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Decline in platelet microparticles contributes to reduced hemostatic potential of stored plasma

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Abstract

Introduction—In an effort to administer life-saving transfusions quickly, some trauma centers maintain thawed plasma (TP). According to AABB, TP is approved for transfusion for up to five days when stored at 1 – 6 °C. However, the alterations in microparticles (MP) contained in the plasma, which are an integral component of plasma's hemostatic capacity, are not well characterized. We report on MP changes in TP between its initial thaw (FFP-0) and five days (FFP-5) of storage.

Materials and Methods—FFP units (n=30) were thawed at 37 °C and kept refrigerated for five days. Phenotypes of residual cells, which include platelets, erythrocytes, leukocytes, monocytes, endothelial cells, and MP counterparts of each cell type, were analyzed by flow cytometry. Functional assays were used for MP procoagulant activity, plasma thrombin generation, and clotting properties (thromboelastography).

Results—In FFP-0 the majority (94%) of residual cells were platelets, along with significant levels of platelet MPs (4408×10³/L). FFP-5 showed a decline in MP count by 50% (p<0.0001), and procoagulant activity by 29% (p<0.0001). FFP-5 exhibited only 54% (p<0.0001) of the potential for thrombin generation as FFP-0, while thromboelastography indicated a slower clotting response (p<0.0001) and a longer delay in reaching maximum clot (p<0.01). Removal of MP by filtration resulted in reduced thrombin generation, while the MP replacement restored it.

Conclusions—Decline in MP with storage contributes to FFP-5's reduced ability to provide the hemostatic potential exhibited by FFP-0, suggesting the presence of platelet MPs in freshly TP may be beneficial and protective in the initial treatment of hemorrhage.

Keywords

Fresh-frozen plasma; Platelets; Microparticles; CAT; Thromboelastography; Transfusion; Trauma

Hemorrhage remains a major cause of potentially preventable death following traumatic injury. Optimal management of the critically injured patients with hemorrhage shock dictates emergent interventions to stop bleeding and simultaneous transfusion of blood

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Conflict of interest statement

None.

products [1–3], including red blood cells (RBC), fresh frozen plasma (FFP), cryoprecipitate, and platelets. Recent data supports a more aggressive protocol when using FFP. Transfusing earlier and in greater amounts of FFP was associated with improved survival rates [4]. The improvement in survival with this more aggressive use of FFP is purported to be due to its hemostatic potential by the delivery of clotting factors and other components necessary for hemostasis. To accommodate this change in practice, and improve the rapid availability of plasma in emergency situations, many centers are using thawed plasma (TP). Research illustrating “adequate” concentrations of coagulation factors in plasma has led to the establishment of guidelines by the American Association of Blood Banks (AABB) allowing the use of TP for transfusion up to five days after thawing in clinical settings, as long as it is stored between 1–6 °C [5,6]. However, appropriate functional measures are needed to document the hemostatic potential of freshly thawed FFP (FFP-0) vs. plasma stored for five days (FFP-5). Accordingly, the true clinical effectiveness of FFP-5 is still uncertain, as clinical outcome data with this product does not exist.

The hemostatic potential of plasma, as it relates to the ability to correct the hypocoagulable state of bleeding patients, is a result of several parameters including: coagulation factor activities, presence or absence of inhibitors, and the procoagulant activity of cellular microparticles (MPs). Quantitative or qualitative declines of these plasma components may result in reduced plasma hemostatic potential. MPs are generated from a variety of blood and vascular cells and are considered essential for hemostasis as they expose phospholipids on their surface which provide binding sites for activated clotting factor complexes. We and others reported reductions in clotting factor levels in TP under various storage conditions and/or preparation and preservation methods [7–11]. Previously, we have analyzed TP and concluded that the hemostatic potential and clot forming ability of plasma significantly declines with storage [11]. Thus, we seek to expand upon this work and discover the mechanisms and particles responsible. However, the information about residual cells and cellular MPs in stored plasma products is limited [12,13]. Since MPs appear to be an integral component of FFP and contribute to clot formation, our objective was to characterize alteration in cellular and MP phenotypes and MP-associated procoagulant activity during five days of TP storage, and to relate any significant changes seen to variance in plasma’s hemostatic potential and the capacity to form a clot. We hypothesized that FFP-5 has reduced hemostatic potential and lower MP-associated procoagulant activity when compared with FFP-0. Our research is particularly relevant at trauma centers whose current protocols use TP in a growing number of trauma situations. In this study, we applied a comprehensive set of functional measures to characterize the impact of residual cells and MPs on FFP-0 and FFP-5’s capacity to generate thrombin and to form a fibrin clot.

Materials and Methods

Plasma

FFP units from 30 blood donors were obtained from the Gulf Coast Regional Blood Center (Houston, TX), as previously reported [11]. Blood was collected in citrate-phosphate-dextrose anticoagulant and plasma was prepared by centrifugation and separation from cellular components [6]. The mean donor age was 42.5 years (range 17 – 66 years), included different blood groups (13 O, 11 A, 4 B and 2 AB) and 47% were male blood donors. FFP was thawed in a water bath at 37 °C according to standard AABB operating procedures [6] and kept at 4 – 6 °C for five days. Residual cells were analyzed immediately while for other assays, aliquots were taken from each of the 30 donor units on day zero (FFP-0) and day five (FFP-5), frozen, and kept at –80 °C until used for simultaneous testing. For MP analysis, platelet-free plasma was prepared by serial centrifugations (15 minutes at 1,500 ×g and 2 minutes at 13,000 ×g) before freezing at –80 °C [14]. When required for analysis, plasma aliquots were thawed for 10 minutes at 37 °C and analyzed within 2 hours.

Residual cells

Phenotypes and concentrations of residual cells were assayed by flow cytometry [15]. (Coulter EPICS XL, Beckman Coulter, Miami, FL) using cell-lineage specific monoclonal antibodies to identify platelets (Fluorescein isothiocyanate [FITC]-conjugated anti-human glycoprotein IIb, CD41), RBC (Phycoerythrin [PE]-conjugated anti-human glycophorin A, CD235a), leukocytes (Phycoerythrin-Cyanin 5 [PC5]-conjugated anti-human pan-leukocyte CD45), monocytes (PC5-conjugated anti-human lipopolysaccharide receptor, CD14), and endothelial cells (PE-conjugated anti-human VE-cadherin, CD144). FITC-, PE- or PC5-conjugated isotype control mouse immunoglobulin G1 were used as negative controls. All antibodies were from Beckman Coulter. Aliquots of plasma (10 μ L) were incubated with antibodies or isotypes (10 μ L) for 15 minutes in the dark, diluted to a final volume of 1 mL, and analyzed immediately. Data were acquired over 120 seconds at the flow rate of 0.5 μ L/s, and results are expressed as cell counts per 10^6 /L of plasma. List mode data files, acquired by flow cytometry, were analyzed using EXPO32 ADC Software.

Cellular microparticles

Microparticle counts and phenotypes were analyzed by multicolor flow cytometry [11,14]. MPs were defined as particles less than 1 μ m in size. Phenotypes include platelet, RBC, leukocyte, monocyte, endothelial cell, tissue factor-positive, and total AnnexinV-positive MPs, which were assayed using the following antibodies: CD41-PE, CD235a-PE, CD45-PC5, CD14-FITC, CD144-PE, CD142-PE, and AnnexinV-FITC, respectively. The flow cytometry protocol for MPs was optimized using fluorescent beads measuring 0.5, 0.9, and 3 μ m (Megamix, Biocytex, Marseille, France), according to the manufacturer's instructions and the recommendations from the International Society on Thrombosis and Haemostasis SSC Collaborative workshop [16]. This bead acquisition allows the settings of the cytometer to study MPs within a constant size region (0.5 μ m – 1 μ m). Briefly, 30 μ L of plasma was stained with 10 μ L of lineage-specific monoclonal antibodies or with AnnexinV. After a 30 minute incubation at room temperature in the dark, samples were diluted with 0.22 μ m-filtered sheath fluid (Isoflow, Beckman Coulter) or AnnexinV binding buffer. The flowcount beads (Flow-Count, Beckman Coulter) with known concentration were added to samples before reading and used to calculate the absolute MP count ($\times 10^3$ /L) in plasma.

MP-associated procoagulant activity was measured using a functional prothrombinase assay according to manufacturers's protocol (Zymuphen MP-activity, Hyphen BioMed, Neuville-sur-Oise, France). Briefly, plasma samples supplemented with calcium, factor Xa, and thrombin inhibitors were added to a microtitre plate pre-coated with AnnexinV. A reagent containing calcium and bovine FXa-FVa was added, followed by human prothrombin. Thrombin generation was detected by a chromogenic substrate. The results were expressed as nM/Phosphatidylserine (PS) equivalents.

Thrombin generation assay

Hemostatic potential of FFP-0 and FFP-5 was evaluated by thrombin generation assay, using the Calibrated Automated Thrombogram (CAT) (Thromboscope, Maastricht, Netherlands), a global functional test of hemostasis [11,17]. The trigger reagents (Platelet Poor Plasma [PPP]-low, Platelet Rich Plasma [PRP], and MP) contained tissue factor (TF) and phospholipids in different proportions resulting in final concentrations of 1 pM TF/4 μ M PL, 1 pM TF/no phospholipid, and no TF/4 μ M phospholipid respectively, enabling increased assay sensitivity for detection of plasma MPs. Upon addition of fluorescent thrombin substrate and calcium chloride, CAT renders the a thrombin generation curve that provides information on several parameters including the lag time (representing the time until initial thrombin had formed (min), the thrombin peak height (nM thrombin), the time to peak (ttPeak, min), the endogenous thrombin potential (ETP) (reflects the area under the

curve), the rate of thrombin generation (mean slope=peak height/[time to peak- lag time]), and the start tail (min).

Thromboelastography (TEG)

The clotting properties of thawed FFP-0 and FFP-5 were evaluated using modified TEG analysis (TEG 5000 Thromboelastograph Analyzer, Haemoscope, Niles, IL), which measures the kinetics of clot formation and clot strength and stability [18]. Clot formation was triggered with a recombinant TF (Recombiplastin, Instrumentation Laboratory) final concentration of 3pM [11]. The analyzed TEG parameters included split point (representing the time to initial fibrin formation), reaction time to clot initiation, the speed of clot propagation (α -angle), final clot strength as a maximum amplitude, time to maximum amplitude, and the maximum elastic modulus. The analysis also included a set of parameters from the mathematical first derivative of the standard TEG: maximum rate of thrombus generation, time to maximum rate of thrombus generation, and total thrombus generation, as potentially more sensitive measures of clot growth [19].

Platelet factor 4 (PF4)

PF4 in plasma was measured using a commercial ELISA kit (IMUCLONE, American Diagnostica Inc) according to manufacturer's instructions.

Plasma filtration

To evaluate the effect of MP count reduction on procoagulant activity and plasma's clot forming abilities, the MP assay, CAT, and TEG were performed on a subset (n=16) of filtered plasma samples, and data were compared to unfiltered samples. We used a microplate filtration device with low protein binding filter to remove MPs larger than 0.2 μ m from the plasma (Ceveron MFU-500, Technoclone, Austria). Furthermore, to see if the recovery of the MP count to a baseline (pre-filtration) will restore hemostatic potential, we performed additional experiments. MPs were recovered from filters and returned to both the filtered and unfiltered plasma, generating 4 groups of samples: unfiltered, filtered, unfiltered +MP, and filtered+MP. We then ran the thrombin generation assay on those samples and compared thrombogram data.

Statistical analysis

Results are expressed as median (interquartile range [IQR]) levels and/or mean $\pm$ standard error of means (SEM) when appropriate. Statistical differences between FFP-0 and FFP-5 were evaluated with Student's *t*-test for paired samples. Statistical comparisons of mean activities in blood type O vs. non-O, and males vs. females were accomplished using two-sample *t* test assuming either equal or unequal variance, as appropriate. Pearson's correlation test was used to determine correlation coefficients (*r*) and *p*-values. A *p*-value of less than 0.05 was considered statistically significant.

Results

Residual cells

Flow cytometry results were recorded and analyzed for residual cells. The median concentrations (IQR) and percentages of residual cells are presented in Table 1. The majority of residual cells (94%) were platelets, and less of other cell types. Residual platelets were activated as evidenced by their light scatter properties and increased expression of the glycoprotein IIb (presented as median fluorescent intensity): 198 (IQR 184 – 302) vs. 116 (IQR 103 – 186), *p*<0.01, compared to non-activated platelets. A representative example of platelet activation and staining with anti-glycoprotein IIb (CD41)

antibody in TP compared to non-activated fresh platelets is presented in Fig. 1(A, B). Platelet activation was also demonstrated by the elevated plasma levels of PF4 (median 0.58 $\mu\text{g/ml}$, IQR 0.34 – 0.69 $\mu\text{g/ml}$), which strongly correlated with platelet counts ($r=0.97$, $p<0.001$) and expression of CD41 ($r=0.82$, $p<0.001$).

Cellular microparticles

Next, we analyzed the presence of MPs in FFP. The median total AnnexinV⁺ MP concentration in FFP-0 was $5130 \times 10^3/\text{L}$. Several cellular MP phenotypes were identified based upon their source of origin: 87.5% platelet, 4.9% erythrocyte, 6.9% leukocyte, 0.2% monocyte, and 0.5% endothelial (Table 2). Quantities of platelet MPs correlated with the number of residual platelets in thawed plasma ($r=0.76$, $p<0.001$). The majority of platelet MPs (92%) bound AnnexinV on their surface (CD41⁺/AnnV⁺), thereby demonstrating expression of PS. However, only a small proportion (2.2%) of TF bearing MPs were detected. A representative example of platelet MP dual staining with AnnexinV and CD41 in FFP-0 and FFP-5 is presented in Fig. 2. After 5 days of plasma storage, we noted a significant decrease in MP counts, with a 48% decrease in total MPs, 50% decrease in platelet MPs, and up to 11% decrease in other phenotypes (Table 2 and Fig. 3). Analysis of the MP procoagulant activity in FFP-0 revealed the median concentration of PS equivalents of 21 nM (IQR 13.8 – 28.1 nM), which significantly decreased by 29% during storage to 13.5 nM PS (IQR 9.5 – 20.4 nM), $p<0.0001$. There was a significant correlation between MP procoagulant activity and the MP count measured by flow ($r=0.69$, $p<0.001$).

Thrombin generation assay

Analysis of FFP-0 thrombograms with PPP-low reagent demonstrated a wide range of values between donors, particularly for the peak thrombin (range 87–227 nM) and ETP (984–1751 nM thrombin/min). All the values changed significantly in FFP-5 (Table 3). However, peak thrombin and the rate of thrombin generation emerged as the best discriminators between fresh and aged plasma. FFP-5 exhibited only 54% of the potential for thrombin generation of FFP-0. Importantly, when PRP and MP reagents were used, which were designed to detect MP-associated thrombin generation, there was a significant decline in all parameters during five days of plasma storage (Table 3). Representative thrombogram tracings from single donor FFP-0 vs. FFP-5 with PPP-low, PRP and MP reagents are presented in Fig. 4.

Clot formation evaluated by TEG

Storage related abilities of plasma to form a fibrin clot, evaluated by TEG, significantly declined, resulting in a longer time to the initial fibrin formation, longer reaction time, longer time to maximum clot, and a longer time to maximum rate of thrombus generation in FFP-5 compared to FFP-0 (Table 4). Other parameters measuring the speed of clot propagation and clot strength showed a tendency towards lower values, but did not reach a statistically significant level. The TEG reaction time to clot initiation weakly correlated with the CAT lag time ($r=0.35$, $p<0.05$), as did TEG time to maximum rate of thrombus generation to CAT ttPeak ($r=0.40$, $p<0.05$). No significant correlation was found between total thrombin generation and ETP, and TEG maximum rate of thrombus generation with CAT peak or rate. There was no significant difference in residual cell or MP counts, MP procoagulant activity, CAT or TEG parameters when analyzed by age, gender, or ABO blood group (blood group O vs. non-O) (data not presented).

Effects of plasma filtration

To evaluate the contribution of MPs to plasma's hemostatic potential and clot forming abilities, we assayed a subset of plasma samples pre- and post-filtration (0.2 μm) by flow

cytometry, MP procoagulant activity, TEG, and CAT. There was a significant decrease in the number of CD41⁺/AnnexinV⁺ MPs post-filtration: median $110 \times 10^3/\text{L}$ (IQR 93–160) vs. $2427 \times 10^3/\text{L}$ (IQR 1697 – 3086), $p < 0.001$. This indicates filtration removed 95% of platelet MPs. Analysis of MP procoagulant activity in filtered plasma resulted in a 36% decline compared to unfiltered (median 17.5 (IQR 12–20) vs. 27.2 (IQR 22–34.1), $p < 0.0001$). Similarly, comparisons of plasma TEG parameters revealed significant changes in FFP-0 and FFP-5 comparisons, including delayed split point and reaction time ($p < 0.05$), and decreased maximum amplitude and maximum elastic modulus ($p < 0.05$), thus linking MPs to clot formation and strength (Table 5). Similarly, when thrombogram parameters were compared pre-and post-filtration, we found lag time [5.7 min (IQR 5.3 – 6.2 min) vs. 6.2 min (IQR 5.5 – 7.1 min), $p < 0.01$], peak thrombin [(67 nM (IQR 50 – 82 nM) vs. 44 nM (IQR 32 – 60 nM), $p < 0.01$], and ETP [(1043 nM*min (IQR 907–1240) vs. 629 nM*min (IQR 539–784), $p < 0.01$], were significantly lower post-filtration. Importantly, after recovering and returning MPs to filtered plasma, thrombogram parameters were restored to the baseline values. Furthermore, when recovered MPs were returned to the original plasma (unfiltered), thrombogram values were greater than the original. A representative example of thrombograms pre- and post-filtration and after MP replacements is presented in Fig. 5.

Discussion

Our study confirms that TP units from a commercial blood bank vendor contain residual cells, predominately platelets, and high levels of platelet MPs. Although quality control requirements for blood components differ between US and Europe, and AABB does not require testing for residual cells, our results indicate that FFP meets the Council of Europe standards [20]. Residual platelets were highly activated during the freeze-thaw cycle and storage, which was evidenced by light scatter characteristics, increased expression of membrane glycoprotein IIb, and elevated levels of PF4 in TP. In general, pre-activated platelets have reduced post-transfusion recovery and survival times and are less responsive to physiological agonists. In addition, the post-transfusion effects of released platelet cytokines are uncertain. It is well recognized that refrigeration (4–6 °C) activates platelets, and once activated, platelets release procoagulant MPs which support hemostasis by promoting blood coagulation and adherence to the damaged vessel wall. This presence of pro-coagulant MPs would confer an advantage in patients with active bleeding. MPs are small cellular membrane fragments (<1 µm) shed from activated or apoptotic blood and endothelial cells. MPs are considered essential for hemostasis, as they expose phospholipids on their surface providing binding sites for activated clotting factor complexes. The importance of platelet MPs was emphasized by showing that the platelet MP surface is 50–100 fold more procoagulant than activated platelets [21]. Recent animal studies have shown circulating MPs accumulate, initiate, and support thrombus formation at the wounded area [22]. The measurement of MPs is currently a subject of intense investigation, given the range of conditions in which they are reported [23,24].

In the current study, we observed the cellular origin of MPs in TP was similar to that normally seen in the peripheral blood, with platelet MPs being the most abundant. Quantities of platelet MPs correlated with the number of residual platelets, suggesting that the standard freeze-thaw cycle likely contributes to MP formation. The majority of platelet MPs expressed PS, while a small proportion of MPs expressed TF. During the standard five day storage, platelet MP count and activity significantly decreased and this reduction was associated with the decline in hemostatic potential of stored plasma. Return of removed MPs to the original plasma restored its baseline hemostatic potential, while adding the excess of MPs resulted in increased thrombin generation. We found increased levels of platelet MPs and low levels of other MP types, which differs from reports of elevated levels of both platelet- and RBC-derived MPs in FFP prepared after an overnight hold of whole blood [12].

The difference may be due to different protocols for production of blood components, and/or use of different leukodepletion filtration systems, or storage time before separation of plasma.

Thrombogram analysis has been increasingly used to evaluate various inherited or acquired bleeding or thrombophilic conditions, and it is often correlated with the risk prediction for bleeding or thrombosis [25–27]. CAT measures the overall potential of plasma to generate thrombin as the central mediator of fibrin generation [17,28], while TEG measures kinetics and strength of fibrin clot formation. We have previously documented decreased plasma clotting properties after five days of storage by both CAT and TEG assays [11]. In the current study, the contribution of MPs was demonstrated by removal of MPs via filtration, resulting in a lower clotting capacity. Plasma filtration did not affect clotting factor levels (data not presented). Hence, in addition to widely reported declines in clotting factors in stored plasma [7–9,11], we now report on significant decrease in MP counts and activity. Our findings are consistent with previous studies showing the ability of MPs to propagate thrombin generation [12,29,30]. Here we further demonstrate that recovery of MPs restores or even increases plasma's hemostatic properties.

The reason for MP decrease during plasma storage is not clear. MPs in stored TP may degrade into smaller size particles over five days of refrigeration by multiple mechanisms, including the activity of extra-cellular phospholipases, such as phospholipase A₂ [31]. Platelet MPs were also reported to undergo opsonization with proteins such as complement proteins, and can be cleared from circulation by phagocytosis [32]. Plasma filtration resulted in decreased MP procoagulant activity, as expected, however, some activity still remained, suggesting smaller MPs (<0.2 µm in size) may be present. It may be assumed that MP procoagulant activity is influenced by MP size, not only by their number. Conversely MPs may create larger aggregates or fuse with activated platelets which fall outside the MP size gate and have less activity. AABB guidelines suggest transfusing through 170–260 µm filters, which likely would not remove MP aggregates. The lifespan of MPs in stored blood products and in various clinical conditions will need to be determined.

Our finding of MP decline during standard TP storage may potentially be relevant for the capacity/potential of stored plasma to correct impaired hemostasis in patients who are massively transfused. Clinical relevance of MPs in supporting coagulation was documented in leukemic and thrombocytopenic patients, showing patients with high levels of circulating MPs were free of bleeding, despite low platelet counts [33]. Also, the presence of high levels of MPs in cryo-precipitate is considered to contribute to its therapeutic effects in bleeding patients [34]. The potential clinical relevance of MPs in stored TP used for massively transfused patients remains to be evaluated. Trauma results in major perturbations of the coagulation system and significant and systemic damage to the endothelium. Multiple studies have already shown an association between poor outcome and age of RBC transfusions [35]. Administering aged TP units with diminished hemostatic potential may provide adequate hemostasis for minor surgery, however, they may be insufficient to correct bleeding and coagulopathy in cases of massive transfusion. Importantly, the balance between hemostasis and thrombosis, as well as pro- or anti-inflammatory and pro-thrombotic properties will need to be evaluated. As mentioned earlier, the use of freshly thawed plasma may be beneficial for the initial treatment of massively bleeding trauma patients by promoting hemostasis, but may subsequently contribute to thrombosis. The balance between benefit and risk of reduced platelet MP content in thawed stored plasma in the clinical setting is completely unknown. It is also of great relevance to take into consideration other aspects and potential side effects of transfusing ABO non-identical FFP, which was reported to be associated with dramatic increases in Acute Respiratory Distress Syndrome and sepsis [36] or increased morbidity and mortality [37]. In addition to its

hemostatic potential, plasma may also have other biological activities when transfused. Our recent study [9] showed freshly TP was less inflammatory, while aged plasma exacerbated the permeability and inflammation of endothelial cells *in vitro*. Prospective studies are needed to evaluate the clinical significance of our findings.

This study has some limitations. First, all analytes, except residual cells, were measured in twice-frozen samples which could affect results. However, since storage at ultra low temperatures minimizes analyte degradation and all samples in our study were treated in the same manner, we do not believe that the refreezing process had any significant impact on analyte activity. Second, we performed FFP analysis only at two time points, at day 0 and 5. Including a more detailed time course may help identify specific kinetics of the MP lifespan and decline, and possibly the way to prevent it. Third, although flow cytometry is the most commonly used method for enumeration of MPs in clinical samples, it has size detection limits. Hence, MPs of smaller sizes remain undetected. Finally, the *in vivo* correlates and the clinical risks vs. benefits associated with transfusion of aged plasma need to be evaluated. At this point, no data exist as to whether these *in vitro* findings change clinical outcomes, for better or worse.

In summary, our results document the presence of residual cells and cellular MPs in FFP. After five days of storage, MPs significantly decrease in number, as does the plasma thrombin generating potential and clot forming capacity. Hence, we speculate that the presence of platelet MPs in TP may be beneficial and protective in the initial treatment of patients with hemorrhagic shock.

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Abbreviations

AABB	American Association of Blood Banks
CAT	Calibrated Automated Thrombogram
ETP	Endogenous Thrombin Potential
FITC	Fluorescein Isothiocyanate
FFP	Fresh Frozen Plasma
IQR	Inter Quartile Range
MFI	Median Fluorescence Intensity
MP	Microparticle
PC5	Phycoerythrin-Cyanin 5
PE	Phycoerythrin
PF4	Platelet Factor 4
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PS	Phosphatidylserine

RBC	Red Blood Cell
TEG	Thromboelastography
TF	Tissue Factor
TP	Thawed Plasma

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Fig. 1.

A representative example of platelet (PLT) activation in thawed plasma analyzed by flow cytometry. A. Dot plot of forward scatter (FS) and CD41 fluorescence of freshly isolated (non-activated) and activated PLT from thawed plasma. B. Histograms gated on PLT to show the fluorescence intensity of CD41. MFI=median fluorescence intensity.

Fig. 2.

Analysis of microparticles (MPs) in FFP-0 and FFP-5 by flow cytometry. MPs are gated within predefined size gate (0.5 μm – 1 μm) in a dot plot of forward scatter (FS) vs. side scatter (SS). Platelet MPs are identified as events double labeled with CD41 and AnnexinV in a dot plot of FL1 vs. FL2. FFP-5 shows lower number of MPs compared to FFP-0. FFP-0, thawed plasma at day 0; FFP-5, thawed plasma at day 5.

Fig. 3. Decline in cellular microparticle (MP) counts between FFP-0 and FFP-5. TMP, total AnnexinV positive MP; PMP, platelet MP; RMP, red blood cell MP; LMP, leukocyte MP; MMP, monocyte MP; EMP, endothelial cell MP. FFP-0, thawed plasma at day 0; FFP-5, thawed plasma at day 5. * $p < 0.0001$.

Fig. 4.

Thrombograms of FFP-0 and FFP-5 with three different trigger reagents (PPP-low, PRP, and MP) showing thrombin generation decline in plasma stored for 5 days. FFP-0, thawed plasma at day 0; FFP-5, thawed plasma at day 5.

Fig. 5.

Thrombograms of thawed plasma, pre and post-filtration, and after recovery of MPs. FFP, thawed fresh frozen plasma; FFP-filtered, plasma after passing through 0.2 μ m filter; FFP-filtered + MP, MPs were recovered from the filter and returned to filtered plasma; FFP + MP, MPs were recovered from the filter and returned to the original FFP.

Table 1

Residual cell phenotypes in FFP-0.

	Cells ($\times 10^9/L$)		%
	Median	IQR	
Platelets	8.45	(3.55 – 21.52)	94.1
Red blood cells	0.14	(0.08 – 0.39)	1.6
Leukocytes	0.11	(0.07 – 0.26)	0.9
Monocytes	0.08	(0.07 – 0.15)	1.2
Endothelial cells	0.19	(0.09 – 0.49)	2.2

Table 2

Microparticle phenotypes in FFP-0 and FFP-5, median (IQR).

MP count ($\times 10^3/L$)	FFP-0	FFP-5	% change	p
TMP (AnnV ⁺)	5130 (2650 – 11240)	2814 (1296 – 4613)	47.8	≤ 0.0001
PMP (CD41 ⁺)	4408 (2060 – 9046)	2441 (855 – 3040)	50.1	≤ 0.0001
CD41 ⁺ /AnnV ⁺	3848 (1563 – 7798)	1885 (757 – 2622)	51.5	0.002
RMP (CD235a ⁺)	246 (162 – 285)	184 (151 – 272)	7.1	NS
LMP (CD45 ⁺)	346 (166 – 693)	307 (200 – 629)	10.8	NS
MMP (CD14 ⁺)	13 (8 – 18)	10 (7 – 15)	3.6	NS
EMP (CD144 ⁺)	23 (16 – 40)	23 (16 – 33)	0	NS
TFMP (CD142 ⁺)	109 (68 – 172)	106 (88 – 151)	7	NS

Table 3

Thrombin generation results in FFP-0 and FFP-5 (all ABO groups), median (IQR).

	FFP-0	FFP-5	% change	P
<i>PPP-low reagent</i>				
Lag time (min)	3.5 (3.3 – 4)	4.4 (4–4.8)	22.3	≤0.0001
ETP (nM thrombin*min)	1300 (1239–1519)	1052 (836–1246)	23.2	≤0.0001
Peak thrombin (nM)	124 (91–155)	65 (53–105)	41.2	≤0.0001
Time to peak (min)	10 (8.6 – 10.7)	11.2 (9.8 – 13.3)	14.6	≤0.0001
Rate (nM/min)	20.6 (13.5 – 28.4)	10.7 (6.4 – 16.3)	45.6	≤0.0001
<i>PRP reagent</i>				
Lag time (min)	7 (6.8 – 7.7)	9.8 (8.7 – 11)	41.3	≤0.0001
ETP (nM thrombin*min)	1217 (1065–1348)	1062 (894–1111)	14.3	≤0.0001
Peak thrombin (nM)	85 (78–98)	56 (51–70)	36.7	≤0.001
Time to peak (min)	18 (15.5 – 19)	21 (20.3 – 22)	23.2	≤0.0001
Rate (nM/min)	7.6 (6.5 – 10.2)	5.3 (4.2 – 6.3)	40.1	≤0.001
<i>MP reagent</i>				
Lag time (min)	17.8 (16.6 – 23.5)	24.2 (21.6 – 29.8)	26.1	≤0.0001
ETP (nM thrombin*min)	618 (565–687)	345 (303–437)	40.3	≤0.0001
Peak thrombin (nM)	34 (30–42)	19 (15–25)	35.8	≤0.001
Time to peak (min)	25.8 (24–33)	36.4 (30–42)	23.5	≤0.0001
Rate (nM/min)	4.2 (3.1 – 5.2)	1.9 (1.3 – 2.8)	34.8	≤0.001

P=statistical significance of the difference between FFP-0 and FFP-5.

Table 4

Thrombelastography (TEG) values for FFP-0 and FFP-5 (all ABO groups), median (IQR).

Variable	FFP-0 (n=30)	FFP-5 (n=30)	P
Split point, SP (min)	2.1 (1.8–2.3)	3.2 (2.7–3.6)	≤0.0001
Reaction time, R (min)	2.8 (2.3–3.1)	3.9 (3.1–4.3)	≤0.0001
Alpha angle (degrees)	49.7 (44.8–55)	51.6 (41.5–55.8)	NS
Maximum amplitude, MA (mm)	25.9 (22.7–28.8)	26.3 (21.2–29.8)	NS
Time to MA (min)	14.4 (12.7–15.8)	16.1 (14.5–18.1)	≤0.01
Maximum elastic modulus, G (dyn/cm ²)	1.8 (1.5–2)	1.8 (1.3–2.1)	NS
Maximum rate of thrombus generation, MRTG (mm/min)	6.4 (4.8–7)	6.3 (4.6–7.2)	NS
Time to MRTG (min)	4 (3.3–4.5)	5.0 (4.1–5.6)	≤0.001
Total thrombus generated, TTG (mm/min)	345 (300–376)	347 (293–392)	NS

Table 5

Thrombelastography (TEG) values for FFP-0 and FFP-5 (all ABO groups), pre-filtration vs. post-filtration, median (IQR).

Variable	FFP-0	FFP-0	FFP-5	FFP-5
	Pre-filtration (n=16)	Post-filtration (n=16)	Pre-filtration (n=16)	Post-filtration (n=16)
Split point, SP (min)	2.0 (1.6 – 2.2)	2.2 (1.9 – 2.5) *	3.3 (2.5 – 3.6)	3 (2.8 – 3.3)
Reaction time, R (min)	2.8 (2.1 – 3.1)	2.8 (2.6 – 3.3)	4.1 (3 – 4.7)	3.5 (3.2 – 4.1)
Alpha angle (degrees)	50.8 (43.5 – 58.5)	50.5 (42.5 – 54.2)	52.1 (37.5 – 57.8)	49.2 (41.3 – 57.4)
Maximum amplitude, MA (mm)	24.8 (22.4 – 27.5)	22.8 (19.7 – 24.2) *	25.3 (20.7 – 28.3)	22.5 (17.4 – 23.7) **
Time to MA (min)	14.4 (11.8 – 15.5)	12.3 (10.5 – 16.2)	15.9 (14.5 – 18)	13.8 (11.9 – 16.5)
Maximum elastic modulus, G (dyn/cm ²)	1.7 (1.5 – 1.9)	1.5 (1.2 – 1.6) *	1.7 (1.3 – 2)	1.4 (1.2 – 1.5) **
Maximum rate of thrombus generation, MRTG (mm/min)	6.6 (4.9 – 7.8)	5.9 (4.4 – 7.4)	6.4 (3.9 – 7.8)	5.7 (4.3 – 7.3)
Time to MRTG (min)	3.6 (3.1 – 4.5)	3.7 (3.4 – 4.5)	5.3 (3.8 – 5.7)	4.3 (3.9 – 5.4)
Total thrombus generated, TTG (mm/min)	331 (299 – 362)	305 (262 – 325) *	328 (277 – 370)	286 (224 – 319) **

* p≤0.05, for the difference between pre-filtration vs. post-filtration.

** p≤0.01, for the difference between pre-filtration vs. post-filtration.