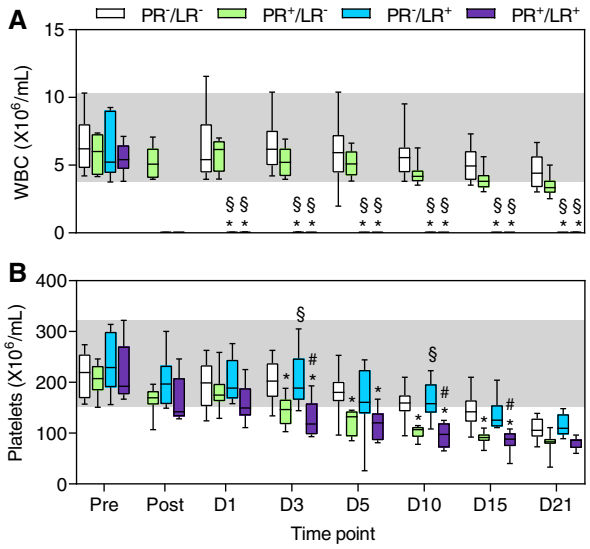

Fig. 1. Decreased white blood cells after leukoreduction. White blood cells (A) and platelets (B) were measured in samples from the designated time points using a hematology analyzer. Shaded areas indicate the entire range of all treatment group day 0 (D0) values. Significance of intergroup comparisons at each time point are denoted as follows: *, $p < 0.05$ when compared to PR^-/LR^-; §, $p < 0.05$ when compared to PR^+/LR^-; #, $p < 0.05$ when compared to PR^-/LR^+.

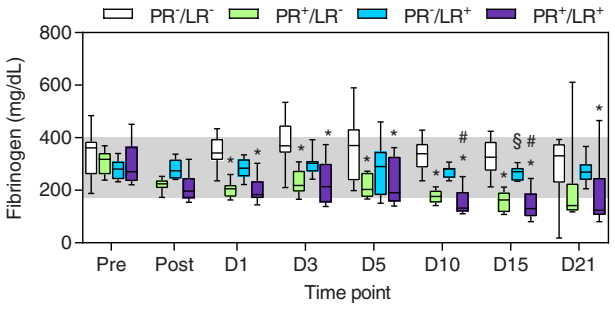

Fig. 2. Pathogen reduction reduced fibrinogen levels shortly after treatment. Coagulation tests were performed on samples drawn from the units at the given time points, and fibrinogen levels were reported. Shaded area represents reference range (170–400 mg/dL[13,14]) as dictated by Barnes-Jewish Hospital (bjhlab.testcatalog.org). Significance of intergroup comparisons at each time point are denoted as follows: *, $p < 0.05$ when compared to PR^-/LR^-; §, $p < 0.05$ when compared to PR^+/LR^-; #, $p < 0.05$ when compared to PR^-/LR^+.

concentration was decreased immediately post-treatment in response to both types of treatment, with significant differences for PR^+/LR^- and PR^+/LR^+ groups starting at D1 (Fig. 2, Table S2). Median fibrinogen levels dropped out of normal reference ranges for PR^+/LR^- and PR^+/LR^+ at D15 and D10, respectively (Fig. 2). Overall, PR, but not LR, resulted in increased INR values and diminished fibrinogen levels.

Both treatments additively affect viscoelastic measures of hemostasis

Formation of fibrin, that is, CT, increased over the 21 days of storage for all treatment groups, but there were little to no differences between treatment groups at the study time points (Fig. 3A, Table S1). In contrast, by D5 of storage PR^+/LR^+ units had increased CFT when compared to the other three treatment groups (Fig. 3B). This additive effect of PR^+/LR^+ on CFT was maintained throughout storage. At D21, either treatment alone had increased CFT (PR^+/LR^- 304.5 [255–438] s, PR^-/LR^+ 306 [260–510] s) compared to PR^-/LR^- units (187 [127–311] s), but the combination of both treatments had an additive effect on prolonged CFT (PR^+/LR^+, 356 [255–670] s) (Fig. 3B). This graded effect was also observed in measurements of MCF and alpha angle (Fig. 3C, Fig. S4A). Both PR^+/LR^- and PR^-/LR^+ units had slight decreases in MCF, yet these did not reach significance until D15 of storage, whereas PR^+/LR^+ units had significantly decreased MCF by D5, albeit still within normal range (Fig. 3C). Both treatments, either alone or in combination, resulted in decreased MCF outside of the normal range by D21 of storage (Fig. 3C). Alpha angle measurements mirrored MCF for all treatment groups (see Fig. S4A, available as supporting information in the online version of this paper). While PR or LR alone contributed to impaired hemostasis as measured by ROTEM, it was the combination of these two treatments that significantly increased CFT and diminished overall clot firmness.

Thrombin generation is not substantially impaired by time and treatment

Next we measured the effects of either treatment on thrombin generation over the course of 21 days of storage. We found that the ETP was not different between treatment groups in response to 1 pM TF (Fig. 4, Table S1). While there were no major changes within a single group over time with respect to peak thrombin generation, there were small, yet noticeable decreases between treatment groups in the peak thrombin formation in response to 1 pM TF (D3 and D15; Fig. 4B, Table S1). Time to peak generation induced by 1 pM TF was not greatly altered in response to either treatment over the entire storage period (see Fig. S5A, available as supporting information in the online version of this paper). Notably, LR appeared to affect thrombin generation more than PR, as PR^-/LR^+ and PR^+/LR^+ groups were consistently different when compared to PR^-/LR^- and PR^+/LR^- units (Fig. 4, Fig. S5, Table S1). However, it is worth noting that despite slight differences between study groups at each time point, the median ETP (i.e., total amount of thrombin generated) stayed within the range of normal values from D0 pretreatment to D21.

Platelet aggregation is impaired by leukoreduction

We found that LR played a prominent role in immediate diminished platelet aggregation in response to four different

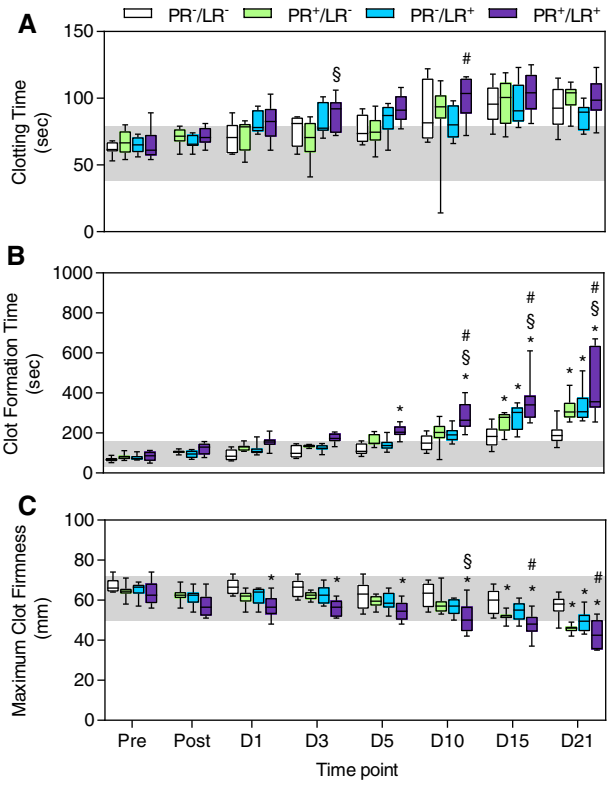

Fig. 3. Both pathogen reduction and leukoreduction impact clot formation. (A) Clotting time (CT), (B) clot formation time (CFT), and (C) maximum clot firmness (MCF), were measured by rotational thromboelastometry in samples from the designated time points. Shaded areas represent reference ranges (CT: 38–79 s; CFT: 34–159 s; MCF: 50–72 mm[15]). Significance of intergroup comparisons at each time point are denoted as follows: *, $p < 0.05$ when compared to PR^-/LR^-; §, $p < 0.05$ when compared to PR^+/LR^-; #, $p < 0.05$ when compared to PR^-/LR^+.

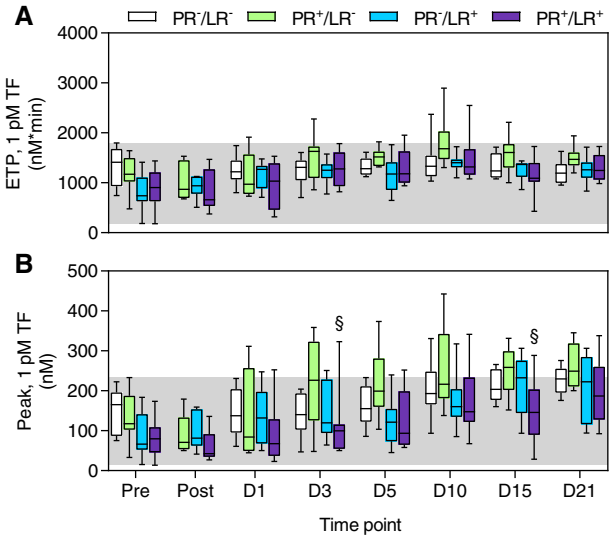

Fig. 4. Thrombin generation is not substantially impaired in response to either pathogen reduction or leukoreduction. (A) Endogenous thrombin potential (ETP) was measured in samples in response to stimulation with 1 pM of tissue factor (TF). (B) Peak thrombin generation in response to 1 pM TF. Shaded area indicates entire range of all treatment group day 0 (D0) values.

platelet agonists (Fig. 5, Table S2). Platelet activation with adenosine diphosphate (ADP) decreased over time as expected, with a median AUC of 11 (2–22) by D21 in PR^-/LR^- units (Fig. 5A, Table S1). PR^+/LR^- units had a similar decrease in platelet aggregation over time with a median AUC of 10.5 (0–18). In contrast, by D21, LR units had almost no measurable platelet aggregation in response to ADP, with PR^-/LR^+ and PR^+/LR^+ units having a median AUC of 1.5 (1–5) and 0 (0–2), respectively. This phenomenon occurred for platelet activation by collagen (Fig. 5B), thrombin receptor activating peptide 6 (TRAP, Fig. 5C), and arachidonic acid (ASPI, Fig. 5D). Of note, concomitant treatments (PR^+/LR^+) resulted in > 50% decreases in platelet aggregation by all agonists at D3 post treatment when compared to D0 pretreatment values (Mann–Whitney test; ADP, $p = 0.008$; ASPI, $p = 0.016$; TRAP, $p = 0.002$; collagen, $p < 0.001$). Interestingly, aggregation induced by ADP, collagen, or TRAP was highly sensitive to the combined treatment, as one day after treatment there was an average 63% reduction in the AUC for those tests (ADP = 59%, TRAP = 62%, collagen = 68%), whereas activation by ASPI was only impaired by a 37% reduction in AUC. Lastly, platelet aggregation in PR^+/LR^+ units was significantly impaired compared to PR^-/LR^- groups out to D10 (for collagen and ASPI) and D15 (for ADP and TRAP) of storage. These data demonstrate that LR, but not PR, is responsible for severe impairment in platelet function both immediately after treatment and for the majority of the storage period.

Either treatment does not alter soluble mediator release

To assess platelet degranulation in response to either treatment, we measured the concentration of platelet factor IV (PF4/CXCL4) and soluble CD40 L (sCD40L/sCD154) in the unit samples from all groups over the course of 21 days. By D21, levels of PF4 had plateaued at approximately 600 pg/mL, irrespective of treatment group (Fig. 6A), with an overall 65% reduction from pre-treatment levels. In contrast, sCD40L levels slowly elevated with time in all treated units, resulting in a 580% increase over pretreatment levels (Fig. 6B). These data suggest limited platelet activation within the WB unit in response to either treatment.

DISCUSSION

In spite of great strides in improving transfusion safety through donor screening and testing, there remains a residual risk for transmitting infectious diseases, hence the

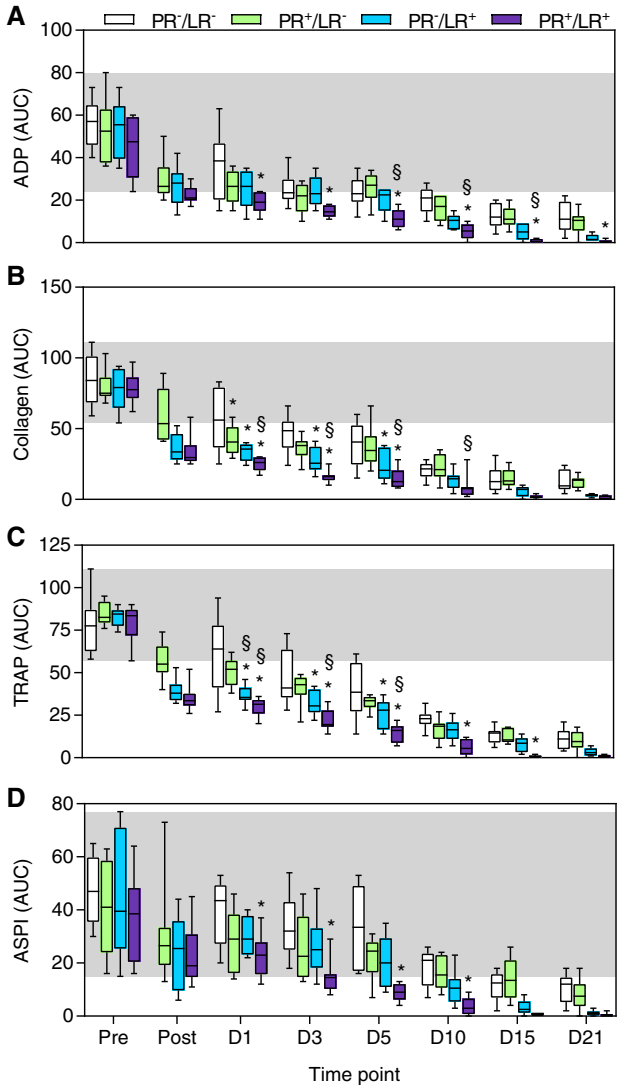

Fig. 5. Leukoreduction results in decreased platelet aggregation responses to multiple agonists. Using impedance aggregometry (Multiplate), platelet aggregation in samples at designated time points was measured after stimulation with (A) adenosine diphosphate (ADP), (B) collagen, (C) thrombin receptor activating peptide 6 (TRAP), and (D) arachidonic acid (ASPI). Data are reported as area under the curve (AUC) for each test. Shaded areas indicate entire range of all treatment group day 0 (D0) values. Significance of intergroup comparisons at each time point are denoted as follows: *, $p < 0.05$ when compared to PR^-/LR^-; §, $p < 0.05$ when compared to PR^+/LR^-.

development of PRT. For the past two decades the use of LR has become standard to reduce the risks associated with WBC in blood products. This study found that PR alone led to slightly increased aPTT values compared to PR^-/LR^- and PR^-/LR^+ groups (see Fig. S3, available as supporting information in the online version of this paper), and led to an immediate posttreatment reduction in fibrinogen levels (Fig. 2), which likely contributed to a reduction in clot formation (Figs. 3 and 4, Fig. S4). In contrast, platelet aggregation was diminished over storage time (from D0 posttreatment to D21) after PR alone, but no more so than PR^-/LR^- units (Fig. 5; $p < 0.001$ Table S1). Our findings are similar to those reported in a study by Pidcoke et al., which showed increased aPTT values and decreased platelet aggregation in response to PR of WB over a period of 21 days of storage.[16] However, there are no reports of combinatorial PR and LR on the hemostatic function of stored WB; therefore, the studies presented herein are the first to determine the impact of these two treatments concomitantly.

The effects of LR alone were as expected based on previous literature. LR led to an immediate two-fold reduction in platelet aggregation, irrespective of PR (Fig. 5), and delayed clot formation by D15 of storage (Fig. 3). Another study using the Imuflex platelet-sparing filter demonstrated LR-impaired collagen-induced platelet aggregation with limited effect on coagulation for up to 21 days of storage.[17] Furthermore, Strandenes et al. reported that clot firmness of LR WB, as measured by ExTem/ROTEM, was not significantly reduced over a period of 14 days of cold storage.[18] In contrast, Siletz et al. reported a reduction in overall clot strength and impaired thrombin generation in LR WB by D10 of cold storage.[19] Possible explanations for the differences between our study and the Siletz study are primarily due to the use of non-platelet-sparing filters by Siletz.

Of the hemostatic tests performed, platelet aggregation was the most impaired function after LR treatment when compared to untreated WB, as LR had a major impact on aggregation (Fig. 5, Table S1). This effect was seen almost immediately, as posttreatment aggregation measurements were approximately 50% decreased from the pretreatment measurements. This reduction in platelet aggregation is not explained by changes in platelet concentration, as the decrease in platelet count pre- and post-LR, while statistically significant, was less than a 1.3-fold change with < 20% coefficient of variation (Fig. 1B, Tables S1 and S2). Leukocytes are known to both activate and inhibit platelet function,[20] and therefore may be important for optimal aggregation. While Grau et al. demonstrated that neither polymorphonuclear or mononuclear leukocytes played a role in collagen-induced aggregation,[21] Faraday et al. showed leukocyte numbers positively correlated with ADP-, collagen-, and arachidonic acid-stimulated platelet aggregation.[22] Moreover, known positive feedback interactions between platelets and leukocytes (P-selectin:PSGL-1 interactions[23]; increased thromboxane production[24]) would suggest that platelet function would benefit from the presence of leukocytes. If this is the case, then LR may actually not be beneficial for supporting maximal platelet responses during transfusions associated with traumatic hemorrhage.

ROTEM-based clotting time was not immediately impaired in response to either treatment, but there was a gradual increase in CT and CFT, as well as a decrease in MCF, over the 21-day storage period (Fig. 3; statistical data

in Table S1). In contrast to gradual changes in ROTEM parameters, there were immediate 50% reductions in platelet aggregation (Fig. 5). These different assays of hemostatic function, separated as coagulation (ROTEM, thrombin generation, and INR/PTT/fibrinogen) versus aggregometry (Mulitplate), may simultaneously underrepresent and overrepresent, respectively, the hemostatic capacity of platelets stored in WB. This hypothesis may explain divergent results with platelet hemostatic function between methods: with ROTEM analysis, bioavailability but not physical structure dictates TF-induced clot formation under constant rotational force, whereas with impedance aggregometry, platelet structure and an intact cytoskeleton are necessary for platelet aggregation in response to single agonists.[25] Further studies using assays that examine platelet aggregation and thrombus formation in WB under physiological flow conditions[26,27] are required to truly measure the function of platelets in cold-stored WB. Importantly, the aggregation response of platelets to a single agonist is likely to be an insufficient measure of physiological platelet responses. Platelets receive multiple stimulatory inputs during the process of hemostasis and can alter the strength of their response based on the precise combination of signals received.[28] Further work outlining how stimulation by multiple agonists simultaneously effects platelet aggregation may highlight differences in platelet function after PR or LR.

While many groups have shown that the concentration of these soluble mediators increases with storage time in platelet concentrates,[4,29–31] there is limited information with respect to these concentrations in WB. Khan et al. measured sCD40L in human packed red blood cell units over time and found that LR resulted in decreased release of sCD40L compared to those units that were not LR.[32] These data showed that neither PR nor LR markedly altered secretion of PF4 and sCD40L (Fig. 6) over the period of 21 days of storage, suggesting minimal platelet degranulation in response to either treatment.

With respect to the combined effects of PR and LR on whole blood, we found that using both treatments led to impairment of hemostasis when compared to PR^{-}/LR^{-} units. PR^{+}/LR^{+} units had the greatest impairments in fibrinogen levels (Fig. 2), ROTEM CFT and MCF (Fig. 3), and platelet aggregation (Fig. 5). These data indicate that, whereas both PR and LR impair platelet aggregation, there is a greater impairment observed due to LR compared to PR alone, and combination of both PR and LR results in the greatest effect on platelet aggregation. Notably, combination treatment also resulted in significant impairment of hemostasis (as measured by ROTEM) by D10 and continuing to D21 of storage. These results suggest that for maximum coagulation potential, perhaps WB should not be treated by both LR and PR. In addition, the in vivo effects of these manipulations, administered both separately as well as in combination, should be studied in clinical trials.

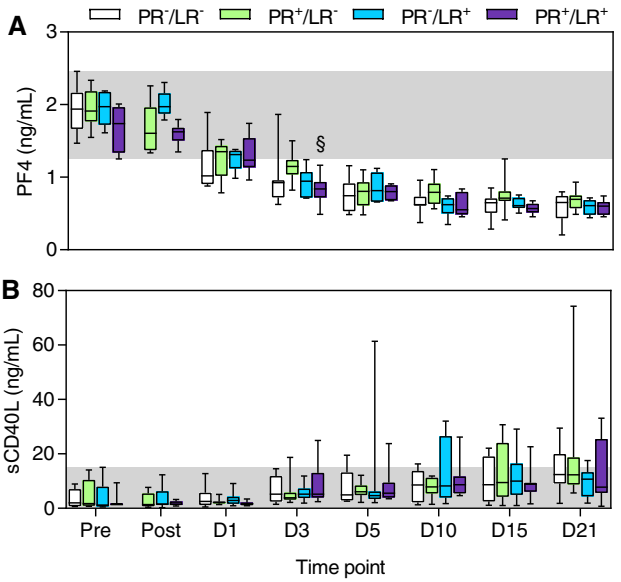

Fig. 6. Release of soluble mediators from platelets is not preferentially induced in response to either pathogen reduction or leukoreduction. ELISAs were performed to measure (A) platelet activating factor IV (PF4), and (B) soluble CD40 ligand (sCD40L), in samples from stored units at the designated time points. Shaded areas indicate the entire range of all treatment group day 0 (D0) values. Significance of intergroup comparisons at each time point are denoted as follows: §, $p < 0.05$ when compared to PR^{+}/LR^{-}.

The clinical translation of our findings needs to take into account local practice standards and patient population risk factors. In areas where LR is mandatory, it appears acceptable to use LR WB because the reduction in hemostatic function is not extreme and not consistent in all hemostatic assays. In areas where PRT is optimal due to high risk of TTD transmission, the benefit of increased safety would also appear to outweigh the potential risk of mild to moderate reduction in hemostatic capacity. Conversely, in areas where LR is not required and there is low risk of TTD, it also appears reasonable to not LR or PRT WB to retain the maximal hemostatic effect for patients with life-threatening hemorrhage. The use of both LR and PRT does have a large enough effect on reduced hemostasis that unless the risk of TTD was very high, the combined methods should be used with caution for patients with life threatening hemorrhage. If our findings are replicated by other investigators, it would be important to explore the mechanisms by which LR and PR affect platelet function and plasma-based measures of hemostasis, adding to the literature already published on PRT effects on platelet function.[33,34]

It is interesting to note that in all study groups there is a reduction in most hemostatic parameters over the 21 days of storage. According to US Food and Drug Administration regulations, WB can be cold stored out to 35 days in additive

solutions, but this is based off of RBC survival and recovery, not hemostatic efficacy. However, the majority of US trauma centers are using cold-stored WB for up to 21 days of storage,[2] presumably based on data regarding the loss of hemostatic potential over time.[16] Of note, a few programs have chosen to use the entire permitted storage duration of 35 days. Licensing criteria for the storage duration of WB should be based on functional measures of hemostasis and oxygen delivery capacity that are clinically relevant. Unfortunately, these parameters are neither clinically available nor well established in laboratory settings. In the future, if RBC and platelet functional quality metrics are established and validated, these metrics can then guide licensing of storage solutions and also should be applied to quality assessment of WB units.

CONCLUSIONS

The combination of LR with a platelet-sparing filter and PR significantly reduces hemostatic function compared to either treatment alone or untreated WB. The clinical consequences of LR and PR of WB in patients with severe bleeding should be examined in trials before both are used in combination in patients.

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DISCLAIMERS

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.

CONFLICT OF INTEREST

M.H.Y. has received an honorarium for speaking for Terumo BCT.

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SUPPORTING INFORMATION

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

Fig. S1. Leukoreduction (Imuflex) and Pathogen reduction bag setup. For leukoreduction with Imuflex system, WB was collected into the collection bag, which was gently inverted several times to mix blood, then (A) hung 85 cm $\pm$ 2.5 cm above the bench top with the primary receiving bags laying on the benchtop. (B) After filtration, the tubing was sealed below the filter, and above the primary receiving bag. For pathogen reduction, WB either from the collection bag (for the LR$^-$ groups) or the primary receiving bag (from the LR+ groups) was (C) transferred to the PRT Bag through tube fusing and gravitational flow. (D) After transfer, Riboflavin (Rf) was added to the PRT bag through gravitational flow, followed by (E) pathogen reduction in the PRT illuminator.

Fig. S2. Neither treatment alters RBC counts. Red blood cells were measured in samples from the designated time points using a hematology analyzer. Shaded area indicates entire range of all treatment group day 0 (D0) values.

Fig. S3. Pathogen reduction has an effect on coagulation. Coagulation tests were performed on samples drawn from the units at the given time points. (A) international normalized ratio (INR) and (B) activated partial thromboplastin time (aPTT) were reported. Shaded areas represent reference ranges (INR: 0.80–1.21; aPTT: 25–37 s) as dictated by Barnes-Jewish Hospital (bjhlab.testcatalog.org). Significance of intergroup comparisons at each time point are denoted as follows: *, $p < 0.05$ when compared to PR^-/LR^-; §, $p < 0.05$ when compared to PR^+/LR^-; #, $p < 0.05$ when compared to PR^-/LR^+.

Fig. S4. With time, pathogen reduction and leukoreduction impact clot formation. (A) alpha (α) angle and (B) maximum lysis (ML), were measured by ROTEM in samples from the designated time points. Shaded area represents reference range (α angle: 63–83; ML: < 15% (therefore no shading, entire y axis). Significance of intergroup comparisons at each time point are denoted as follows: *, $p < 0.05$ when compared to PR^-/LR^-; §, $p < 0.05$ when compared to PR^+/LR^-; #, $p < 0.05$ when compared to PR^-/LR^+.

Fig. S5. Time to peak thrombin generation is not substantially impaired in response to either pathogen reduction or leukoreduction. Endogenous thrombin potential (ETP) was measured in samples in response to stimulation with 1 pM of tissue factor (TF). Time to peak in response to 1 pM TF (C) was recorded. Shaded areas indicate entire range of all treatment group day 0 (D0) values. Significance of intergroup comparisons at each time point are denoted as follows: §, $p < 0.05$ when compared to PR^+/LR^-.

Table S1. Kinetic and Inter-group Statistical Analyses P values after Kruskal Wallis test either comparing groups at specific time point (Pre, Post, D1, D3, D5, D10, D15, D21) or comparing time points within a specific group (PR^-/LR^-, PR^+/LR^-, PR^-/LR^+, PR^+/LR^+). Tests are grouped by their

category: coagulation (Coag), rotational viscoelastometry (Clot), platelet aggregation by multiplate impedance (PLT Agg), thrombin generation (TG), platelet activation (release of soluble mediators) (Act), complete blood count/hematocrit (Hematocrit), blood gases/nutrients/ions (Blood Gases), and leukoreduction (LR).

Table S2. Pre and Post Treatment Statistical Analyses P values after paired Wilcoxon Rank Sum test on intra-group comparisons between Pre and Post treatment time points. Tests are grouped by their category: coagulation (Coag), rotational viscoelastometry (Clot), platelet aggregation by multiplate impedance (PLT Agg), thrombin generation (TG), platelet activation (release of soluble mediators) (Act), complete blood count/hematocrit (Hematocrit), blood gases/nutrients/ions (Blood Gases), and leukoreduction (LR).