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Impaired platelet-dependent thrombin generation associated with thrombocytopenia is improved by prothrombin complex concentrates in vitro

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Abstract

Background: Impaired thrombin generation (TG) in patients with acquired coagulopathy, is due to low coagulation factors and thrombocytopenia. The latter is typically treated with platelet transfusions and the former with plasma and occasionally with prothrombin complex concentrates (PCCs). We hypothesized that manipulating the concentrations of coagulation factors might result in restoration of platelet-dependent TG over and above that of simple replacement therapy.

Objective: To investigate the influence of PCCs on impaired TG secondary to thrombocytopenia.

Methods: TG was evaluated by thrombin generation assay using a thrombocytopenia model in which normal plasma samples with varying platelet counts (20-300 × 10^9/L) were spiked with PCCs (25%-150% increase in plasma PCC levels).

Results: PCCs and platelets significantly increased TG in a dose-dependent manner in vitro. Two-way repeated measures of analysis of variance showed variance in peak height, area under the curve, time to peak, and velocity. This variance explained, respectively, by levels of PCC was 47, 59, 25 and 53%; by platelet count was 45, 28, 44, and 14%; by the combination was 80, 67, 70, and 62% variance; and a combination with additional interaction was 91, 84, 76, and 68%. TG at a platelet count 40 × 10^9/L with an approximate 25% increase in PCC concentration was similar to TG at 150 × 10^9/L. Similarly, patient samples spiked ex vivo with PCCs also showed highly significant improvements in TG.

Conclusions: Impaired TG of thrombocytopenia is improved by PCCs, supporting the need for additional studies in complex coagulopathies characterized by mild to moderate thrombocytopenia and abnormal coagulation.

KEYWORDS
acquired coagulopathy, blood coagulation disorders, blood platelets, prothrombin complex concentrates, thrombin, thrombocytopenia

Essentials

- Bleeding tendency of acquired coagulopathy (AC) is due to low coagulation factors and platelets.
- Increasing platelet count and clotting factors independently increases thrombin generation (TG).
- In an in vitro thrombocytopenia model, prothrombin complex concentrates (PCCs) normalized TG at standard doses.
- The effect of PCCs in AC is beyond simple replacement therapy.

1 | INTRODUCTION

Thrombin is the final common enzyme of the coagulation cascade and is responsible for fibrin polymerization and clot formation. Impaired thrombin generation is typically seen in acquired hemostatic abnormalities commonly caused by liver disease, major trauma, critical illness, and dilutional coagulopathy, and can aggravate ongoing bleeding or increase the risk of bleeding. These abnormalities present singly or as variable combinations of coagulopathy, thrombocytopenia, hypofibrinogenemia, and hyperfibrinolysis.[1] Restoration of thrombin generation (TG) and clot formation are vital for arresting or preventing bleeding. Treatments for these abnormalities include plasma or prothrombin complex concentrates (PCCs), platelet transfusions, fibrinogen or cryoprecipitate infusions, and tranexamic acid.

Treatment is instituted either prophylactically to prevent periprocedural bleeding or therapeutically for the management of active bleeding. The pathogenesis of bleeding in these situations is multifactorial, and patients often receive more than 1 form of therapy for correction of abnormalities. Thresholds have been defined by various guidelines for transfusion of plasma, PCC, platelets, cryoprecipitate, fibrinogen, and tranexamic acid.[2–8] However, there are very few data available on the interaction between the various deficient components and TG and clot formation.

Adequate TG is dependent on the interaction of the procoagulant and anticoagulant pathways along with platelets and tissue factor–bearing cells.[9] Increasing platelet count and prothrombin concentration independently demonstrate significant impacts on the rate and magnitude of TG.[10,11] Variation in levels of prothrombin and antithrombin within the normal range cause significant changes in the rate, peak, and total amount of thrombin generated in in vitro models.[12,13]

Similarly, decreasing platelet counts have been associated with lower maximal rate of TG, lower peak, and time to peak (TTP) thrombin in models of thrombocytopenia, particularly at counts < 50 × 10^9/L.[14] The rate of TG and peak TG continue to increase until a platelet count of 300 to 500 × 10^9/L when values plateau, although the area under the curve (AUC), lag time, and TTP plateau earlier at around 100 × 10^9/L.[15] A 3- to 5-fold variation in the ability of platelets to support TG has also been demonstrated.[16]

The use of prohemostatic agents for management of thrombocytopenia is not a new concept. In a small clinical study, administration of recombinant factor VIIa (rFVIIa) stopped overt bleeding in 6 of 8 patients with severe thrombocytopenia.[17]

In the context of impaired platelet-dependent TG, demonstrable with thrombocytopenia, we hypothesized that the addition of PCCs will result in restoration of TG. PCCs were chosen rather than purified coagulation factors as PCCs are regularly used for the management of acquired coagulopathy. Furthermore, standard doses of PCCs provide a greater concentration of clotting factors in comparison with standard doses of plasma, and the effect is similar to that seen with higher volumes of plasma.[18,19] To investigate our hypothesis, we evaluated the influence of PCCs on TG in an in vitro model of thrombocytopenia over a range of platelet counts (20-300 × 10^9/L) by thrombin generation assay (TGA). This was further evaluated by TGA analysis of blood samples from patients with thrombocytopenia and varying degrees of coagulopathy following ex vivo spiking with PCCs.

2 | METHODS

2.1 | Study design

This in vitro/ex vivo spiking laboratory study was performed on venous blood samples from healthy volunteers and patients with thrombocytopenia. The study had ethical approval from the Katharine Dormandy Coagulation Research Plasma Bank (REC No:14/YH/1272) and relevant institutional approvals. Written informed consent was obtained from all patients and volunteers.

2.2 | Healthy volunteers and patients

Five healthy volunteers and 21 patients with thrombocytopenia (platelet count < 80 × 10^9/L) were included. To understand if the effect of PCC was related to underlying coagulopathy, 2 groups of patients with thrombocytopenia were identified. The first was patients with liver disease with acquired coagulopathy (International Normalized Ratio [INR] >1.5), and the second was thrombocytopenia

secondary to myelosuppressive chemotherapy with no active infection.

2.3 | Venous blood collection and sample preparation

Thirty milliliters of venous blood for TGA analysis was collected from antecubital veins with a 21-gauge butterfly needle into citrated S-Monovette tubes (citrate 3.2%; Sarstedt AG, Numbrecht, Germany) with added corn trypsin inhibitor (CTI; final concentration 18 μg/mL; Cambridge Biosciences, Cambridge, UK).

Blood from 5 healthy volunteers was rested for 30 minutes at room temperature, and platelet-rich plasma (PRP) was prepared by centrifugation for 15 minutes at 190 *g*. Platelet-poor plasma (PPP) was prepared from the remaining sample by double centrifugation at 2000 *g* for 15 minutes. PRP with a range of platelet counts was prepared by resuspending the PRP of a known platelet count with autologous double-spun PPP, with counts confirmed using an XS-1000i full blood count analyzer (Sysmex Corporation, Kobe, Japan).

2.4 | PCC concentration for spiking experiments

The PCC used was Beriplex (CSL Behring, Marburg, Germany) containing procoagulant proteins factor II (prothrombin), factor VII, factor IX, and factor X; anticoagulant proteins C and S; and small quantities of antithrombin and heparin.[20] Since even small quantities of heparin interfere with the coagulation assays,[21] it was neutralized with Hepzyme (removes ≤ 2 USP U/mL unfractionated heparin from 1 mL plasma; Dade Behring/Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany), and removal was confirmed by an anti-Xa heparin assay.[22] Clotting factors were measured and found not to be affected by Hepzyme treatment (results not shown). Beriplex was reconstituted according to the manufacturer's instructions and, following treatment with Hepzyme, frozen in aliquots at −45°C.

In line with PCC labeling, the assigned factor IX potency was used as the reference for quantifying the amount of PCC used for spiking; hereafter, the strength of PCC concentration refers to the labeled potency. The concentration of factor IX in the reconstituted lot of PCC after treatment with heparinase was 23 IU/mL, while the concentrations of prothrombin, factor X, factor VII, protein C, and protein S were 22, 30, 19, 30, and 25 IU/mL, respectively, as measured by 1-stage clotting assays (prothrombin, factor VII, factor IX, and factor X), chromogenic assay (protein C activity), and latex antigen (free protein S).

Four concentrations of PCCs were used in the spiking experiments, and the lowest strength was 3.3 μL of reconstituted heparinase-treated PCC at 1:3 dilution in Owren's Buffered Saline (OBS), followed by 3.3 μL at a 1:1 dilution in OBS, and 3.3 μL and 4.9 μL undiluted PCC, equating to an increase of PCC concentration in the plasma of 0.26 IU/mL, 0.52 IU/mL, 1.03 IU/mL, and 1.53 IU/mL.

2.5 | TG and in vitro spiking with PCCs of thrombocytopenic plasma obtained from healthy volunteers

The TG assay was undertaken using a Spectramax i3x microtiter plate reader (Molecular Devices UK Ltd, Berkshire, UK), where TG was measured using the same substrate as the Thrombinoscope method (in the absence of calibrator) in a microtiter plate (Immulon 2B, Thermo Fisher Scientific, Waltham, MA, USA), and recorded in relative fluorescence units (RFU).[12,23,24] Initial TG experiments were conducted on a calibrated automated thrombogram (CAT) machine (Thrombinoscope BV, Maastricht, The Netherlands) including incorporation of an internal calibrator.[25] Increases in PCC at the higher end resulted in depletion of the thrombin-specific fluorescent substrate and an inability to bring assays to closure. In patient samples, the highest dose was dropped and samples analyzed on a CAT.

TG was undertaken in PRP with a range of platelet counts (20, 40, 60, 90, 150, 225, and 300 × 10^9/L).

For the TG assay, 20 μL of 0.5 PM tissue factor (TF) trigger (Dade Innovin, Siemens Healthcare Diagnostic Products GmbH, Marburg, Germany) was added to a 96-well round-bottomed plate in duplicate for each test. Eighty microliters of PRP was added, and the plate was incubated for 5 minutes at 37°C inside the microtiter plate reader. TG was initiated with the addition of 20 μL FluCa (0.1 M $CaCl_2$/Fluorogenic substrate 2.5 mM Z-Gly-Gly-Arg-AMC. HCl; Thrombinoscope BV, Maastricht, The Netherlands) and measured kinetically at 390/460 nM over 60 minutes at 37°C. All samples were measured in duplicate using PRP at each platelet count, while PPP, with and without added PCCs, was run in parallel. The control included PPP in which TG was triggered with the same 0.5 PM TF trigger without added phospholipid. This was done to identify any significant background activity related to PCCs alone and as a comparator to demonstrate the impact of increasing platelet count. Measured and derived TG parameters analyzed included peak height (PH) in RFU, AUC in RFU, TTP in minutes, and the maximum rate of TG in RFU/sec (V_{max}). PH, AUC, and TTP were calculated as previously described,[12,23] and V_{max} was provided by Spectramax i3x software (Soft Max Pro 6.4; Molecular Devices Ltd, San Jose, CA, USA). A single batch of freeze-thawed platelets was run as control for all experiments, and the interrun variability was 11% for the PH and 16% for AUC.

2.6 | Ex vivo spiking of patient samples—thrombin generation assay

Ten milliliters of blood from patients was collected into 3.2% sodium citrate tubes to which CTI had been added, as described above, and left at room temperature for 30 minutes. TG assays were performed on patients' PRP on the Spectramax without adjustment of platelet counts as for the normal donor samples, with and without PCC.

2.7 | Statistical analysis

For the in vitro thrombocytopenia model, TG at a platelet count of 150 × 10^9/L with no added PCC was considered normal and assigned a value of 0. The percent differences (percent increase or decrease) between normal and the range of PRP platelet counts spiked with increasing concentrations of PCCs for TG parameters PH, AUC, TTP, and V_{max} (RFU/sec) were calculated and data presented graphically.

Two-way repeated measures analysis of variance (ANOVA) was carried out on the healthy volunteer samples to assess the effects of PCC concentration and the PRP platelet count simultaneously, together and with the interaction between them (ie, additive and synergistic effects), for each of the 4 thrombin clotting parameters. Variances both between and within samples were summed, and the percentage of variance explained by PCC and platelets separately was calculated, as well as the percentage explained by these two variables in combination.

Similar analyses were carried out on repeated measures of samples from patients with thrombocytopenia to investigate the effect of adding PCC. We assessed the effect of any dose versus no dose at all and the effects of different doses. Repeated measures were available for each sample for every level of PCC concentration.

All analyses were carried out in Stata, version 15.1 (StataCorp LLC, College Station, TX, USA).

3 | RESULTS

3.1 | PCCs improve and increase thrombin generation in thrombocytopenic normal plasma in a dose-dependent manner

TG in PRP was measured over a range of platelet counts (20, 40, 60, 90, 150, 225, and 300 × 10^9/L) with and without the addition of 4 different strengths of PCCs (spiked plasma concentrations of 0.26, 0.52, 1.03, and 1.53 IU/mL). In the absence of PCCs, lower platelet counts were associated with lower PH, AUC, V_{max}, and increased TTP, and the AUC appears to plateau when platelet counts reach normal levels. Two-way repeated measures ANOVA showed that increasing concentrations of PCCs significantly increased PH and AUC, shortened TTP, and increased V_{max}/sec, $P < 0.001$ for all except TTP ($P = 0.01$). Similarly, increasing platelet count significantly increased PH, AUC, shortened TTP, and increased V_{max}, $P < 0.001$ for all except V_{max} ($P = 0.02$). Furthermore, the variance in PH, AUC, TTP, and V_{max} explained by levels of PCC was 47, 59, 25, and 53% respectively, and by platelet count was 45, 28, 44, and 14%, respectively, suggesting important contributions from both components. A combination of PCC and platelet count accounted for 80, 67, 70, and 62% variance and a combination with additional interaction between PCC and platelet count accounted for 91, 84, 76, and 68% variance, respectively.

Changes in TG parameters in relation to platelet count and PCC concentration are presented in Figure 1, where zero represents the "normal baseline TG" at a PRP platelet count of 150 × 10^9/L without the addition of PCCs.

Following ex vivo spiking with PCCs, similar increases of procoagulant factors prothrombin, factor VII, factor IX, and factor X, and anticoagulant proteins C and S were seen, which were proportionate to the dose (data not shown). This is in contrast to the increases seen clinically, where variable increases of different factors are seen due to the differences in the volume of distribution.

Increasing PCC concentration by 0.52 IU/mL with a platelet count of 20 × 10^9/L, and by 0.26 IU/mL with a platelet count of 40 × 10^9/L resulted in TG similar to that observed in normal plasma at a platelet count of 150 × 10^9/L (Figure 2).

3.2 | Thrombocytopenic patient blood samples following ex vivo spiking with PCCs show increases in thrombin generation

The findings in the in vitro thrombocytopenic model generated from healthy volunteers were replicated in PRP samples from patients with thrombocytopenia and varying severity of coagulopathy. Whole blood samples from patients with normal INR and low platelet counts secondary to chemotherapy between 7 and 73 × 10^9/L were analyzed without PCC and with PCC (spiked plasma concentrations 0.26, 0.52, and 1.03 IU/mL) similar to the healthy volunteer samples. Whole blood samples from patients with liver disease included a range of INRs between 1.6 and 2.2, platelet counts (26-72 × 10^9/L), and fibrinogen levels (<0.4-6.0 g/L). This group was included to assess the impact, if any, of concurrent coagulopathy.

The baseline values and results following spiked PCCs of patient samples in TG are presented in Table 1 and Figure 3. In samples from patients with liver disease with thrombocytopenia, spiking with PCC at any concentration was associated with statistically significant increases in PH, AUC, and V_{max} (Figure 3A-C) with no effect on TTP ($P = 0.19$; data not shown). Dose-dependent increases were also significant for PH, AUC, and V_{max} with no effect on TTP; data not shown). The lack of obvious impact on TTP may be related to the already shortened TTP.

Similarly, in samples from patients with thrombocytopenia secondary to chemotherapy spiking with PCC resulted in statistically significant increases in PH, AUC, and V_{max} (Figure 3 D-F) with no effect on TTP ($P = 0.21$; data not shown). Dose-dependent increases were also significant for PH, AUC, and V_{max} with some effect on TTP ($P = 0.011$; data not shown). These findings were consistent with those observed in the healthy volunteer thrombocytopenia model.

4 | DISCUSSION

Thrombin generation in plasma is influenced by both platelets and coagulation factors. High levels of coagulation factors, in particular prothrombin, and high platelet counts are associated with an

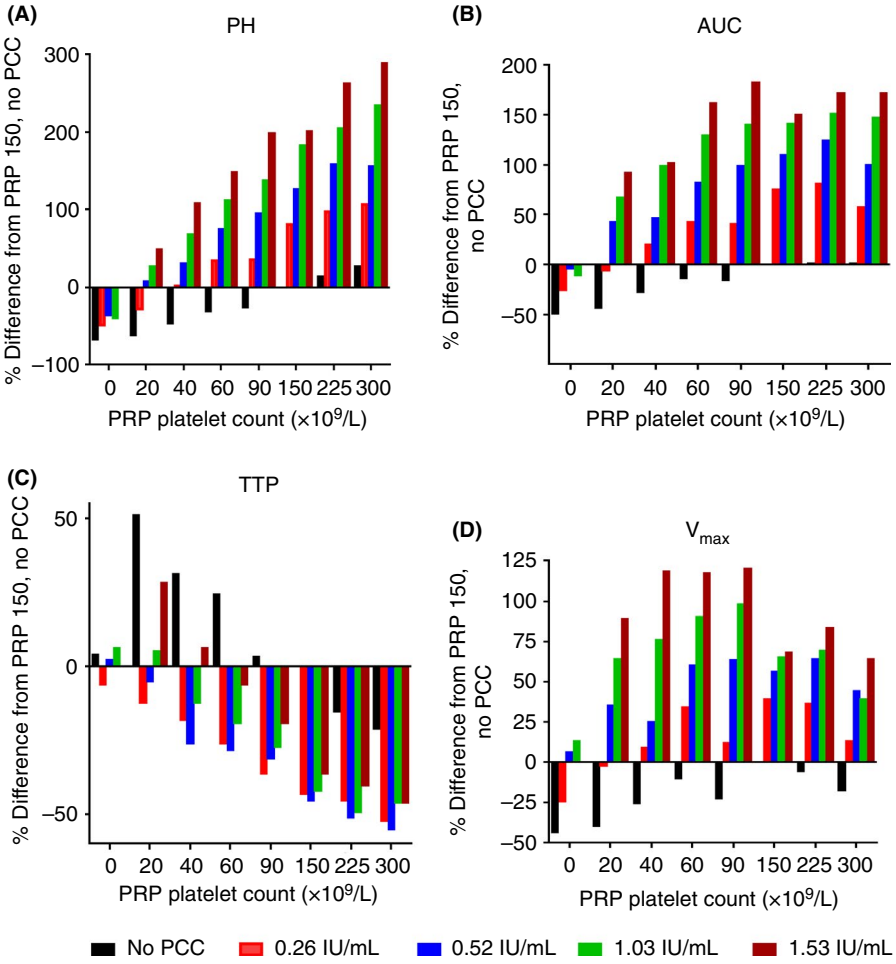

FIGURE 1 Percent change in PRP thrombin generation parameters (A) PH, (B) AUC, (C) TTP, and (D) V_{max} without PCC and with PCC over a range of platelet counts compared to "normal baseline" defined as TG at a PRP platelet count of 150 × 10^9/L without PCC. Normal baseline was defined as TG at a PRP platelet count of 150 × 10^9/L without PCC triggered with 0.5 PM tissue factor and is represented by the horizontal line at zero. Percent change in TG from baseline "0" before and after the addition of PCC was calculated over a range of PRP platelet counts (20-300 × 10^9/L) (n = 5 normal donors) and PPP without phospholipid, all triggered with 0.5 PM tissue factor. Four concentrations of PCC were added corresponding to an increase in plasma PCC concentration of 0.26 (red), 0.52 (blue), 1.03 (green), and 1.53 IU/mL (brown). No PCC is represented by black color. At around a platelet count of 90 × 10^9/L, the effect of increasing platelet count appears to plateau. PCCs at 0.26 IU/mL resulted in normalization of the TG assay, and at 0.52 IU/mL resulted in about a 1-fold increase in TG when compared to TG achieved at 150 × 10^9/L without PCC. At the same platelet count, the 2 higher concentrations resulted in TG parameters that were suggestive of hypercoagulability. AUC, area under the curve; PCC, prothrombin complex concentrate; PH, peak height; PPP, platelet-poor plasma; PRP, platelet-rich plasma; TG, thrombin generation; TTP, time to peak; V_{max}, maximum velocity

increased rate of TG (V_{max}), shorter TTP, higher peak thrombin, and increased quantity of thrombin (AUC).[10,11,13,26] We have demonstrated in an in vitro model of thrombocytopenia that increasing the concentration of procoagulants, through addition of PCCs available for clinical use, improved the decreased TG secondary to thrombocytopenia to within and above the normal range. The TG pattern seen following the addition of PCCs appears to be similar to that with increasing prothrombin concentration[26] and increasing platelet count.[15] The improvement in TG was confirmed in thrombocytopenic patient blood samples following spiking with PCCs. This effect might explain the slightly superior clinical effectiveness of PCCs demonstrated in acquired coagulopathy when compared to plasma.[27]

In our in vitro spiking experiments, the TG curve showed a dose-dependent improvement in all parameters in relation to increasing platelet count and increasing PCC concentration. The improvement with increases in platelet counts appears more modest compared to the change with higher concentrations of PCC, where no plateau was demonstrated despite the concurrent increase in proteins C and S. Further, higher PCC doses were associated with TG beyond the normal range. Our results show that TG is extremely sensitive to an increase in procoagulants, even in the presence of normal levels of clotting factors. In our study, the variance in PH, AUC, TTP, and V_{max} explained by PCC concentration or platelet count suggested important contributions from both components. The variance explained by PCC was 47, 59, 25, and 53% for PH,

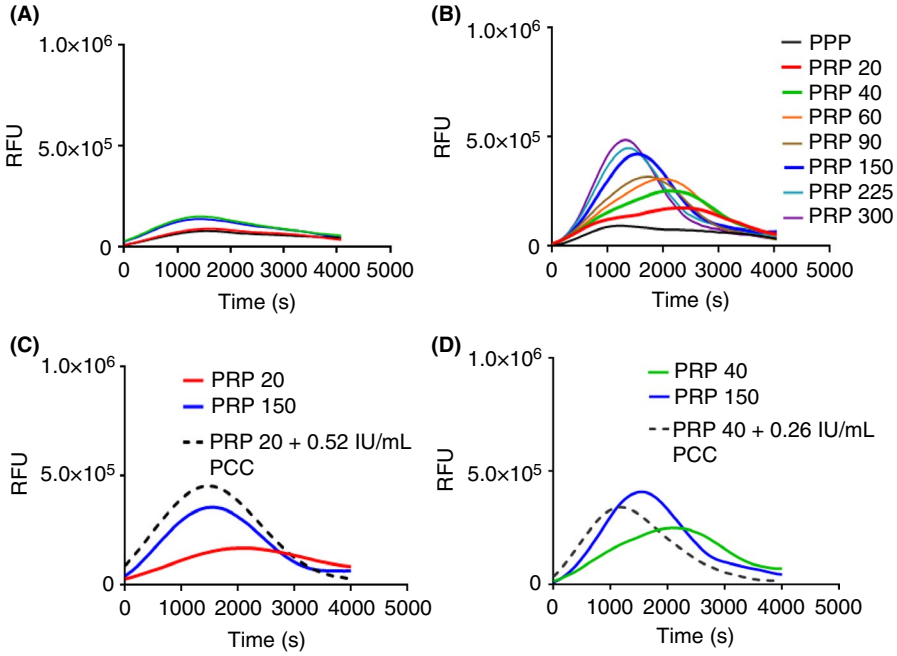

FIGURE 2 TG curves in (A) PPP, (B) PRP from one representative donor across a range of platelets counts (20-300 × 10^9/L), (C) PRP with platelet count of 20 × 10^9/L without PCC and added PCC compared to platelet count of 150 × 10^9/L without PCC, and (D) PRP with platelet count of 40 × 10^9/L without PCC and added PCC compared to platelet count of 150 × 10^9/L without PCC. TG is measured as change in RFU over time. PRP TG was measured over a range of platelet counts in parallel to PPP without phospholipid after triggering with 0.5 PM tissue factor. (A) TG in PPP at baseline without PCC (black), and with the addition PCC at 3 concentrations corresponding to 0.26 (red), 0.52 (blue), and 1.03 (green) IU/mL increase in PCC concentration is presented. (B) TG curves for PPP without phospholipid (black) and the range of increasing platelet counts (20, 40, 60, 90, 150, 225, and 300 × 10^9/L) where corresponding colors are indicated. PRP 150 × 10^9/L is colored blue. (C) TG at PRP count 20 × 10^9/L without PCC (red) and after the addition of PCC at 0.52 IU/mL (black hashed) is compared to PRP 150 × 10^9/L without PCC (blue). (D) TG at PRP count of 40 × 10^9/L without PCC (green) and after the addition of PCC at 0.26 IU/mL (black hashed) is compared to PRP 150 × 10^9/L with no PCC added (blue). PCC, prothrombin complex concentrate; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RFU, relative fluorescence unit; TG, thrombin generation

TABLE 1 Thrombin generation measurement before and after the addition of PCC at three concentrations

Spiked plasma concentration of PCC	Normal (n = 5), PRP 150			Normal (n = 5), PRP 90			Normal (n = 5), PRP 40			Thrombocytopenia with prolonged INR[a], n = 9			Thrombocytopenia with normal coagulation[b], n = 10		
	Peak	AUC	V_{max}	Peak	AUC	V_{max}	Peak	AUC	V_{max}	Peak	AUC	V_{max}	Peak	AUC	V_{max}
Baseline	0.41 (0.07)	560 (100)	0.16 (0.04)	0.31 (0.06)	480 (99)	0.13 (0.03)	0.22 (0.06)	420 (94)	0.12 (0.03)	0.26 (0.13)	310 (150)	0.092 (0.05)	0.27 (0.18)	400 (170)	0.12 (0.05)
0.26 IU/mL	0.73 (0.10)	970 (92)	0.22 (0.03)	0.55 (0.08)	780 (91)	0.18 (0.03)	0.41 (0.08)	660 (100)	0.17 (0.03)	0.38 (0.23)	550 (330)	0.116 (0.10)	0.37 (0.19)	560 (290)	0.18 (0.09)
0.52 IU/mL	0.91 (0.10)	1200 (150)	0.25 (0.05)	0.78 (0.12)	1100 (240)	0.26 (0.04)	0.52 (0.09)	820 (110)	0.20 (0.03)	0.57 (0.30)	706 (420)	0.23 (0.11)	0.43 (0.25)	660 (320)	0.22 (0.10)
1.03 IU/mL	1.1 (0.13)	1300 (150)	0.26 (0.03)	0.96 (0.13)	1320 (240)	0.31 (0.04)	0.68 (0.09)	1100 (97)	0.28 (0.03)	0.69 (0.85)	890 (490)	0.30 (0.16)	0.53 (0.36)	890 (540)	0.27 (0.13)

Note: Values for normal donor PRP at 3 platelet counts and patients with thrombocytopenia and abnormal coagulation (liver disease) and thrombocytopenia and normal coagulation (chemotherapy) are included. Values in table are given as × 10^6, presented as mean (1 standard deviation). The unit of measure is relative fluorescence units, where V_{max} is RFU/sec.

Abbreviations: AUC, area under the curve; INR, International Normalized Ratio; PCC, prothrombin complex concentrate; PRP, platelet-rich plasma; V_{max}, maximum velocity.

[a]Liver disease.

[b]Secondary to chemotherapy.

AUC, TTP, and V_{max}, respectively, while the variance accounted for by platelet count was 45, 28, 44, and 14%, respectively, with PCCs having the least impact on TTP and platelets on V_{max}. When these were combined, this increased to 80, 67, 70, and 62% of variance, respectively, and was even higher when a combination with additional interaction between PCC and platelet count was considered

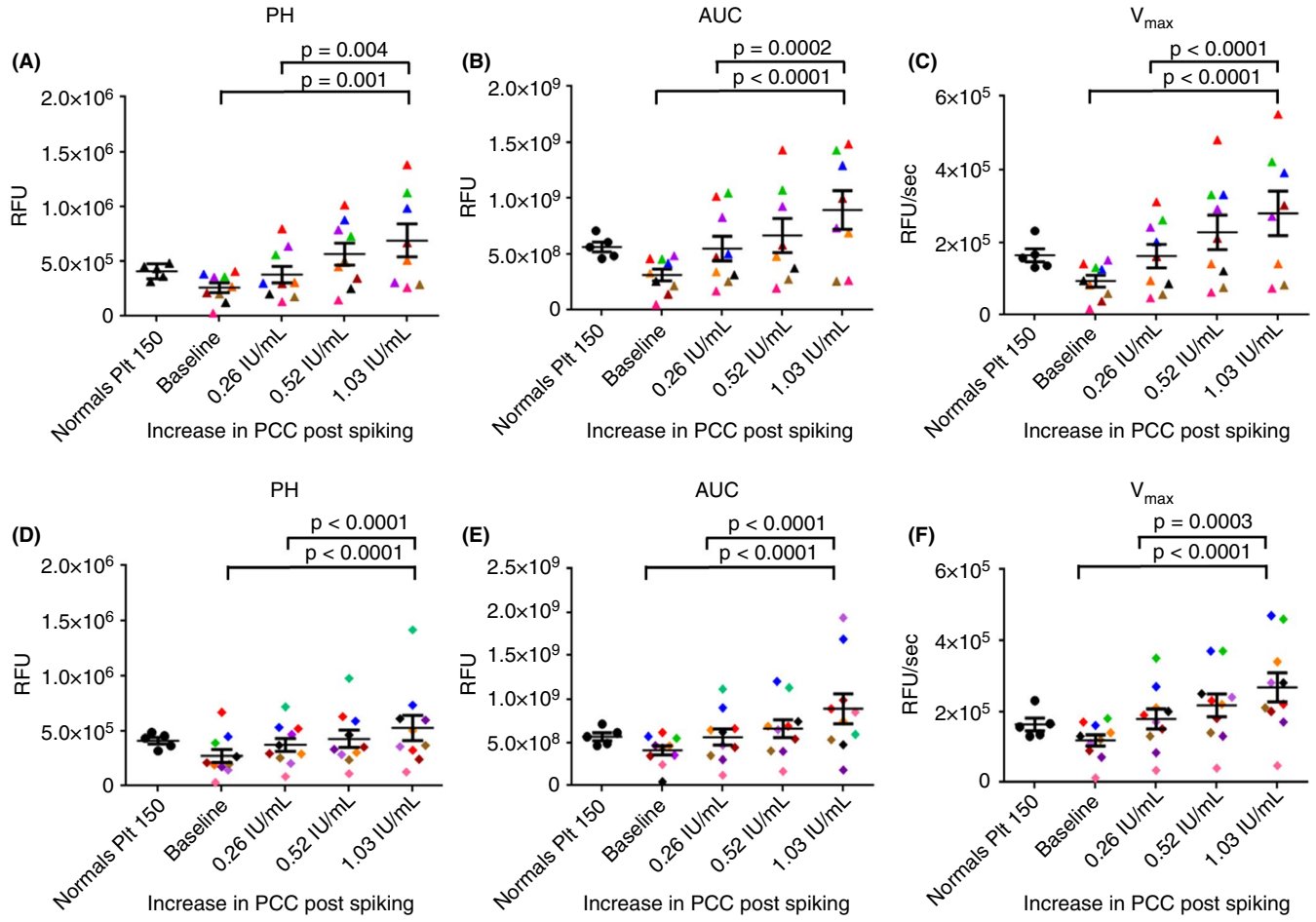

FIGURE 3 TG in thrombocytopenia samples from patients with and without the addition of PCC. TG was performed on PRP from patients with varying degrees of thrombocytopenia with liver disease (n = 9) (A–C) (platelet counts, 17-55 × 10^9/L and INR > 1.5) or thrombocytopenia secondary to chemotherapy (D–F) (n = 10) (platelet count 9-68 × 10^9/L); color indicates individual patient samples. TG parameters included PH measured in RFU, AUC in RFU, and velocity of thrombin generation, V_{max} (RFU/sec). TG was performed without PCC and PCC at 3 concentrations corresponding to 0.26, 0.52, and 1.03 IU/mL increase in PCC concentration. Corresponding values for normal healthy donors (n = 5, PRP 150 × 10^9/L) are included for comparison. Data are presented as individual plots with mean ± standard error of the mean. *P* values < 0.05 are considered statistically significant. *P* values are presented for comparison between no PCC and added PCC and between the doses of PCC. AUC, area under the curve; PCC, prothrombin complex concentrate; PH, peak height; PPP, platelet-poor plasma; RFU, relative fluorescence unit; TG, thrombin generation; V_{max}, maximum velocity

(91, 84, 76, and 68%, respectively). An increase in total and peak thrombin in response to increasing prothrombin concentration has previously been demonstrated by other groups.[28] We believe that the significant effect of the PCCs was related to the prothrombin levels. In a cell-based model of coagulation, near maximal TG was seen with factor X levels of around 10%, and the rate, peak, and AUC of TG continued to increase in a near-linear fashion as the concentration of prothrombin was increased, with no evidence of saturation of the prothrombinase complex.[26] However, in a porcine model of dilutional coagulopathy, PCC administration was associated with a sustained increase in TG, unlike recombinant human prothrombin, which produced a transient increase.[29]

Importantly, an approximate 25% increase in PCC concentration at a platelet count of 40 × 10^9/L, and 50% increase at a platelet count of 20 × 10^9/L, resulted in TG that would normally be associated with a platelet count of 150 × 10^9/L and normal levels of coagulation factors. Thus, PCCs administered at doses in use in current clinical practice produce an improvement of TG across all platelet counts, but the beneficial effect is marked at lower levels of platelets. Higher doses of PCCs were associated with TG beyond the normal range with no ceiling, suggesting potential for hypercoagulability if used inappropriately.

Furthermore, administration of PCC resulted in an improvement in all TG parameters, unlike rFVIIa, which demonstrated shortened lag time and increased rate of TG with no influence on PH or AUC.[14,30]

Our study has some important limitations. It is an in vitro laboratory study and may be disproportionately sensitive to spiking with PCCs. The use of PCCs to compensate for severe thrombocytopenia does not take into consideration the role of platelets in primary hemostasis. While TG tests are informative with regard to mechanisms, they do not necessarily reflect the bleeding situation. We have not undertaken

TG assays with thrombomodulin to evaluate the impact of proteins C and S. In addition, it is possible that TF-containing microparticles present in the patient plasma samples may have slightly impacted on the TG results, but the minimal effect in PPP spiked with PCCs suggest that this may not have been that important. Furthermore, it is important to note the plasma levels achieved after in vivo administration are not similar to levels seen following in vitro spiking. Moreover, an in vitro laboratory study cannot provide estimates of thrombotic risk, a factor that is particularly important to consider in patients with liver disease. For example, a clinical trial to evaluate the use of the thrombopoietin-receptor agonist eltrombopag to increase platelet count in patients with thrombocytopenia and chronic liver disease was terminated due to an increased incidence of portal vein thrombosis in the treatment group.[31] A study in liver transplant patients with prolonged INR confirmed that PCC can effectively restore thrombin generation, but noted that the required doses of PCC are lower than for warfarin reversal, likely as a result of slow thrombin inhibition.[32] A recent study confirmed the efficacy of PCC in patients with liver disease, without an excess of thrombotic events, and also observed lower dosing with PCC compared to anticoagulant reversal.[33] Additionally, this study did not evaluate interactions with other hemostatic modalities, such as desmopressin and antifibrinolytics, and therefore may not fully reflect the multifaceted treatments that patients with thrombocytopenia may receive in clinical practice.[34]

In contrast to our ex vivo studies of spiking with PCCs, the increases in the levels of clotting factors in patients, after administration of PCCs, are variable due to redistribution of the factors to extravascular compartments, which affect the recovery and half-life. Factor IX demonstrates a median recovery of 1.57% and prothrombin 2.11% per IU/kg administered to healthy volunteers.[35] Through extrapolation of the levels from the spiking studies, and by utilizing the median recoveries from the healthy volunteer study, the 4 PCC concentrations achieved with spiking (0.26, 0.52, 1.03, and 1.53 IU/mL) can potentially be demonstrated in patients receiving PCCs at doses of 17, 33, 66, or 97 IU/kg, respectively, using factor IX levels and recovery. The lower 2 concentrations and doses correlate with doses administered to patients in clinical practice, but the higher 2 doses are not recommended for clinical use.

It is critical that the results of the ex vivo spiking study are confirmed in samples from patients receiving PCCs for acquired coagulopathy. Equally additional TGA analysis with thrombomodulin is important to understand the impact of concurrent increase in proteins C and S. Further, similar analysis should be undertaken with viscoelastometric tests as they are increasingly used to determine the need for transfusion of blood components. PCCs have traditionally been considered as a plasma alternative, but our studies show that the influence of PCCs in TG goes beyond simple replacement with plasma. The ratio of procoagulants and anticoagulants is altered with PCCs, and they improved the impaired TG of thrombocytopenia and low coagulation factors. Indeed, judicious use of PCCs along with clear algorithms for transfusion of blood products have produced a reduction in the quantity of allogenic products infused.[36]

In summary, we have demonstrated for the first time the impact of increasing levels of coagulation factors on platelet-dependent TG in the context of thrombocytopenia. Our results in PRP TG demonstrate that increasing the concentration of procoagulant proteins in thrombocytopenic samples improves TG to normal ranges and higher. These data suggest that further clinical studies would be of interest to investigate the role of PCCs in the management of complex coagulopathy characterized by abnormal coagulation and mild to moderate thrombocytopenia.

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AUTHOR CONTRIBUTIONS

PC conceived the idea. PC, CH, KG, AG, JHF, JHM developed the experimental methods. TAF and EB consented patients and collected samples. CH performed the assays. CH, PC, and RM analyzed the data. RM performed the statistical analysis. PC and CH wrote the first draft of the manuscript. All authors critically reviewed the manuscript through its development and had access to the final version of the manuscript and approved the final version and figures.

RELATIONSHIP DISCLOSURE

PC received research funding from CSL Behring, Pfizer, NovoNordisk, SOBI, and Bayer; and is on the advisory boards and speaker bureau for Baxalta, Bayer, Biogen, CSL Behring, Chugai, Pfizer, Freeline, NovoNordisk, Roche, SOBI, and Shire. PC also provided funding via Royal Free Charity (TF35) for editing of the manuscript by Meridian HealthComms. All other authors declare nothing to report.

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