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Innate coagulability changes with age in stored packed red blood cells

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Introduction

Trauma remains the leading cause of morbidity and mortality among adults less than 45 years old in the United States, with an associated 23% increase in trauma-related deaths over the last decade (1). Up to 50% of deaths are secondary to hemorrhagic shock and occur within the first 6 hours of hospital admission. As many as 35% of injured patients arrive to the emergency department with a coagulation imbalance known as trauma induced coagulopathy (2–4). This impairment in coagulation status is often compounded by acidosis, hypothermia, hypocalcemia, and hemodilution that occurs following hemorrhage. Prehospital and in-hospital resuscitation strategies of hemorrhage control and blood transfusion have been implemented in order to reduce the morbidity and mortality associated with hemorrhagic shock (1, 5, 6). Early implementation of damage control resuscitation principles including the initiation of a massive transfusion protocol with administration of packed red blood cells, plasma, and platelets in a balanced ratio has been associated with improved mortality (7, 8). Packed red blood cell (pRBC) units administered during the resuscitation of hemorrhagic shock are often of varied storage ages. The storage age can range from 1 day to 42 days of cold storage, according to the Food and Drug Administration's (FDA) approved shelf-life of pRBC units. However, packed red blood cells with a storage duration greater than 14 days, classified as aged pRBCs, have been associated with increased in-hospital mortality risk in trauma patients undergoing transfusion of 3 or more RBC units within 24 hours of hospital arrival (9).

Our research has previously demonstrated that pRBCs of increased storage duration accumulate biochemical and structural changes known as the red blood cell (RBC) storage lesion (10–17). Furthermore, pRBC-derived microvesicles have emerged as a major byproduct of pRBC storage that have significant *in vitro*, *in vivo*, and clinical consequences

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following transfusion (10, 11, 13, 18). We have previously studied the independent *in vitro* and *in vivo* thrombotic potential of the transfusion of aged pRBC-derived microparticles (12, 15). Similarly, recent clinical studies have demonstrated an association between pRBC transfusion and the incidence of venous thromboembolism. Spinella et. al demonstrated that increased duration of pRBC storage is associated with an increased incidence of deep vein thrombosis as well as in-hospital mortality from multi-organ failure in trauma patients (19). Similarly, Goel et. al supported the association between perioperative pRBC transfusion with the development or progression of postoperative venous thromboembolism (20).

Despite these associations between aged pRBC transfusion and venous thromboembolic complications, the impact of storage age on the innate coagulability of pRBCs themselves has not been studied. We therefore undertook this investigation to more thoroughly comprehend the coagulation status of murine packed red blood cells over the 14-day standard storage duration. We sought to investigate the effect of storage age on the innate coagulability and aggregability of stored pRBCs.

Material and Methods

Murine blood banking

All murine experiments were performed following approval by the Institutional Animal Care and Use Committee of the University of Cincinnati. Genetically identical C57BL/6 mice were utilized in this experiment in order to control for donor-specific factors, such as age and gender, that would confound our findings. Male 8 to 10-week-old donor mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with intraperitoneal pentobarbital (0.1 mg/g body weight). Murine whole blood was obtained from these donor animals via cardiac puncture and banked with standard citrate, phosphate, double dextrose (CP2D; 3.27 mg/mL citric acid, 26.30 mg/mL sodium citrate, 2.22 mg/mL monobasic sodium phosphate, and 46.40 mg/mL dextrose), in our standard murine blood banking protocol (21).The banked whole blood was immediately used for analysis as the fresh WB group. Packed red blood cells were generated from fresh WB via the following protocol. The banked whole blood was centrifuged at 300g for 7 minutes with removal of the platelet rich plasma supernatant. The remaining blood was centrifuged at 1000g for 15 minutes with collection of the remaining platelet poor plasma supernatant. pRBC units were prepared following the addition of Additive Solution-3 (AS-3) to the erythrocyte pellet in a 2:9 ratio. AS-3 (0.42 mg/mL citric acid, 5.88 mg/mL sodium citrate, 2.76 mg/mL monobasic sodium phosphate, 4.10 mg/mL sodium chloride, 10mg/mL dextrose, 0.30 mg/mL adenine) is a preservative solution approved for the long-term storage of human red blood cells. These pRBCs were maintained under standard storage conditions for up to 14 days. We have previously determined this interval to be biochemically equivalent to the current 42 day Food and Drug Administration approved shelf life of human pRBC units (21).

Fresh frozen plasma isolation and storage

In preliminary coagulation studies, we investigated the impact of fresh liquid plasma and fresh frozen plasma (FFP) on non-activated rotational thromboelastometric (NATEM) clotting parameters, including clotting time (CT), clot formation time (CFT), alpha angle (α-

angle), maximum clot firmness (MCF), and fibrinolysis (LI30). We determined that there was no significant difference in the coagulation potential when pRBCs were combined with FFP or fresh liquid plasma. With the use of FFP in trauma resuscitation and lack of pragmatic clinical availability of freshly donated liquid plasma, we elected to utilize FFP for the following coagulation studies. We centrifuged murine whole blood at 4600 rpm for 10 minutes at room temperature. The platelet rich plasma supernatants were collected taking care not to include the buffy coat. The collected plasma was centrifuged again at 10,200 rpm for another 10 minutes at room temperature to isolate and remove the platelet pellet. The plasma was then pooled to generate a stock of homogenous murine plasma to remove the confounder of individual plasma performance. Half of the plasma stock was separated into 160μL aliquots that were immediately frozen in −80°C upright freezer (Thermo Fisher Scientific, Waltham, MA) to generate FFP. The other half was stored as liquid platelet poor plasma (PPP) for immediate use in analysis.

Platelet-rich plasma isolation

Platelet-rich plasma (PRP) was isolated following centrifugation of murine whole blood at 300g for 7 minutes at 4°C. The plasma supernatants were pooled and stored for immediate analysis. The addition of normal saline to fresh WB was utilized as the control group.

Thromboelastometry

Rotational thromboelastometry (TEM Systems Inc., Durham, North Carolina) analyses were performed to investigate alterations in the coagulation status. Prior to analysis, the blood samples were run on the Ac•T diff hematology analyzer (Beckman Coulter, Brea, California) and determined to have equivalent red blood cell count, hemoglobin, and hematocrit. NATEM assays were run on freshly stored whole blood, freshly stored pRBCs, and pRBCs at the end of their 14-day storage duration (aged pRBCs). NATEM assays were also performed on pRBCs combined with FFP for complete comparative analysis incorporating a 1:1 ratio of transfused pRBCs:FFP that is consistent with damage control resuscitation strategies. Specifically, 300μL of fresh whole blood (WB) collected in CP2D in a 1:7 ratio was analyzed via NATEM assay. Subsequently, viscoelastic parameters were obtained for 300μL aliquots of fresh WB, fresh and aged pRBC units alone (Fresh pRBC, Aged pRBC) as well as fresh and aged pRBC units in a 1:1 volume ratio with FFP (Fresh pRBC + FFP, Aged pRBC + FFP). Specifically, the pRBC + FFP group was generated by mixing 150 μL of pRBCs and 150 μL of FFP prior to running on the instrument. The viscoelastic coagulation parameters of CT, CFT, α-angle, MCF, and LI30 were analyzed to determine innate *in vitro* blood component coagulability. Given the significant *in vivo* impact of microvesicles on thrombus formation, microvesicles were isolated as previously described (22) from aged pRBCs and added to fresh pRBCs, with subsequent analysis via NATEM assay.

Aggregation Analysis

Multiplate impedance aggregometry (Verum Diagnostics, Munich, Germany) was utilized to measure RBC and platelet aggregation. Analysis was performed on WB, fresh pRBC alone, aged pRBC alone, and fresh and aged pRBC in combination with varying ratios of PPP and PRP to simulate different ratios of blood products as they would be transfused. Platelet

aggregation was induced by 6.5 μM adenosine diphosphate (ADP), 0.5 mM arachidonic acid (AA) or 0.2 M calcium chloride ($CaCl_2$) as agonists. Contrary to $CaCl_2$, ADP and AA are standard agonists utilized to initiate platelet aggregation. In this experiment we chose to utilize $CaCl_2$ in order to investigate the impact of additional calcium administration with fresh and aged pRBCs. In a massively transfusion trauma patient, administration of $CaCl_2$ is often utilized in order to overcome resuscitation associated coagulation changes, such as those induced by the anticoagulant effects of citrate in the storage solutions.

The agonists were prepared as dictated by manufacturer instructions (Roche Diagnostics, Mannheim, Germany; DiaPharma Group Inc., West Chester, OH). Following instrument set-up, 300 μL of diluent (0.9% NaCl or 0.9% NaCl with 3mM CaCl2) along with 300 μL of blood sample were inserted into the test cell channels via electronic pipette and underwent a 3-minute warming/equilibration incubation period. The blood samples were as follows: WB, fresh pRBCs 1:1 ratio with PRP, and aged pRBCs in a 1:1 ratio with PRP. Subsequently, 20 μL of the indicated agonist was pipetted into the bottom of the test cell followed by a 6-minute incubation period. Aggregation was measured by sensing the change in electrical impedance that is automatically converted into arbitrary aggregation units (AU). The area under the aggregation curve (AUC) was calculated by the total height of the aggregation curve and the slope as determined by the speed and final strength of aggregation (23).

Statistical analysis

GraphPad Prism was utilized (San Diego, CA) to perform statistical analysis of data via ANOVA or t-test where indicated. $P<0.05$ was deemed statistically significant. Data is presented as mean ± standard error of the mean.

Results

Non-activated rotational thromboelastometry

When compared to fresh whole blood, fresh pRBCs demonstrated significant prolongation in CT, CFT, as well as significantly reduced α-angle and MCF. At the end of the 14-day storage duration, NATEM assays demonstrated that when compared with fresh pRBCs, aged pRBCs showed a significant exacerbation of the prolongation in CT, CFT, as well as reduction in α-angle and MCF (Figure 1).

Fresh pRBCs, aged pRBCs, fresh pRBCs + FFP, aged pRBCs + FFP each had prolonged CT and CFT, along with reduced α-angle and MCF when compared to WB. Following the addition of FFP to the fresh pRBCs in a 1:1 ratio, there was a significant reduction in CT and CFT as well as an increase in α-angle and MCF as compared to fresh pRBCs alone. There were similar findings when comparing aged pRBCs + FFP to aged pRBCs alone. (Figure 2).

Compared to fresh pRBCs + FFP, aged pRBCs + FFP had no significant difference in α-angle and demonstrated a reduced CT, but still showed significant prolongation in CFT, and decreased MCF (Figure 3).

The investigation of the impact of microvesicle presence on the innate coagulability of pRBCs, demonstrated no differences in NATEM parameters when comparing microvesicle from aged pRBCs added to fresh pRBCs versus to the addition of saline to fresh pRBCs (Figure 4).

Multiplate impedance aggregometry

Role of Agonist—When ADP was utilized as an agonist, fresh pRBC + PRP demonstrated minimal platelet aggregation and aged pRBC + PRP demonstrated no platelet activity. When AA was utilized as an agonist, neither fresh pRBC + PRP nor aged pRBC + PRP showed any platelet activation. By contrast, when $CaCl_2$ was utilized as an agonist, fresh pRBC + PRP had aggregation similar to that of whole blood. Aged pRBC + PRP also demonstrated aggregation in response to the $CaCl_2$ reagent, although it remained significantly less than the activation exhibited by fresh pRBC + PRP (Figure 5).

Role of pRBC:PRP Ratio Variation—Given the results in aggregation using $CaCl_2$, this reagent was utilized as the agonist for the analysis of varying PRP and pRBCs ratios. When the ratio of fresh pRBC:PRP was decreased (1:3) there was a significant reduction of platelet activity compared to fresh pRBC + PRP in a 1:1 ratio. When the ratio of fresh pRBC:PRP was increased (3:1), there was significantly more aggregation than 1:3 and no difference when compared to 1:1. However, with aged pRBC, there was no differences in aggregation with variation in pRBC:PRP ratios. Additionally, the overall platelet aggregation for the older pRBC was consistently lower than that of the fresh pRBCs (Figure 6A). These data suggest that pRBCs, and specifically fresh pRBCs, may contribute to platelet aggregability.

Role of Platelets in Plasma—$CaCl_2$ was utilized as the agonist in this investigation of removing platelets from the plasma. When PPP was utilized instead of PRP there was a significant reduction in platelet aggregation, except with increased pRBC:plasma ratios (Figure 6B). This confirmed that that increased presence of fresh RBCs can overcome the absence of platelets after calcium stimulation.

Discussion

In the present study, we investigated the impact of storage age on pRBC coagulability. We determined that the duration of pRBC storage is an important factor in the coagulation of transfused pRBCs. Our findings suggest that, as pRBCs age, they develop an innate defect in coagulation, which adversely impacts viscoelastic coagulation parameters. Following the addition of FFP to the fresh pRBCs in a 1:1 ratio, NATEM parameters improved as expected when compared with fresh pRBCs alone. Similarly, when compared to aged pRBCs alone, the aged pRBCs + FFP demonstrated significant improvement in the NATEM parameters. However, despite improvement in the parameters for fresh and aged pRBCs, the coagulation potential of these components remained inferior to that of whole blood following the addition of FFP. We also determined that the addition of plasma to aged pRBCs improved NATEM coagulation parameters, but not completely. Furthermore, the addition of plasma did not correct the acquired coagulation differences between the fresh and aged pRBCs.

Trauma-induced coagulopathy has been identified to play a significant role in the sequela following hemorrhagic shock. Clotting factor consumption and dilution are elements that have been implicated in trauma-induced coagulopathy. Plasma, an essential component of trauma resuscitation, is administered in order to combat the pathophysiology of hemorrhage (24, 25). In traumatic hemorrhagic shock, FFP is administered in a 1:1 fashion with pRBCs, in order to achieve hemostasis in a coagulopathic exsanguinating patient (7, 8). Unfortunately, plasma administration alone may not always completely reverse coagulopathy. The current data suggest that as pRBCs age, they may develop an innate loss of their usual hemostatic potential that cannot be completely reversed with FFP utilization.

Our data additionally demonstrates that as pRBCs age, they not only become inherently hypocoagulable, but they also play a role in impaired platelet aggregation. We validated the use of multiplate aggregometry in this study for the analysis for platelet aggregation by demonstrating that the utilization of PPP generated significantly less platelet aggregation when compared to the utilization of PRP. We subsequently found that when aged pRBCs were reconstituted with FFP in a 1:1 ratio they generated significantly less platelet aggregation then fresh pRBCs. This impairment in aggregation was overcome with the addition of a high dose of calcium chloride. Previous studies have shown that extracellular calcium does not induce platelet aggregation in rats or humans, which suggests that there may be a role for red blood cell aggregation in thrombus formation(26, 27). Of note, consistent with our previous findings, platelet aggregation remained diminished in the aged pRBCs +FFP when compared to the fresh pRBCs. This confirms the essential role of calcium administration to help achieve hemostasis in patients who receive a rapid transfusion of a large volume of blood products (28).

Our experimental findings suggest that there are other factors, in addition to pRBCs alone, that play a role in transfusion associated thromboembolic events. Multiple studies have recognized an association between transfusion of packed red blood cells of greater storage duration and increased risk of adverse outcomes, such as, thrombotic events, multi-organ failure, and death (9, 18, 19, 29). We have previously found that red blood cell-derived microvesicles, an important mediator of post-transfusion immune response (11), also impact the post-transfusion coagulation status. Accumulation of red blood cell-derived microvesicles can lead to a transient hypercoagulable state in the transfusion recipient through the accelerated activation of clotting factors (11). Our data indicate, however, that not only are pRBCs inherently hypocoaguable as they age, but also that the accumulated microvesicles did not contribute to increased coagulability as determined by viscoelastic testing.

These contrary findings suggest that the interaction of the red blood cells and some red blood cell byproducts with the vascular endothelium and platelets is likely, in part, responsible for stimulating thrombogenesis. We have previously shown that microvesicle-mediated thrombogenesis occurs secondary to microvesicle stimulated release of P-selectin from the vascular endothelium (12). Cell-free hemoglobin, another byproduct of red blood cell storage, has been shown to play a role in vascular thrombosis via nitric oxide scavenging, reactive oxygen species formation, and pro-inflammatory characteristics (30, 31). Furthermore, membrane alterations that occur during storage, such as

phosphatidylserine externalization, band-3 expression reduction and clustering, as well as reduced presence of the negative surface charge, sialic acid, have also been implicated in contributing to thrombus formation (26, 32, 33). These in-vitro findings, however, may not always lead to generation of venous thromboembolic events and other adverse events. The clinical trials ABLE, INFORM, and TRANSFUSE evaluated the use of fresh pRBCs versus standard-issue pRBCs demonstrated. These studies demonstrated no benefit in the use of fresh pRBCs (34–36). Similarly, the clinical trial Red-Cell Storage Duration Study (RECESS), comparing transfusion of fresh pRBCs (storage age < 10 days) to older pRBCs (storage age > 21 days) in the perioperative period of complex cardiac surgery patients, found no significant difference in the 7-day change in multiple organ dysfunction score, including the hematologic component of this score (37). While these clinical trials address the impact of storage age on patient outcome, they do not address the implications of transfusion of end-of-shelf-life pRBCs nor massive transfusion of aged red blood cells. Therefore, further studies will need to be performed in order to reconcile the in-vitro and in-vivo findings.

In this study, we found that increasing the ratio of pRBC:PRP resulted in significantly more platelet aggregation in response to calcium stimulation. Erythrocytes have historically been surmised to play a limited role in thrombosis, but recently described capacities in hemostasis (38–41) have prompted a renewed interest in the role of erythrocytes in clot formation. These capacities include the erythrocyte role in platelet margination, protein expression stimulating thrombin generation, and the interaction of platelets and fibrinogen with endothelial cells (42–45). Our data suggest that the red blood cells themselves may contribute to whole blood aggregability independent of interactions with endothelium. Future studies are need to more thoroughly establish the impact of the red blood cell in platelet aggregation. Furthermore, it will be important to investigate the role of red blood cell interactions with the endothelium and other plasma and capillary bed constituents as the red blood cell ages.

Although promising, this study is not without limitations. We utilized genetically identical mice of the same gender and age in order to control for variability in donors and recipients due to differences in age and gender. In the preparation of pRBCs, the plasma cannot be completely removed as this would lead to the damage of the erythrocytes. Although there is residual in the pRBCs, the plasma likely does not appreciably contribute to the functional coagulability of the pRBC units. Additionally, the plasma from the donor mice was pooled in order to control for variation in clotting factors between mice. Our data do not account for potential variability in the results that may have been present due to gender, age, body mass, and genetic differences. While the murine model allows for elimination of donor/recipient variability, these findings may be not completely applicable to humans, who have significant variability from person to person.

In conclusion, stored packed red blood cells, contrary to the clinical thrombotic associations that prompted this investigation, become inherently hypocoagulable as they age. This coagulation impairment, although improved with calcium and FFP administration, cannot be completely reversed. While packed red blood cells develop an innate reduction in coagulation potential, they may still play more of a role in aggregation via their interaction

with platelets and endothelial cells. It will be important to further investigate the role of both native and transfused red blood cells in thrombosis formation in order to more thoroughly understand the sequela of thrombotic events following blood transfusion.

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Highlights

- Stored packed red blood cells become inherently hypocoagulable as they age
- Packed red blood cell hypocoagulability is not completely reversed with plasma
- Microvesicles have been attributed to post-transfusion thrombotic events
- Microvesicles do not change *in-vitro* coagulability of the packed red blood cells

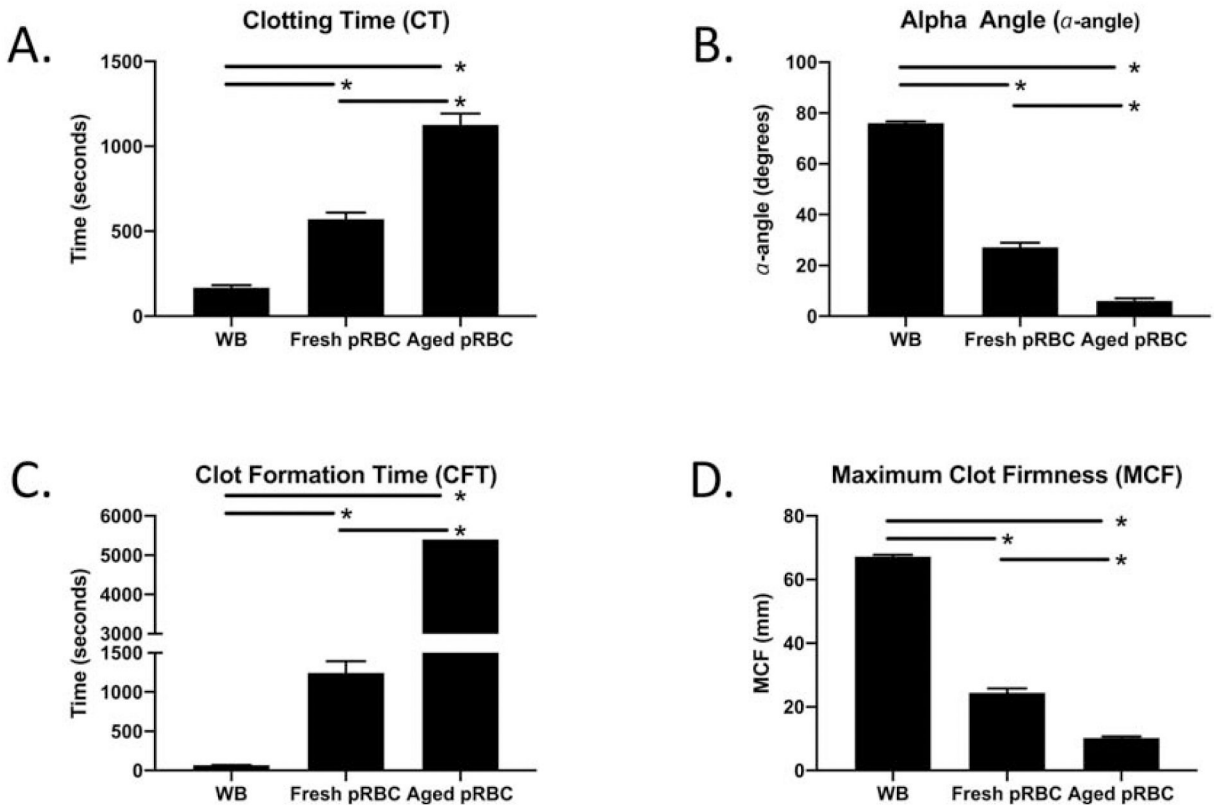

Figure 1.

NATEM parameters of pRBCs stored at the end of the 14-day storage period. When compared with fresh pRBCs, aged pRBCs showed a significant exacerbation of the prolongation in CT (A), reduction in α-angle (B), prolongation in CFT (C), and reduction of MCF (D). n = 5. $P < 0.05$ for all analysis.

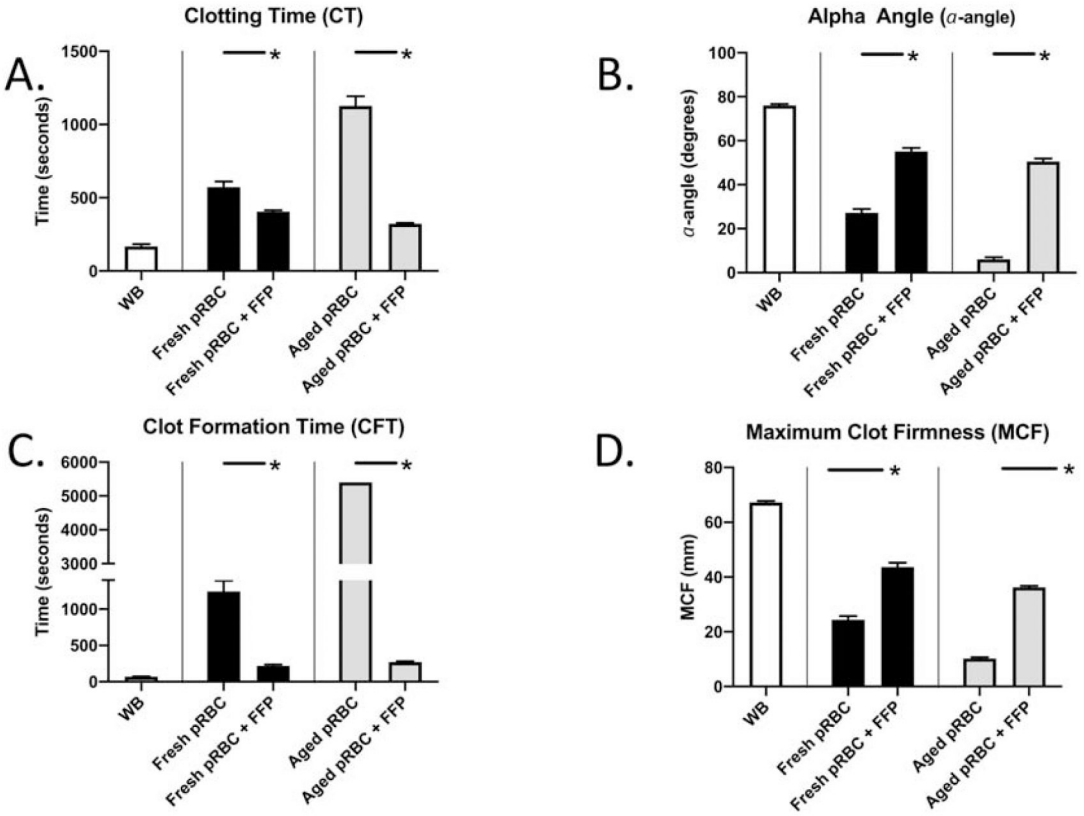

Figure 2.

Fresh pRBC, aged pRBC, fresh pRBC + FFP, aged pRBC + FFP all had inferior NATEM coagulability when compared to WB. The addition of FFP to fresh pRBCs in a 1:1 ratio improved NATEM parameters but remained inferior to WB. n = 5. $P < 0.05$ for all analysis.

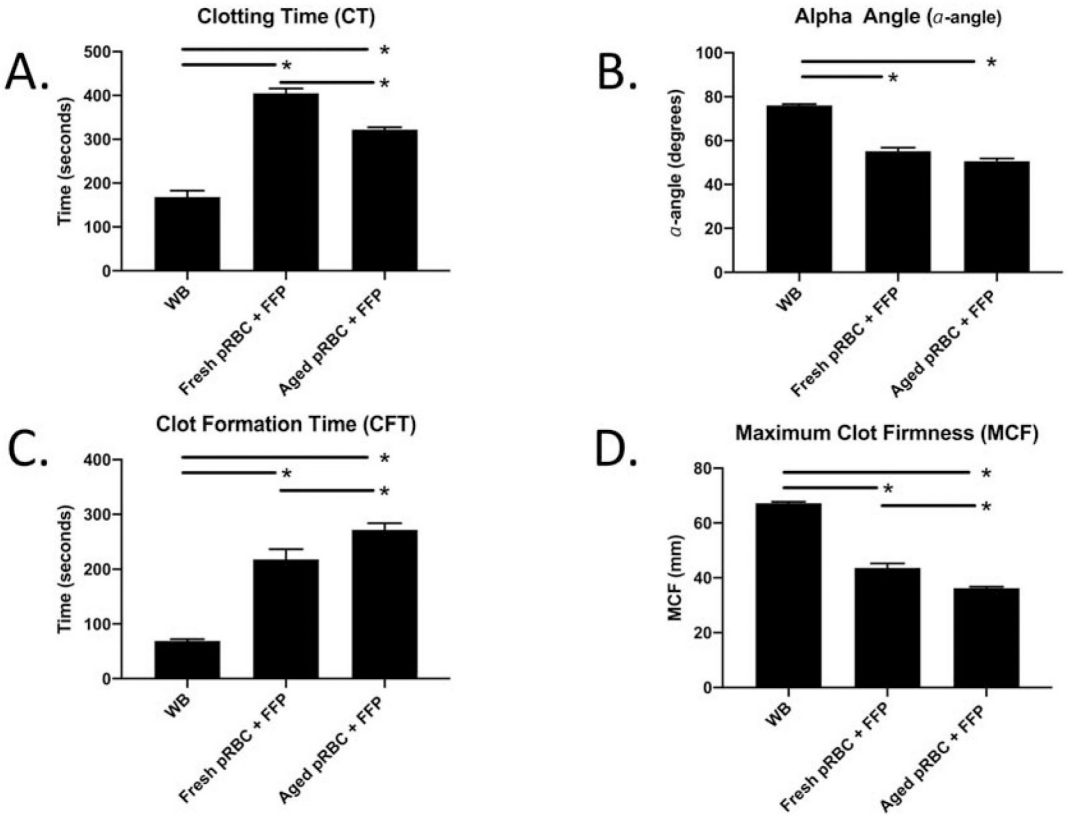

Figure 3.

Aged pRBC + FFP demonstrated prolonged CT (A), CFT (C), and reduced MCF (D) when compared to fresh pRBC + FFP. There was no difference in alpha-angle (B). n = 5. $P < 0.05$ for all analysis.

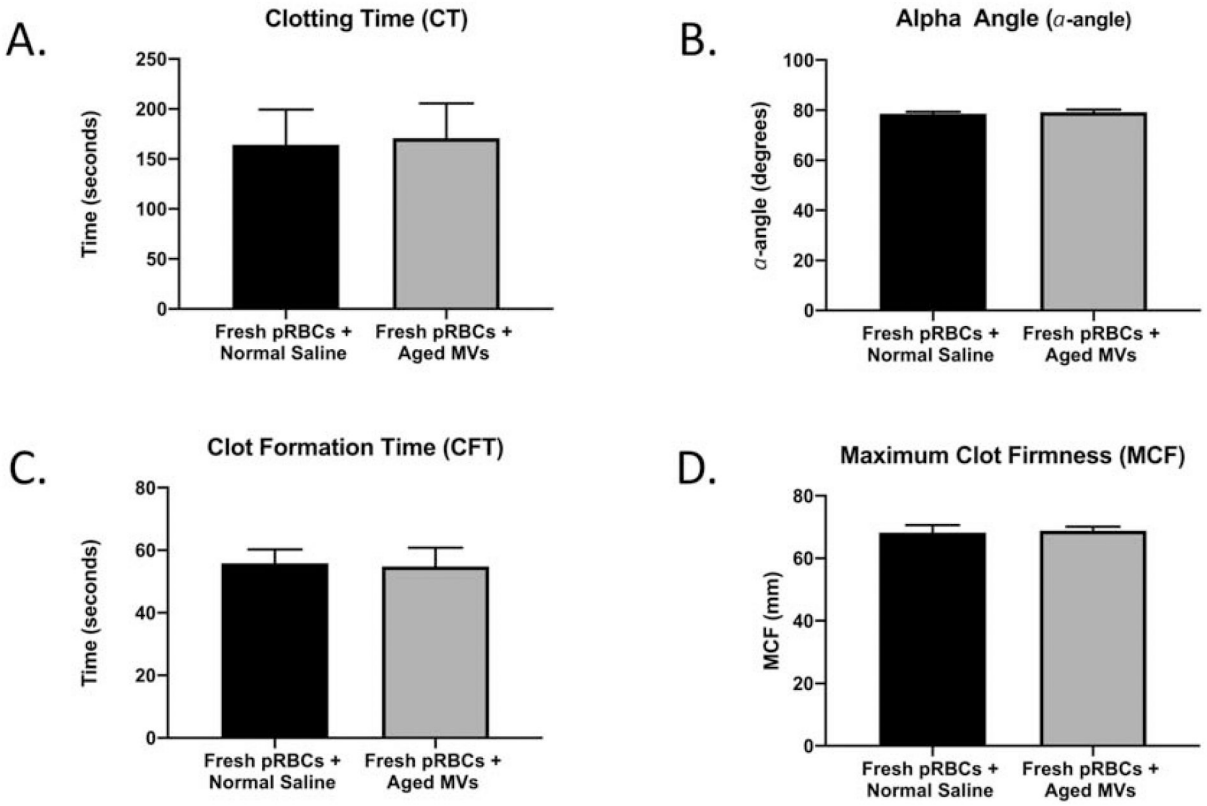

Figure 4.

Microvesicles (MVs) isolated from aged pRBCs that were added to fresh donor pRBCs did not produce a change in NATEM parameters, CT (A), alpha angle (B), CFT (C), and MCF (D), when compared to the addition of normal saline (control). n = 5. $P < 0.05$ for all analysis.

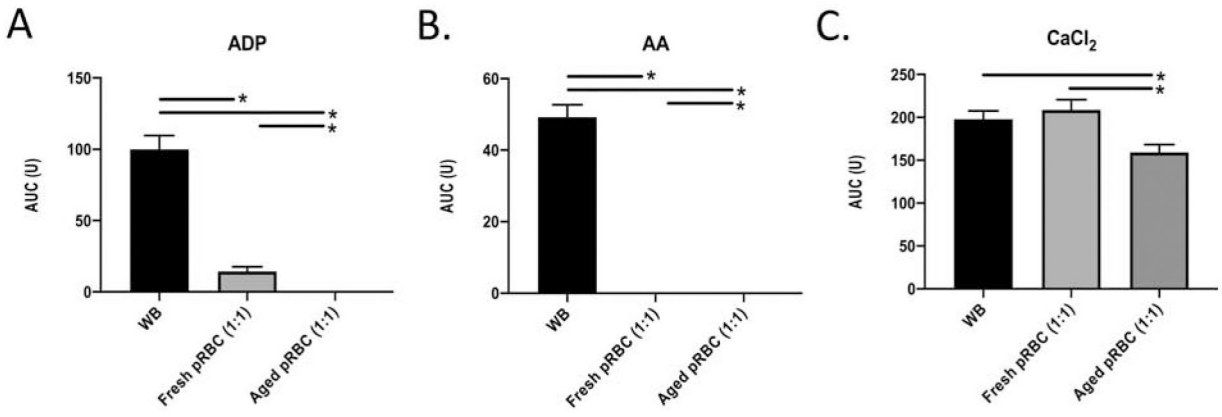

Figure 5.

The addition of $CaCl_2$ (C) as an agonist resulted in augmentation of platelet aggregation, dissimilar to ADP (A) and AA agonists (B). There was less platelet aggregation in response to $CaCl_2$ for aged pRBC +PRP compared to fresh pRBC + PRP (C). n = 5 per group. $P < 0.05$ for all analysis.

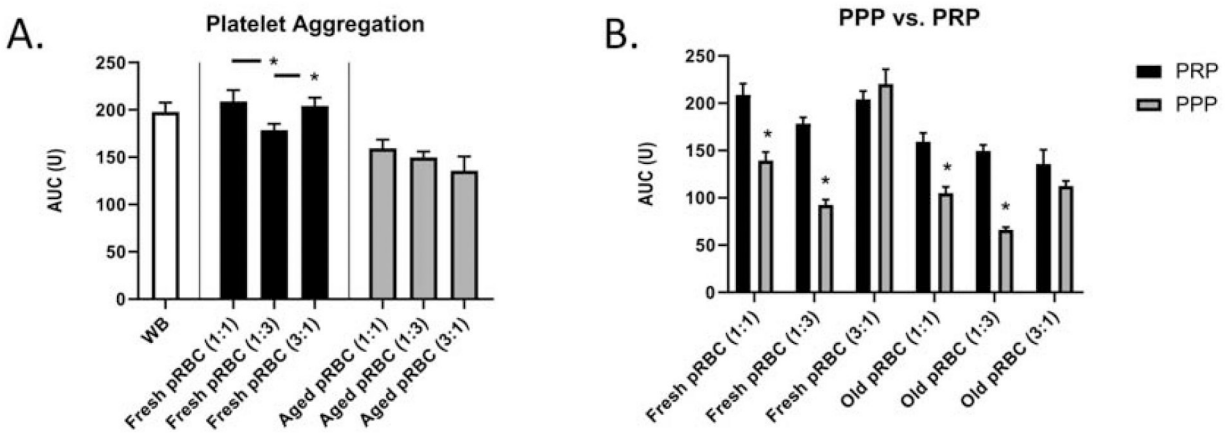

Figure 6.

An increase in the ratio of fresh pRBC:PRP resulted in significantly more platelet aggregation than 1:3 and no difference when compared to 1:1. However, with aged pRBC, there was no differences in platelet aggregation with variation in pRBC:PRP ratios. n = 5. $P < 0.05$ for all analysis.