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Hemostatic *in vitro* Properties of Novel Plasma Supernatants Produced from Late-Storage Low-Titer Type O Whole Blood

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Abstract

Background: The use of low-titer group O whole blood is increasing. To reduce wastage, unused units can be converted to packed red blood cells. Supernatant is currently discarded post-conversion; however, it could be a valuable transfusable product. The aim of this study was to evaluate supernatant prepared from late-storage low-titer group O whole blood being converted to red blood cells, hypothesizing it will have higher hemostatic activity compared to fresh never-frozen liquid plasma.

Methods: Low-titer group O whole blood supernatant (n=12) prepared on storage day 15 was tested on day 15, 21, and 26 and liquid plasma (n=12) on 3, 15, 21, and 26. Same day assays included cell counts, rotational thromboelastometry, and thrombin generation. Centrifuged plasma from units was banked for microparticle characterization, conventional coagulation, clot structure, hemoglobin, and additional thrombin generation assays.

Results: Low-titer group O whole blood supernatant contained more residual platelets and microparticles compared to liquid plasma. At day 15, low-titer group O whole blood supernatant elicited a faster intrinsic clotting time compared to liquid plasma (257 +/− 41 vs. 299 +/− 36 seconds, p=0.044), and increased clot firmness (49 +/− 9 vs. 28 +/− 5 mm, p<0.0001). Low-titer

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Conflicts of Interest: PCS is a consultant for Hemanext, Cerus, Secure Transfusion Solutions, Haima, and Co-Founder and Chief Medical Officer for Kalocyte. DJT consults for Fresenius Kabi and Rialta. MHY has given paid lectures for Terumo BCT, the manufacture of the bags into which low-titer group O whole blood units used in this study were collected, and consults for Grifols, Verax, and Hemanext. MDN consults for Haemonetics, Takeda, Instrumentation Laboratories, and is on the Scientific Advisory Board of Haima Therapeutics.

group O whole blood supernatant showed more significant thrombin generation compared to liquid plasma (day 15 endogenous thrombin potential 1071 +/− 315 vs. 285 +/− 221 nM*min, p<0.0001). Flow cytometry demonstrated low-titer group O whole blood supernatant contained significantly more phosphatidylserine and CD41+ microparticles. However, thrombin generation in isolated plasma suggested residual platelets in low-titer group O whole blood supernatant were a greater contributor over microparticles. Additionally, low-titer group O whole blood supernatant and liquid plasma showed no difference in clot structure, despite higher CD61+ microparticle presence.

Conclusions: Plasma supernatant produced from late-storage low-titer group O whole blood shows comparable, if not enhanced, *in vitro* hemostatic efficacy to liquid plasma.

Summary Statement:

Low-titer group O whole blood supernatant is currently discarded post-conversion to packed red blood cells. However, this plasma supernatant may be a valuable transfusable product, showing similar, if not enhanced, *in vitro* hemostatic properties to comparable products.

Introduction:

The use of whole blood for the resuscitation of bleeding civilian patients has recently experienced a resurgence[1]. Low-titer group O whole blood has been demonstrated to be safe in both pediatric[2] and adult[3] trauma populations, and some evidence suggests its superiority to component therapy in these patient groups as well[4,5]. At this time, over 120 institutions within the United States, and many more worldwide, have implemented low-titer group O whole blood programs for trauma resuscitation[6].

The use of low-titer group O whole blood poses some challenges to the transfusion service. Low-titer group O whole blood collected in citrate phosphate dextrose has a 21-day shelf life (or 35-day shelf life in citrate phosphate dextrose adenine), expiring sooner than packed red blood cells in additive or adenine-containing solutions or plasma that is not frozen (liquid plasma)[7], and has the potential for high wastage due to its singular indication for use in hemorrhaging patients[8]. Additionally, pre-storage leukoreduction is only available for collection in citrate phosphate dextrose, therefore limiting leukoreduced units to a 21-day shelf life. These factors, as well as concerns regarding the function of the platelets in low-titer group O whole blood that has been stored for longer than two weeks[9], contribute to product wastage, which has become an increasingly salient concern in the era of pandemic-induced blood shortages[10,11].

Considering blood shortage, it is critical to utilize a whole blood unit in a cost-effective manner. One strategy adopted by several institutions is to convert unused low-titer group O whole blood units to packed red blood cell units, which have broader indications for use[12,13]. This conversion process may occur after 14 days of low-titer group O whole blood storage. Centers that prefer to keep low-titer group O whole blood through the end of storage (21 days in citrate phosphate dextrose or 35 days in citrate phosphate dextrose adenine) would therefore not consider this conversion process since the red blood cells could no longer be used. This conversion practice also results in the creation of a plasma supernatant

that is currently discarded, despite its status as a transfusable product (liquid plasma) per US blood banking regulations. A unit of liquid plasma is currently separated from whole blood any time from collection to 5 days after the whole blood expires. Therefore, with a 21-day shelf life of whole blood collected in citrate phosphate dextrose, never-frozen liquid plasma can remain in contact with red blood cells and platelets for up to 26 days and is a transfusable product up to day 26 post collection. In the interest of maximizing the benefit of each unit of unused low-titer group O whole blood, the hemostatic and biochemical properties of low-titer group O whole blood supernatant (whole blood supernatant) were compared to liquid plasma. We hypothesized that whole blood supernatant, compared to liquid plasma, would have enhanced hemostatic efficacy and coagulation parameters without increasing free hemoglobin due to longer storage conditions in the presence of platelets and red blood cells.

Materials and Methods:

Blood Products and Sampling

All blood products were obtained from a local U.S. Food and Drug Administration-accredited blood collector (Vitalant; formerly Institute for Transfusion Medicine). This protocol was approved by the University of Pittsburgh Institutional Review Board. The University of Pittsburgh Institutional Review Board has deemed research on donated blood products to be not human subjects research as donor is not directly identifiable by the research team. Donations were collected from eligible volunteer donors in accordance with the standards of the Association for the Advancement of Blood and Biotherapies. All group O whole blood units were collected into citrate phosphate dextrose anticoagulant. The whole blood was leukoreduced using an in-line platelet-sparing leukoreduction filter (Immuflex, Terumo, Somerset, NJ, USA). The whole blood units were then stored between 1–6°C without agitation for up to 14 days in monitored refrigerators. If unused on day 15, the whole blood supernatant, produced via gravity sedimentation, was expressed into a collection bag (Fenwal, Lat Zurich, IL, USA) and sent to the research laboratory in a validated, ice-containing cooler that maintained the ambient air temperature between 1–10°C. The supernatants (N=12) were received for testing on storage day 15, and were stored for the duration of the study at 1–6°C without agitation. Sample size was determined based on unit availability and deemed sufficient based on the coefficient of variation of assays performed. Figure 1A depicts study workflow. The supernatant units were aseptically sampled for the assays described below on storage days 15, 21, and 26. The control group for the experiments were group-O fresh, never-frozen liquid plasma units (N=12) that were prepared by centrifugation from whole blood collected in citrate phosphate dextrose, stored at 1–6°C, and received for testing on storage day 3. Plasma units were stored for the duration of the study at 1–6°C without agitation and aseptically sampled for the assays described below on storage days 3, 15, 21, and 26.

All spiking and sampling of blood products took place within a BSL-2 certified biological safety cabinet using aseptic technique. Following spiking using a needle-free bag spike adapter (Covetrus, Portland, ME, USA), all exposed connections were covered with plastic sealing film (Parafilm, Bemis, Sheboygan Falls, WI, USA). Sampling was performed by

slow, gentle aspiration using 5 mL and 10 mL syringes after vigorously disinfecting the luer-lock port with an alcohol pad. A total of 12mL was sampled per unit at each timepoint, with 3 mL used for same-day assays. The remaining 9 mL was centrifuged twice at 2,500 xg for 15 min at room temperature to remove residual platelets, after which the platelet poor plasma was aliquoted and frozen at −80°C.

Cell Counts

Erythrocyte, leukocyte and platelet counts, and mean platelet volume, were determined in freshly sampled units using the ADVIA 2120i hematology system (Siemens Medical Solutions USA, Inc., Malvern, PA, USA).

Rotational Thromboelastometry (ROTEM)

Hemostatic properties of whole blood supernatant and liquid plasma units were measured via viscoelastic testing performed using rotational thromboelastometry (ROTEM) (Werfen North America, Bedford, MA, USA) on freshly sampled units according to the manufacturer's whole blood EXTEM and INTEM protocol (000503–05-US and 000503–02-US respectively). Briefly, an aliquot of sample at 37°C was recalcified with STAR-TEM solution and exposed to either EXTEM or INTEM solution to initiate coagulation via the extrinsic or intrinsic coagulation pathway. Parameters measured include time to initiation of clot to an amplitude of 2 mm ("clotting time"; s), time until clot firmness reaches an amplitude of 20 mm ("clot formation time"; s), the maximal clot amplitude achieved ("maximum clot firmness"; mm), angle between the horizontal axis and tangential line through the 2 mm amplitude point ("alpha", °), and percentage of maximum clot firmness present at 60 min ("lysis index-60"; %). Samples were run until determination of lysis index-60. If a sample did not produce a clot formation time, the clot formation time value was recorded as 3600 s.

Conventional Coagulation Assays

A Stago Compact Max device (Diagnostica Stago, Inc., Asnieres-sur-Seine, France) was used to measure prothrombin time (PT), activated partial thromboplastin time (aPTT), Clauss fibrinogen, and factor V, factor VII, factor VIII, antithrombin (ATIII), and protein S activity levels. All assays, including STA-Neoplastine CI Plus 10 (00667), STA-PTT Automate 5 (00595), STA-Fibrinogen 5 (00674), STA-Deficient V (00744), STA-Deficient VII (00743), STA-Deficient VIII (00725), STA-Stachrom AT III (00596), and STA-Staclot Protein S (00746), were performed according to the manufacturer's instructions on platelet poor plasma samples.

Thrombin Generation Assay

Thrombin generation was measured using the calibrated automated thrombogram (CAT; Diagnostica Stago) and the manufacturer's instructions for determination of thrombin generation in both freshly-sampled (utilizing the platelet rich plasma reagent without a source of exogenous phospholipids) and frozen platelet poor plasma (utilizing the platelet poor plasma reagent)[14]. Briefly, 80 μL of sample was mixed with 20 μL reconstituted platelet rich plasma or platelet poor plasma reagent, as appropriate, and mixed with 20

μL FluCa solution containing calcium and fluorogenic substrate just prior to measurement. Three technical replicates were performed for each sample. Absorbance was measured at 460 nm every 20 s for a total of 60 min. Lag time (min), endogenous thrombin potential (nM*min), maximal thrombin generation ("Peak", nM), and time to peak ("Time to Peak", min) were reported.

Microparticle Detection, Enumeration, and Characterization

Microparticle concentrations and particle size were measured in PPP samples with nanoparticle tracking analysis using the NanoSight NS300 platform (Malvern Panalytical, Malvern, UK). Individual samples were vortexed and diluted 1:1000 in sterile phosphate buffered saline to a total volume of 1 mL and loaded into the instrument's syringe pump. The syringe pump was set to a speed of 1000 μL/min and 400 μL was advanced through the fluidics to ensure the sample was in the optical cell. The syringe pump speed was reduced to 30 μL/min and the automatic focus tool was used to set analysis-optimal camera parameters including focus, gain, and camera level. Three 30-second video captures were recorded for each sample and particle sizes and concentrations were determined by nanoparticle tracking analysis software analysis of light scattering and Brownian motion. Final sample concentrations were calculated by multiplying nanoparticle tracking analysis reported concentrations by the dilution factor.

Cellular origin of microparticles was determined by flow cytometry. Flow cytometry was performed on platelet poor plasma samples using a NovoCyte 3000 3-laser flow cytometer (Agilent, Santa Clara, CA, USA). Briefly, samples were prepared by incubating 100 μL of platelet poor plasma with 100 μL of 1X annexin binding buffer (BD Biosciences, Franklin Lakes, NJ USA) and 2 μL each of allophycocyanin (APC)-CD41a, phycoerythrin (PE)-CD235a, and Brilliant Violet 421 (BV421)-Annexin V (all fluorescent tags obtained from BD Biosciences). Samples were incubated at room temperature in the dark for 20 min prior to addition of 300 μL of annexin binding buffer to terminate the reaction immediately before being analyzed. Megamix-plus SSC beads (Diagnostica Stago, Asnieres-sur-Seine, France) were used in accordance with manufacturer instructions to set appropriate scatter threshold settings for the detection of microparticles. Compensation was performed daily using UltraComp eBeads (Invitrogen, Waltham, MA, USA) according to manufacturer instructions (a BV421-CD41a antibody was used for BV421 compensation). 20 μL of each prepared sample was run at slow aspiration speed (14μL/min) with 2 rinse cycles and 1 agitation cycle between each individual run to ensure representative sampling. Gating was established by comparing positive and negative control samples and was applied uniformly without alteration once determined. All flow cytometry data analysis was performed using NovoExpress software (Agilent).

Plasma Free Hemoglobin Measurement

Plasma free hemoglobin was quantified in platelet poor plasma samples using a commercial colorimetric assay (Hemoglobin Colorimetric Detection Kit, Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The kit specifies a lower limit of detection of 21 mg/dL. Briefly, aliquots of hemoglobin standard or sample platelet poor plasma were plated on a 96 well plate and incubated with hemoglobin detection reagent for

30 min prior to measuring absorbance at 560 nm using a plate reader. Sample concentrations were calculated using a curve fit from the absorbance of serially diluted hemoglobin standards.

Clot Structure Analysis

Clot structure was analyzed via confocal microscopy (Olympus Fluoview 3000). Platelet poor plasma samples were incubated with 5 mM $CaCl_2$, 0.1 mg/mL Alexa-Fluor 488-labeled fibrinogen for visualization (Thermos Fisher Scientific), and 0.5 U/mL thrombin (Chrono-log Corp.). Clots were formed between a glass slide and coverslip and were allowed to polymerize for at least 3 hours prior to imaging. At 60x magnification, z-stacks of 4.5 μm thickness and 7 slices with a step size of 0.75μm were acquired. ImageJ was utilized to create 3D projections and binary images and quantify fiber density (black to white pixel ratio) from 3 z-stack images per clot.

To visualize microparticle incorporation, Alexa-Fluor 647 anti-human CD61 antibody (Biolegend 336407) antibody was added to PPP samples (2 uL antibody per 50 uL sample) in addition to 5 mM $CaCl_2$, 0.1mg/mL Alexa-Fluor 488-labeled fibrinogen, and 0.5 U/mL thrombin. Clots were prepared and imaged as above. ImageJ was utilized to create 3D clot projections and microparticles were quantified from 3 z-stack images per clot via ImageJ Particle Analysis.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9 (GraphPad, San Diego, CA). All hypothesis testing was two-tailed. Data were analyzed using a mixed-effects model using a restricted maximum likelihood (REML) method. Pairwise comparisons were performed for estimated cell means using Šidák correction (m=4) for multiple testing. The fixed effects in the model were storage age and product type, and a random intercept was fitted for variation due to the specific unit. The random effects were retained in the model for all analyses. Sphericity was not assumed and therefore the Geisser-Greenhouse correction was used. The significance threshold was $p < 0.05$. All data are presented as arithmetic mean ± standard deviation. Mean difference and 95% confidence interval (CI) of difference is report for critical interpretations as (mean difference [95% CI]).

Results:

Cell counts, microparticle characterization, and free hemoglobin

The residual platelet concentration in whole blood supernatant was significantly higher than in liquid plasma at day 15 (mean difference: 52 [95% CI: 20, 83] $\times 10^3$ cells) and day 21 (mean difference: 23 [95% CI: 2, 44] $\times 10^3$ cells), though the differences were not significant at day 26 (mean difference: 17 [95% CI: −2, 37) $\times 10^3$ cells) (Figure 1). No significant differences were observed between groups in terms of red blood cell concentration, and leukocyte concentration was significantly lower in whole blood supernatant units at day 15 and 26 (Figure 1).

Whole blood supernatant had significantly higher total microparticle concentrations than liquid plasma at day 15 (mean difference: 1.36 [95% CI: 0.32, 2.40] $\times 10^{11}$ per mL), day 21 (mean difference: 1.35 [95% CI: 0.17, 2.58] $\times 10^{11}$ per mL), and day 26 (mean difference: 1.70 [95% CI: 0.61, 2.79] $\times 10^{11}$ per mL) (Figure 2). The whole blood supernatant mean microparticle size was significantly larger at day 15 and day 26 compared to liquid plasma microparticles (Figure 2). Flow cytometry results demonstrated that the microparticle population in both groups consisted of CD235a+ (red cell derived), CD41+ (platelet derived), and phosphatidylserine+ microparticles. However, significantly more phosphatidylserine+ CD41+ microparticles were present in whole blood supernatant compared to liquid plasma at day 15 (mean difference: 4.64 [95% CI: 0.84, 8.43] %), day 21 (mean difference: 4.46 [95% CI: 0.56, 8.35] %), and day 26 (mean difference: 4.25 [95% CI: 0.29, 8.21] %). No significant difference was found in phosphatidylserine+ CD235a+ microparticles between whole blood supernatant and liquid plasma at any point during storage: day 15 (mean difference: 1.69 [95% CI: −1.86, 5.24] %), day 21 (mean difference: 1.38 [95% CI: −2.01, 4.78] %), or day 26 (mean difference: 1.33 [95% CI: −2.44, 5.09] %) (Figure 3).

Plasma free hemoglobin was not significantly different between the whole blood supernatant and liquid plasma groups at day 15 (137.0 ± 91.8 versus 90.0 ± 48.3 mg/dL, p = 0.354), day 21 (127.6 ± 86.2 versus 100.7 ± 64.2 mg/dL, p = 0.783), or day 26 (124.9 ± 86.4 versus 96.7 ± 62.6 mg/dL, p = 0.751) (Supplemental Figure 1).

Rotational thromboelastometry (ROTEM)

Liquid plasma group contained four samples (two at day 15, one at day 21, and one at day 26) that did not obtain an EXTEM clot formation time and five samples (one at day 15, two at day 21, and two at day 26) that did not obtain an INTEM clot formation time. These data were recorded as an assay cap of 3600 s. While there were no between-group differences seen in the extrinsic pathway clotting time during storage, the whole blood supernatant had a significantly shorter intrinsic pathway clotting time at day 15 (mean difference: −42 [95% CI: −83, −1] s), which was not conserved at day 21 (mean difference: −17 [95% CI: −71, 36] s) or day 26 (mean difference: −14 [95% CI: −64, 36] s), (Figure 4). The coefficient of variation percent was 16% for whole blood supernatant and 12% for liquid plasma for intrinsic pathway clotting time at day 15. Day 15 whole blood supernatant also had significantly higher maximum clot firmness with both intrinsic (mean difference: 21 [95% CI: 13, 29] mm) and extrinsic (mean difference: 7 [95% CI: 1, 14] mm) pathways, though this significant difference persisted to day 21 with the intrinsic pathway only (mean difference: 9 [95% CI: 1, 17] mm). The coefficient of variation percent for intrinsic maximum clot firmness was 19% for whole blood supernatant and 19% for liquid plasma at day 15. At day 26, there were not significant differences in maximum clot firmness between groups. Extrinsic lysis index-60 was significantly lower in day 15 whole blood supernatant compared to liquid plasma, indicating greater clot lysis in the supernatant group. However, no differences were seen at day 21 or 26, nor were any significant between-group differences observed for intrinsic lysis index-60. Additionally, no significant differences were observed for the intrinsic clot formation time and alpha, as well as the extrinsic clot formation time and alpha.

Conventional coagulation assays and clotting factor levels

There was a small but statistically significant increase in the PT in whole blood supernatant that persisted at day 15 (mean difference: 1.2 [95% CI: 0.3, 2.0] s), day 21 (mean difference: 1.3 [95% CI: 0.1, 2.6] s), and day 26 (mean difference: 1.6 [95% CI: 0.4, 2.7] s) (Figure 5). No significant between-group differences were seen at the different storage days in aPTT, fibrinogen, factor VII activity, and factor VIII activity. Factor V activity was not statistically different between groups at day 15 (mean difference: −14 [95% CI: −29, 1] %), but was significantly lower in whole blood supernatant at day 21 (mean difference: −18 [95% CI: −32, −4] %) and day 26 (mean difference: −19 [95% CI: −31, −6] %). There were no between-group differences in the activity of endogenous anticoagulant proteins ATIII and protein S at any point during storage (Figure 5). Coefficient of variation percent for day 15 PT and aPTT was 7% and 6% respectively for whole blood supernatant and 4% and 8% respectively for liquid plasma.

Thrombin generation

Thrombin generation includes missing data for liquid plasma (PRP reagent) traces, Lag time, ETP, Peak, time to peak (one at day 21 and one at day 26), as well as missing data for liquid plasma (PPP reagent) traces, Lag time, ETP, Peak, time to peak (one at day 26) due to machine error. The thrombin generation curves and the derived parameters for both whole blood supernatant and liquid plasma (with platelet rich plasma reagent used on same-day samples and platelet poor plasma reagent on banked plasma samples) are shown in Figure 6. For same-day whole blood supernatant and liquid plasma samples, endogenous thrombin potential was significantly higher in whole blood supernatant at day 15 (mean difference: 785.9 [95% CI: 495.0, 1076.0] nM*min), day 21 (mean difference: 643.6 [95% CI: 282.2, 1005.0] nM*min), and day 26 (mean difference: 473.9 [95% CI: 129.6, 818.2] nM*min). Thrombin peaks were similarly significantly higher in whole blood supernatant compared to liquid plasma. Kinetic parameters of the thrombin generation reaction (lag time and time to peak) were significantly lower in whole blood supernatant at day 15 and day 21 compared to liquid plasma, indicating more rapid reaction progress.

For banked platelet poor plasma samples, endogenous thrombin potential was not significantly different between the groups at days 15 (mean difference: 174.9 [95% CI: −74.1, 423.9] nM*min) and day 21 (mean difference: 93.2 [95% CI: −110.6, 296.9] nM*min), though endogenous thrombin potential was significantly higher in whole blood supernatant at day 26 (mean difference: 256.7 [95% CI: 39.0, 474.3] nM*min). Lag time was prolonged in the whole blood supernatant samples but between-group differences did not reach significance. Peak and time to peak were not significantly different between groups.

Clot structure analysis

A subset of whole blood supernatant (n=6) and liquid plasma (n=6) units were evaluated for clot structure analysis. Clots made from whole blood supernatant and liquid plasma platelet poor plasma samples had similar fibrin network structure and whole blood supernatant clots contained significantly more CD61+ microparticles within the clot network at all storage days (Figure 7). Quantitatively, the clot density of the samples, measured by the ratio of

fibrin fibers to background, was not significantly different between groups at any storage day.

Discussion:

In this study, we characterized whole blood supernatant salvaged from whole blood units. Whole blood supernatant showed improved *in vitro* hemostatic properties over never-frozen liquid plasma. Furthermore, increased residual platelet and microparticle concentrations were found in whole blood supernatant units without a concomitant increase in the free hemoglobin concentration. In viscoelastic tests, while whole blood supernatant did have increased clot lysis compared to liquid plasma at day 15, we posit this is inconsequential as liquid plasma at processing showed similarly increased clot lysis compared to later storage days, and both products appear to stabilize with respect to lysis over the duration of storage.

These findings are concordant with another comprehensive evaluation of plasma quality in whole blood performed by Huish and colleagues[15], in which platelet-rich whole blood, platelet-depleted whole blood, and liquid never-frozen plasma were stored over 35-days, sampled intermittently, and derived sample plasma tested *in vitro*. Viscoelastic parameters were improved in the plasma in the whole blood group, and thrombin generation parameters were also improved in plasma from both whole blood products compared to liquid plasma. They also identified a kinetic decrease in factor V activity specific to platelet-containing whole blood units, which was likely due to factor V sequestration on the surface of platelet microparticles. Our data support this by also demonstrating the late decrease in factor V activity while simultaneously showing elevated levels of microparticles in the whole blood supernatant samples.

In this study, liquid plasma was chosen as a comparator instead of frozen plasma products to control for potential differences in manufacturing and storage. Thawed plasma is suitable for clinical use for 5 days after thawing per Association for the Advancement of Blood and Biotherapies standards, while liquid plasma can be stored between 1–6°C for up to 26 days if collected in citrate phosphate dextrose or 40 days if collected in citrate phosphate dextrose adenine. While thawed plasma is more commonly used in the clinical setting, there is evidence suggesting that never-frozen liquid plasma has better *in vitro* hemostatic parameters[16,17]. A propensity score-matched analysis of the Trauma Quality Improvement Project (TQIP) database also demonstrated a faster time-to-transfusion when never-frozen liquid plasma over frozen plasma was used in the resuscitation of severely-injured trauma patients[18]. Whole blood supernatant, similar to never-frozen plasma, would be advantageous in the clinical setting as units would be immediately availabile. Future studies should examine various processing techniques for whole blood supernatant units, in addition to the sedimentation process used here, to evaluate how manipulation of the whole blood unit might influence clotting parameters pre- and post- separation. The findings in this study may also suggest, however, that it could be advantageous to store whole blood for the entirety of its shelf life, even if whole blood hemostatic function degrades over time, because whole blood supernatant units are comparable to, if not better than, to liquid plasma hemostatically. For centers with little blood product waste, separating a whole blood unit into components only to transfuse it later as red blood cells, a plasma product, and added platelets in a 1:1:1

transfusion strategy, consideration should be made not to convert low-titer group O whole blood, and transfuse it as whole blood over its entire shelf life, adding additional platelets as necessary.

An important difference between the whole blood supernatant and the never-frozen liquid plasma units studied here is the use of a platelet-sparing leukoreduction filter for preparation of whole blood units. At our blood collection center, whole blood units are routinely leukoreduced, while liquid plasma does not pass through a leukoreduction filter, potentially resulting in the higher leukocyte concentrations observed in the liquid plasma units. We do not suspect that cell death is responsible for reduced WBC concentration in supernatant units compared to liquid plasma. Leukoreduction however could play a role in microparticle production. Many groups have published on the deleterious effects of non-platelet-sparing leukoreduction as it pertains to whole blood coagulation parameters, with processes that also remove platelets having the most pronounced effects on global hemostasis[19,20]. Our group and others have also noticed minor impairment of platelet function in the 14–21 day old units in the setting of platelet-sparing leukoreduction[21–23], though some reports have not noted an impact of global metrics of *in vitro* hemostasis[24]. There are comparatively few studies evaluating the impact of modern platelet-sparing leukoreduction on plasma quality, though recent work by Tan and colleagues identified a significant amount of leukocyte cellular debris in liquid plasma units obtained from a regional blood center, and showed significant increases in interleukin-6, a surrogate marker for inflammation, when plasma from these units was incubated with fresh whole blood[25]. An excess of leukocyte cellular debris in plasma products may contribute to immunomodulation, and it has been conjectured that leukoreduction of plasma may produce a more immunologically neutral product[26].

This study found that liquid plasma microparticles were smaller compared to microparticles in whole blood supernatant, potentially due to the presence of extracellular vesicles or differences in cellular origin since liquid plasma had significantly less platelet-derived microparticles[27]. Increased platelet-derived microparticles in whole blood supernatant are likely due to increased residual platelets in these units and/or prolonged storage in the presence of platelets. Various mechanisms may contribute to the formation of platelet-derived microparticles, such as inevitable platelet activation during unit production and storage. The contributions of platelet-derived microparticles to hemostasis are well documented[28,29]. Comprising the majority of circulating subcellular particles, platelet-derived microparticles can express platelet receptors such as CD62P, CD42B, and glycoprotein IIb/IIIa, which allow interaction with both platelets and other microparticles. Furthermore, they disproportionately express phosphatidylserine in their membranes, which confers procoagulant properties as the anionic lipids form a scaffold for the X-ase and prothrombinase complexes which propagates thrombin generation[30,31]. Furthermore, both platelet- and erythrocyte-derived microparticles can initiate intrinsic-pathway coagulation via a factor XII-dependent mechanism[32,33]. While the preponderance of microparticles in the whole blood supernatant units may explain the improvements in thrombin generation and viscoelastic parameters compared to the liquid plasma units, one important consideration for clinical implementation of these whole blood supernatants is the potential for thrombotic complications. Platelet-derived microparticles can enhance venous thrombus formation, reducing bleeding time and increasing thrombus weight, in murine models[28] and

higher platelet-derived microparticle levels were associated with venous thromboembolic disease in one observational study[34]. While whole blood supernatant may have important hemostatic benefits in the actively bleeding trauma patient, this population is also notably hypercoagulable in the days to weeks following the initial injury[35]. Future work should aim to quantify the relative risk of thromboembolic complications with whole blood supernatant plasma compared to standard liquid plasma or FFP.

This study has several important limitations. First, this was an *in vitro* hemostatic function study conducted under static (i.e., non-flow) conditions, which does not completely account for the hemostatic contributions of vascular components and von Willebrand Factor (vWF) interactions, but is an important aspect in understanding a transfusion product's native function. In future studies, it will be critical to evaluate *in vivo* hemostatic efficacy of whole blood supernatant, in addition to evaluating the safety profile with thrombotic and immune risks at multiple storage durations. We are also pursuing testing in more advanced and novel ex vivo benchtop assays to further the capability of assessing transfusion efficacy in a high-throughput fashion. Another limitation was an inability to directly compare the two groups at early-storage timepoints (prior to day 15) as the whole blood units were in clinical use during that timeframe. However, since this study was designed to replicate their intended clinical use, whole blood supernatant production after 14 days of whole blood storage replicates real-world practice and thus would be the earliest timepoint that a supernatant would be clinically used. Variability in whole blood supernatant products due to separation technique was a potential limitation in this study. However, coefficient of variance percent from functional metrics measured at storage day 15 showed acceptable coefficient of variation less than 20% in the whole blood supernatant products. Perhaps most significantly, this study was limited in its ability to evaluate donor-specific variation. It is known that there is significant heterogeneity in blood donor characteristics and that this accounts for a nontrivial proportion of the variation between blood product parameters[36]. However, this study was not designed to evaluate for these differences, and granular donor data was not available for analysis.

Conclusion

Low-titer group O whole blood supernatant meets the current Association for the Advancement of Blood and Biotherapies standards for transfusion. Plasma supernatants produced during the conversion of low-titer group O whole blood to packed red blood cell units have improved thrombin generation and viscoelastic parameters relative to never-frozen liquid plasma units of similar storage age. These *in vitro* hemostasis findings occur in the context of higher residual platelet concentrations and microparticle concentrations in whole blood supernatant units. Clotting factor activity, clot structure analysis, and free hemoglobin generally did not differ between liquid plasma and whole blood supernatant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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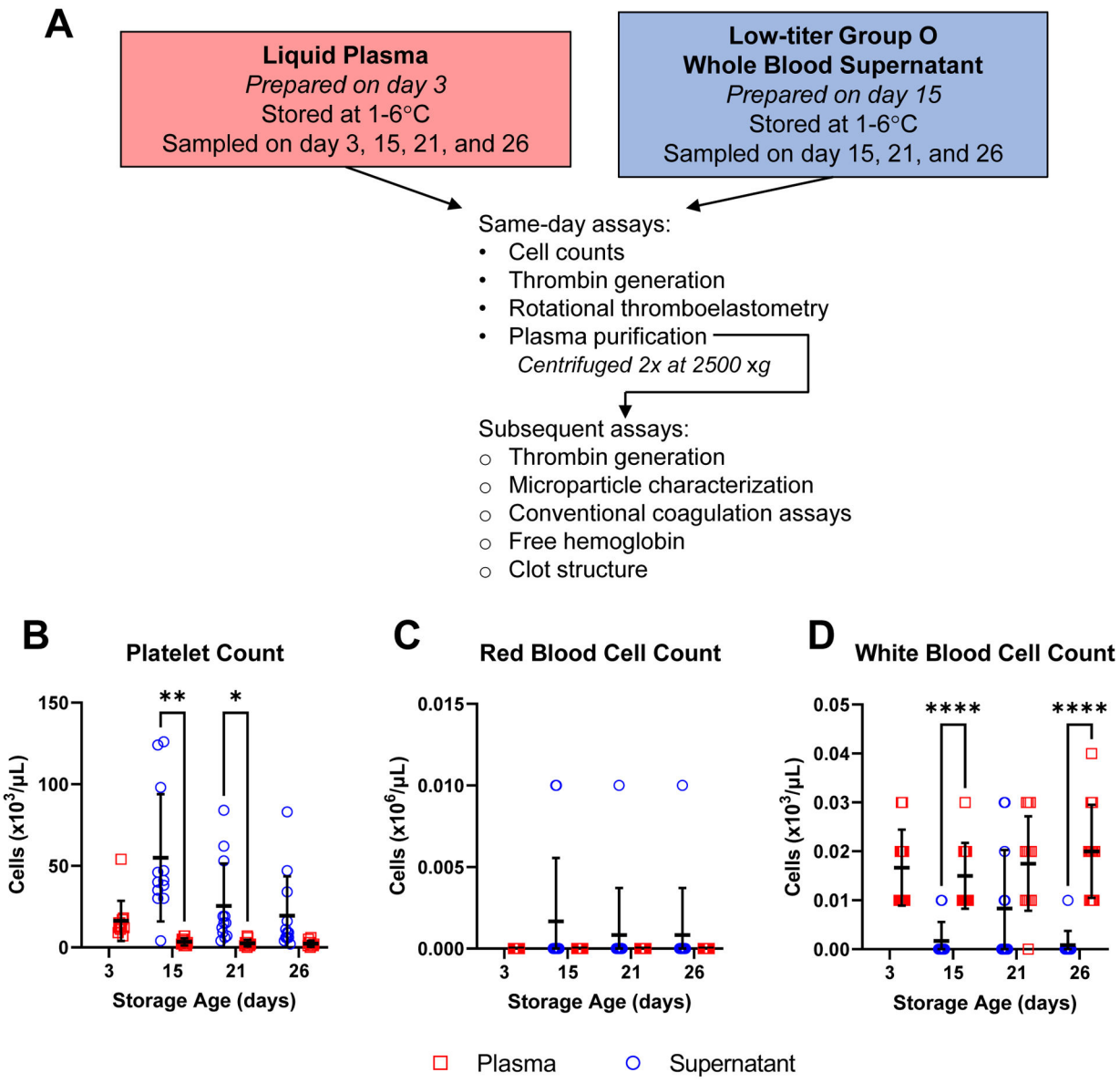

Figure 1.

Flow diagram of low-titer group O whole blood supernatant and liquid plasma storage testing for same-day assays and subsequent testing on banked plasma (A). Cell counts in low-titer group O whole blood supernatant and liquid plasma units over the course of storage (B-D). Plasma units (red squares, N=12) were assayed on days 3, 15, 21, and 26 of storage and supernatants (blue circles; N=12) were assayed on days 15 (1 day post-recycling), 21, and 26. Platelet counts (B), erythrocyte counts (C), and leukocyte counts (D) are shown. Data are presented as individual values with bars representing mean +/− standard deviation. Comparisons within storage days are shown: * p<0.05; ** p<0.01; **** p<0.0001.

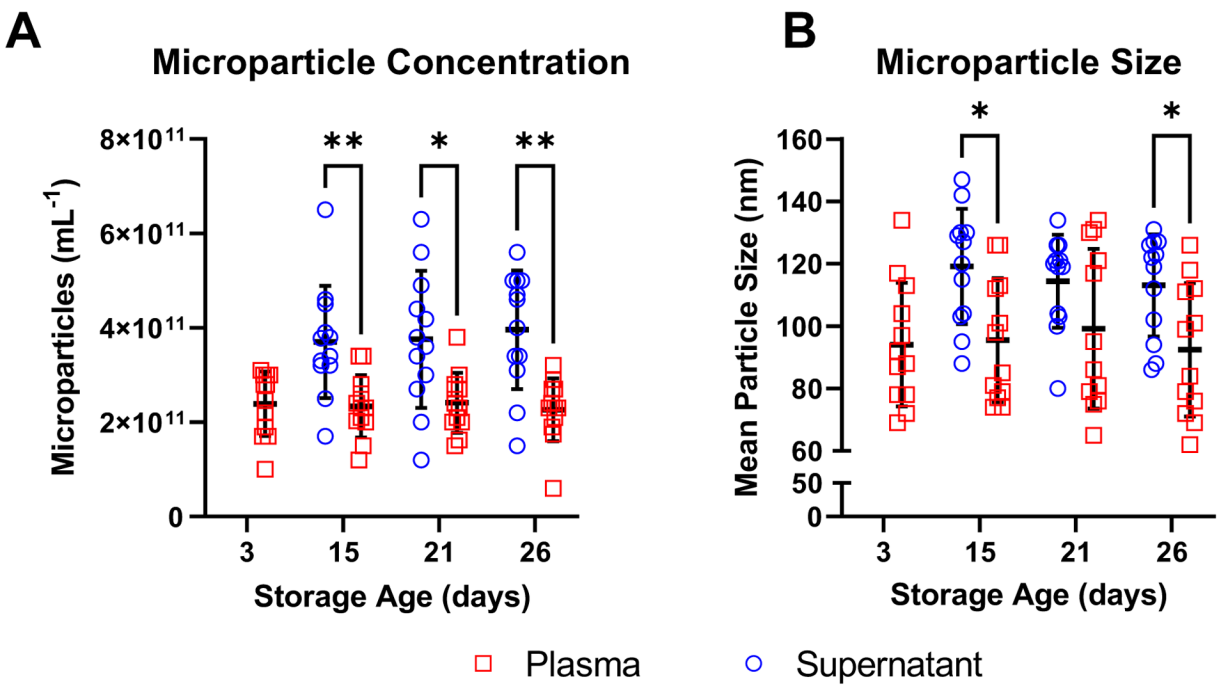

Figure 2.

Microparticle detection in platelet poor plasma derived from low-titer group O whole blood supernatant and liquid plasma units using nanoparticle tracking analysis. Plasma units (red squares; N=12) were sampled on days 3, 15, 21, and 26 of storage and supernatant units (blue circles; N=12) were sampled on days 15 (1 day post-recycling), 21, and 26. Microparticle concentrations (A) and sizes (B) were measured from platelet poor plasma derived from the units on the corresponding storage day. Data are presented as individual values with bars representing mean +/− standard deviation. Comparisons within storage days are shown: * $p<0.05$; *** $p<0.001$.

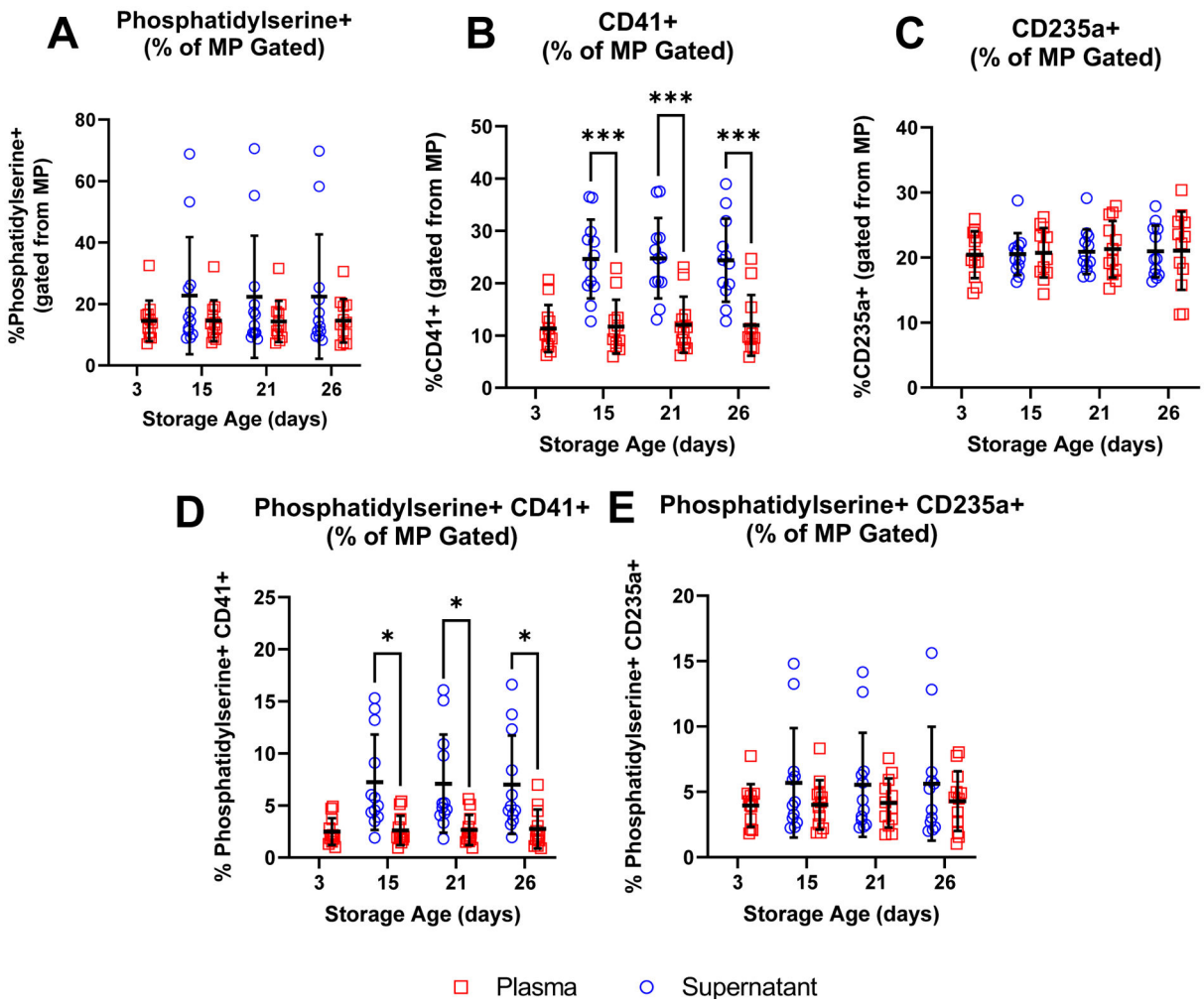

Figure 3.

Microparticle characteristics determined by flow cytometry. Liquid plasma units (red squares; N=12) were sampled on days 3, 15, 21, and 26 of storage and low-titer group O whole blood supernatant units (blue circles; N=12) were sampled on days 15 (1 day post-recycling), 21, and 26. Microparticle content was measured from PPP derived from the units on the corresponding storage day. Phosphatidylserine+ (A), CD41+ (B), and CD235+ (C) microparticles as a percent of microparticles gated are shown, as well as phosphatidylserine+ and CD41+ (D) and phosphatidylserine+ and CD235+ (E) microparticles as a percent of microparticles gated. Data are presented as individual values with bars representing mean +/− standard deviation. Comparisons within storage days are shown: * $p<0.05$; *** $p<0.001$.

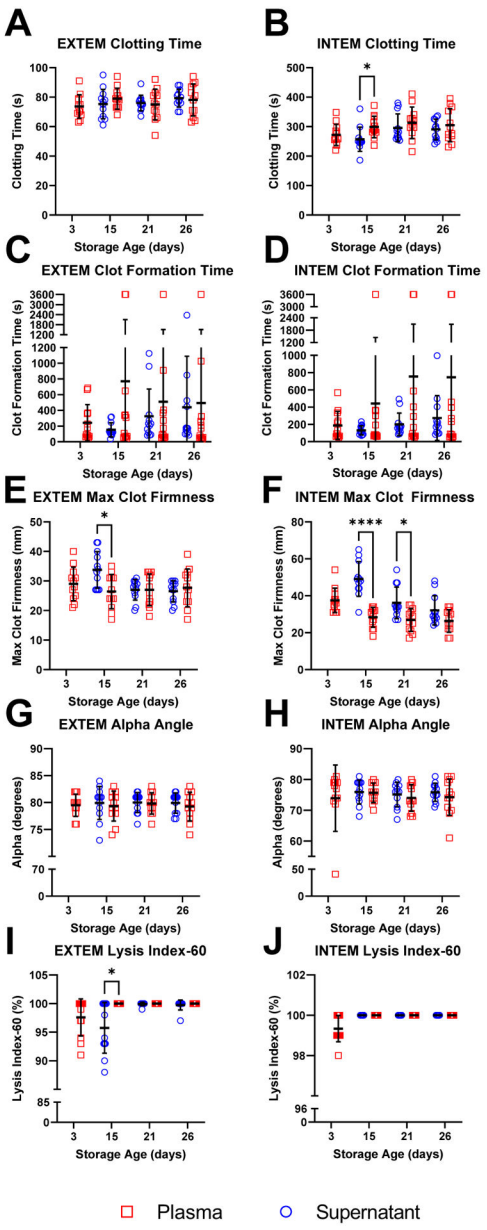

Figure 4.

Hemostatic properties of low-titer group O whole blood supernatant and liquid plasma units from ROTEM assays. Plasma units (red squares; N=12) were measured on days 3, 15, 21, and 26 of storage and supernatant units (blue circles) were measured on days 15 (1 day post-recycling), 21, and 26. ROTEM assays measuring hemostatic properties via the extrinsic pathway (left) and intrinsic pathway (right) were conducted. Clotting time (A-B), clot formation time (C-D), maximum clot firmness (E-F), alpha angle (G-H), and lysis (I-J) at 60 minutes, where 100% represents no lysis, are shown for both agonists. Samples that did not produce clot formation time were recorded as 3600s (assay maximum). Data are presented as individual values with bars representing mean +/− standard deviation.

Comparisons within storage days are shown: * $p<0.05$; ** $p<0.01$, *** $p<0.001$; **** $p<0.0001$.

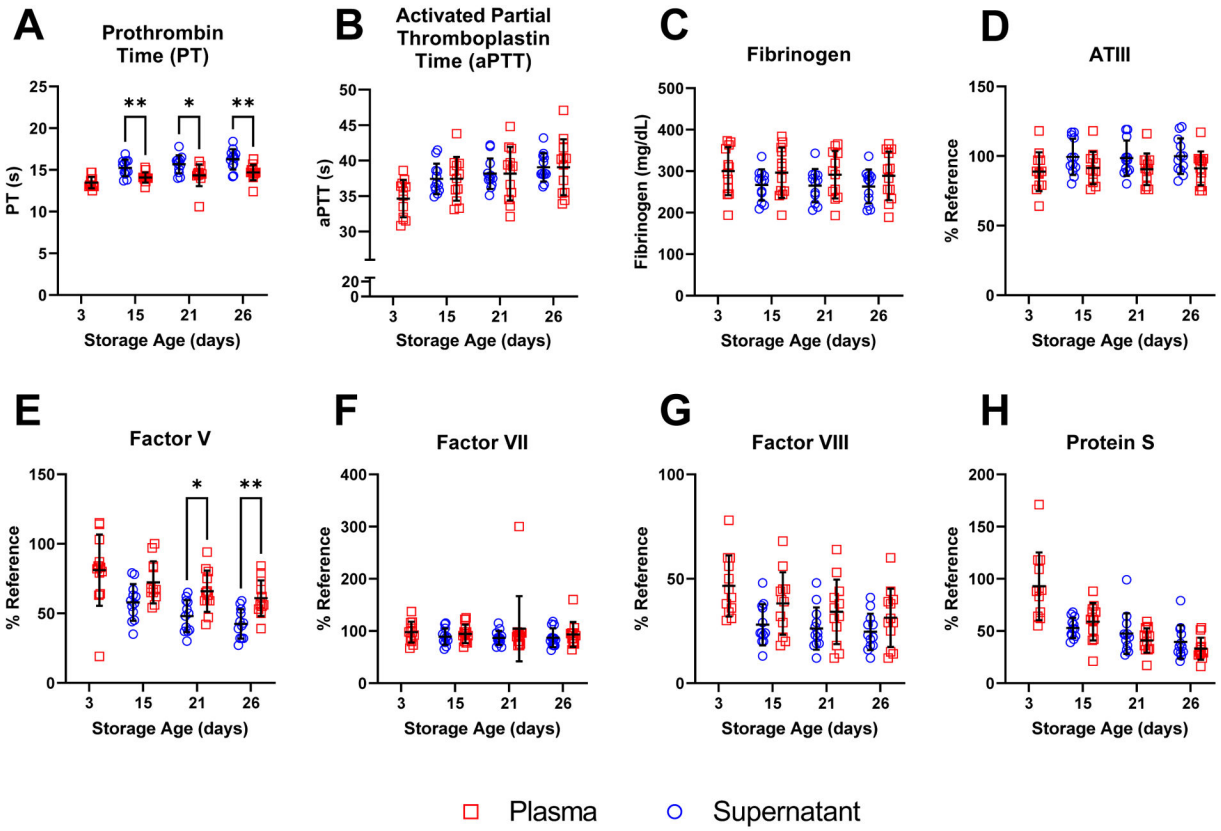

Figure 5.

Conventional coagulation assays and factor levels of low-titer group O whole blood supernatant and liquid plasma units. Plasma units (red squares; N=12) were sampled on days 3, 15, 21, and 26 of storage and supernatant units (blue circles) were sampled on days 15 (1 day post-recycling), 21, and 26. Assays were run on batched platelet poor plasma derived from the units on the corresponding storage day. Prothrombin time (A), activated partial thromboplastin time (B), Fibrinogen (C), Antithrombin III (D), Factor V, VII, VIII (E-G), and Protein S (H) are shown. Data are presented as individual values with bars representing mean +/− standard deviation. Comparisons within storage days are shown: * $p<0.05$; ** $p<0.01$, *** $p<0.001$; **** $p<0.0001$.

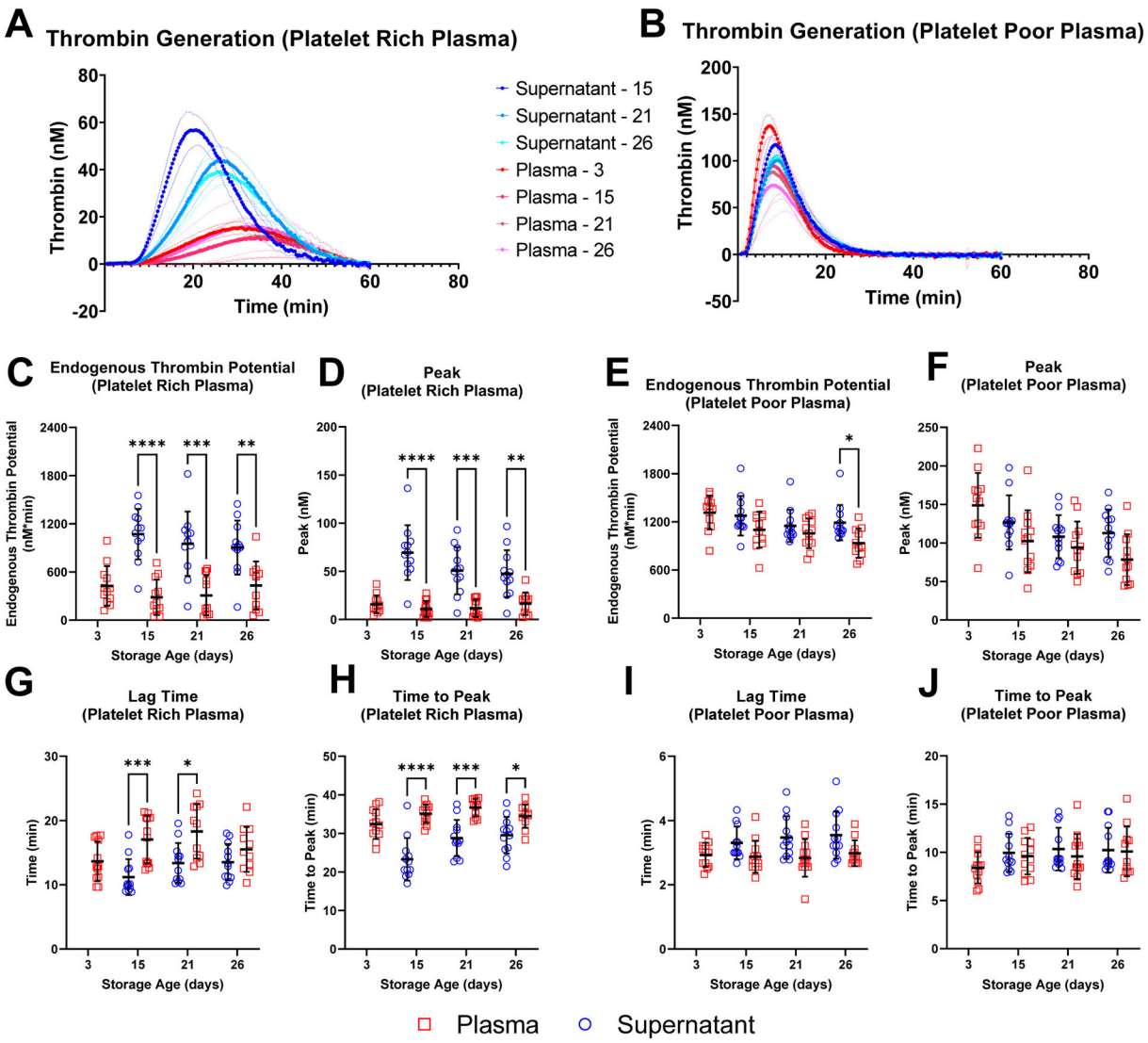

Figure 6.

Thrombin generation in low-titer group O whole blood supernatant and liquid plasma units measured by a Calibrated Automated Thrombogram (CAT). Plasma units (red squares; N=12) were measured on days 3, 15, 21, and 26 of storage and supernatant units (blue circles) were measured on days 15 (1 day post-recycling), 21, and 26. Thrombin generation with the platelet rich plasma reagent (left) (A, C, D, G, H) was performed for same-day assays, and thrombin generation with the platelet poor plasma reagent (right) (B, E F, I, J) was performed on banked plasma. Thrombin generation traces are illustrated with respective quantification of endogenous thrombin potential (C, E), peak thrombin generation ("Peak") (D, F), Lag Time (G, I), and time to peak thrombin generation ("Time to Peak") (H, J). Traces are presented as mean with 90% confidence interval indicated with the dotted lines. Data are presented as individual values with bars representing mean +/− standard deviation. Comparisons within storage days are shown: * $p<0.05$; ** $p<0.01$, *** $p<0.001$; **** $p<0.0001$.

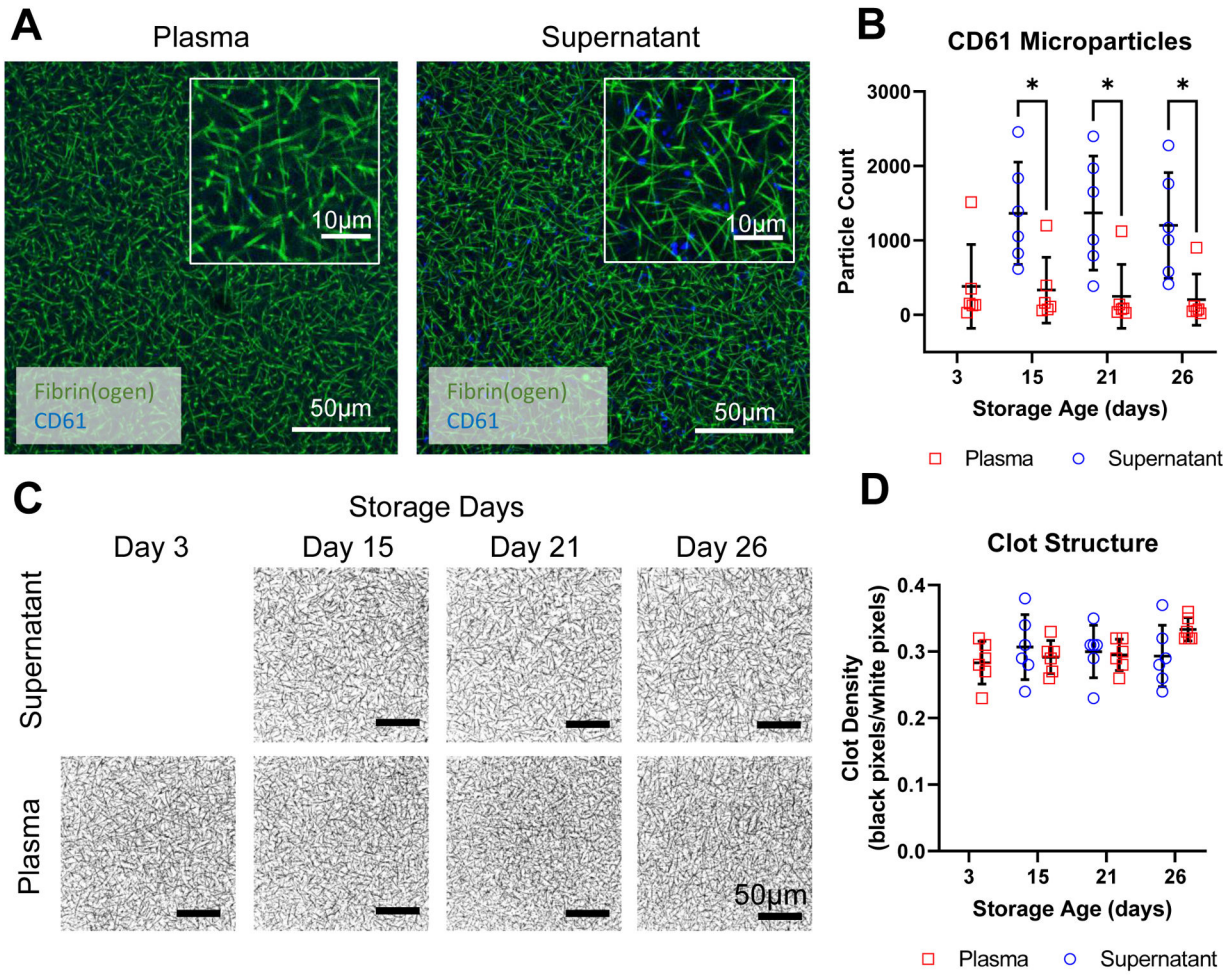

Figure 7.

Detailed microparticle incorporation and fibrin network analysis. Representative images of clots made from banked plasma prepared from storage day 26 low-titer group O whole blood supernatant and liquid plasma controls are shown (A), as well as quantified CD61+ microparticles imaged in the clots (B). Representative binary fibrin(ogen) network images are shown from storage day 3, 15, 21, and 26 samples (C), and corresponding quantified clot density (D). Individual values were obtained by averaging 3 images per clot. Data are presented as individual values with bars representing mean +/− standard deviation. Comparisons within storage days are shown: *p<0.05.