A Step Toward Balance: Thrombin Generation Improvement via Procoagulant Factor and Antithrombin Supplementation

Alexander Y. Mitrophanov, PhD,* Fania Szlam, MMSc,† Roman M. Sniecinski, MD,† Jerrold H. Levy, MD,‡§ and Jaques Reifman, PhD*

BACKGROUND: The use of prothrombin complex concentrates in trauma- and surgery-induced coagulopathy is complicated by the possibility of thromboembolic events. To explore the effects of these agents on thrombin generation (TG), we investigated combinations of coagulation factors equivalent to 3- and 4-factor prothrombin complex concentrates with and without added antithrombin (AT), as well as recombinant factor VIIa (rFVIIa), in a dilutional model. These data were then used to develop a computational model to test whether such a model could predict the TG profiles of these agents used to treat dilutional coagulopathy.

METHODS: We measured TG in plasma collected from 10 healthy volunteers using Calibrated Automated Thrombogram. TG measurements were performed in undiluted plasma, 3-fold saline-diluted plasma, and diluted plasma supplemented with the following factors: rFVIIa (group rFVIIa); factors (F)II, FIX, FX, and AT (group "combination of coagulation factors" [CCF]-AT); or FII, FVII, FIX, and FX (group CCF-FVII). We extended an existing computational model of TG to include additional reactions that impact the Calibrated Automated Thrombogram readout. We developed and applied a computational strategy to train the model using only a subset of the obtained TG data and used the remaining data for model validation.

RESULTS: rFVIIa decreased lag time and the time to thrombin peak generation beyond their predilution levels ($P < 0.001$) but did not restore normal thrombin peak height ($P < 0.001$). CCF-FVII supplementation decreased lag time ($P = 0.034$) and thrombin peak time ($P < 0.001$) and increased both peak height ($P < 0.001$) and endogenous thrombin potential ($P = 0.055$) beyond their predilution levels. CCF-AT supplementation in diluted plasma resulted in an improvement in TG without causing the exaggerated effects of rFVIIa and CCF-FVII supplementation. The differences between the effects of CCF-AT and supplementation with rFVIIa and CCF-FVII were significant for lag time ($P < 0.001$ and $P = 0.005$, respectively), time to thrombin peak ($P < 0.001$ and $P = 0.004$, respectively), velocity index ($P < 0.001$ and $P = 0.019$, respectively), thrombin peak height ($P < 0.001$ for both comparisons), and endogenous thrombin potential ($P = 0.034$ and $P = 0.019$, respectively). The computational model generated subject-specific predictions and identified typical patterns of TG improvement.

CONCLUSIONS: In this study of the effects of hemodilution, CCF-AT supplementation improved the dilution-impaired plasma TG potential in a more balanced way than either rFVIIa alone or CCF-FVII supplementation. Predictive computational modeling can guide plasma dilution/supplementation experiments. (Anesth Analg 2016;123:535–46)

A pathologic reduction in plasma concentrations of coagulation factors can occur in trauma patients because of plasma dilution with resuscitation fluids and factor depletion resulting from hemorrhage.[1,2] Acute traumatic coagulopathy is an early condition independent of hypothermia and acidosis and is associated with both procoagulant and anticoagulant factor level reduction.[3–5] When associated with inflammation and shock, this could lead to increased thrombin generation (TG) and, in combination with reduced levels of antithrombin (AT) and other inhibitors, result in a hypercoagulable state.[6,7] Certain complex surgical procedures (such as the use of cardiopulmonary bypass[8,9]) may also result in significant blood plasma dilution. Therefore, it is imperative to develop a detailed understanding of the effects of clotting factor depletion, dilution, and subsequent supplementation on blood coagulation.

The fundamental event of blood coagulation is the clotting factor–driven network of biochemical reactions facilitating TG,[10,11] which is an essential indicator of blood coagulation in trauma patients.[6,7] The influence of dilution

From the *DoD Biotechnology High Performance Computing Software Applications Institute, Telemedicine and Advanced Technology Research Center, US Army Medical Research and Materiel Command, Fort Detrick, Maryland; †Department of Anesthesiology, Emory University School of Medicine, Atlanta, Georgia; and Departments of ‡ Anesthesiology and §Surgery, Duke University School of Medicine, Durham, North Carolina.

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Address correspondence to Alexander Y. Mitrophanov, PhD; Jaques Reifman, PhD, DoD Biotechnology High Performance Computing Software Applications Institute, Telemedicine and Advanced Technology Research Center, US Army Medical Research and Materiel Command, ATTN: MCMR-TT, 504 Scott St, Ft. Detrick, MD 21702. Address e-mail to alex@bhsai.org; jaques.reifman.civ@mail.mil.

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and clotting factor supplementation on TG in human plasma has been investigated in several experimental studies.[8,12–18] In particular, analyses of supplementation with prothrombin complex concentrates (PCCs) are of interest because their use for hemorrhage control is an emerging concept in trauma[19] and surgery.[20]

A number of PCCs with different compositions are commercially available.[20] They can be broadly categorized as 3-factor PCCs, which contain factors (F)II, FIX, and FX, or 4-factor PCCs, which have the addition of FVII. Most commercially available PCCs additionally contain small amounts of anticoagulants, such as proteins C and S or AT. Because of the complexity of the multiple blood coagulation biochemical reactions in which the PCC components are involved, the optimal PCC composition for any given pathologic condition is still an open question.[19,20]

A 2012 study using computational modeling compared the ability of 3 different clotting factor supplementation scenarios to restore normal TG in diluted human plasma.[21] That comparison suggested that the use of a combination of coagulation factors (CCF; here designated as CCF-AT) consisting of FII, FIX, and FX, and AT restores normal TG after hemodilution. This restoration was more accurate than that observed with the supplementation with FVIIa or with the supplementation only with FII, FVII, FIX, and FX (here designated as CCF-FVII).[21] The possibility of coadministration of AT and PCCs was also suggested in a review of therapeutic strategies for the treatment of trauma-induced coagulopathy as a means to reduce possible prothrombotic complications.[19]

The objective of this in vitro experiment was 2-fold. First, we sought to compare the effects of recombinant (r)FVIIa, CCF-AT, and CCF-FVII supplementation in diluted human plasma on TG. Second, we investigated whether TG in this experimental model could be predicted a priori with sufficient accuracy for individual subjects. This second aim was defined in light of the rapidly evolving paradigm that computational predictive models hold promise for optimizing therapeutic approaches for blood coagulation abnormalities.[8,21–26] Our first hypothesis was that CCF-AT supplementation results in a more complete restoration of normal TG in diluted plasma than recombinant factor VIIa (rFVIIa) and CCF-FVII supplementations alone. Our second hypothesis was that our computational modeling strategy can predict the effects of dilution and clotting factor supplementation on TG in human plasma.

METHODS

Subject Group

This study was approved by the IRB of Emory University (Atlanta, GA) and by the Human Research Protection Office, Office of Research Protections, US Army Medical Research and Materiel Command (Fort Detrick, MD). The subjects were recruited at the Emory University School of Medicine. The experimental phase of this research was performed at the Emory University School of Medicine, whereas the computational modeling phase was performed at the Department of Defense Biotechnology High Performance Computing Software Applications Institute. All subjects (healthy volunteers) gave written informed consent to participate in this study. The subjects consisted of 10 individuals (7 women and 3 men) with no preexisting clotting abnormalities and who were not taking any medications known to affect coagulation testing.

Sample Collection

Venous blood samples (25 mL) were collected from each subject. The first 2 mL of blood was discarded. Blood was drawn into 3.2% citrate-containing glass Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Complete blood count was determined using an AcT-8 Series Beckman Coulter Analyzer (Coulter Corporation, Miami, FL). Blood samples were then centrifuged at 2000*g* (room temperature) for 20 minutes to obtain platelet-poor plasma (PPP). Each centrifuged plasma sample was checked for residual platelets (by doing another blood count in the PPP), and only samples with a platelet count <1000 1/μL were retained for further analysis and stored at −80°C. We chose this 1-step centrifugation protocol because it has been routinely and successfully used in our laboratory for several years.[12,13]

Sample Preparation for Measurements and Clotting Factor Supplementation Dosing

A flow diagram of the study procedures is shown in Figure 1. Thawed plasma samples were diluted 3-fold with normal saline (0.9% NaCl). The amount of dilution was chosen as an approximation of the extent of dilution that leads to coagulopathy using in vivo (porcine) models of coagulopathy.[21,27] The diluted plasma was used to prepare samples supplemented with rFVIIa, CCF-AT, or CCF-FVII. These samples, along with the undiluted and diluted unsupplemented samples, were used for TG measurement. In rFVIIa supplementation experiments, rFVIIa was added to the hemodiluted specimens to obtain a final concentration of 40 nM. This supraphysiologic concentration (~400-fold higher than the normal average value) was chosen because it reflects the expected rFVIIa plasma concentration after the administration of a typical therapeutic rFVIIa dose (ie, 90 μg/kg body weight).[28] In experiments involving supplementation with coagulation factors FII, FVII, FIX, FX, or AT, the amounts of individual factors were added to diluted plasma samples to result in concentrations that were restored to measured baseline (ie, to their respective predilution values).[21,29,30] In this study, we did not use preformulated, brand name PCC products that contain several coagulation factors in manufacturer-defined proportions, which allowed us to more precisely restore the factors of interest back to baseline levels. We obtained commercially available individual coagulation factors and chose the supplementation scheme according to the design of our study (Supplemental Digital Content, Table S1, http://links.lww.com/AA/B432). rFVIIa was obtained from Novo Nordisk (Bagsværd, Denmark); all other procoagulant and anticoagulant factors used for supplementation were obtained from human factors from Hematologic Technologies, Inc. (Essex Junction, VT).

Coagulation Factor Measurement

To obtain baseline (ie, predilution) clotting factor levels (which were subsequently used for mathematical modeling), we measured factor levels in all undiluted plasma samples. We then performed the same analyses in diluted samples

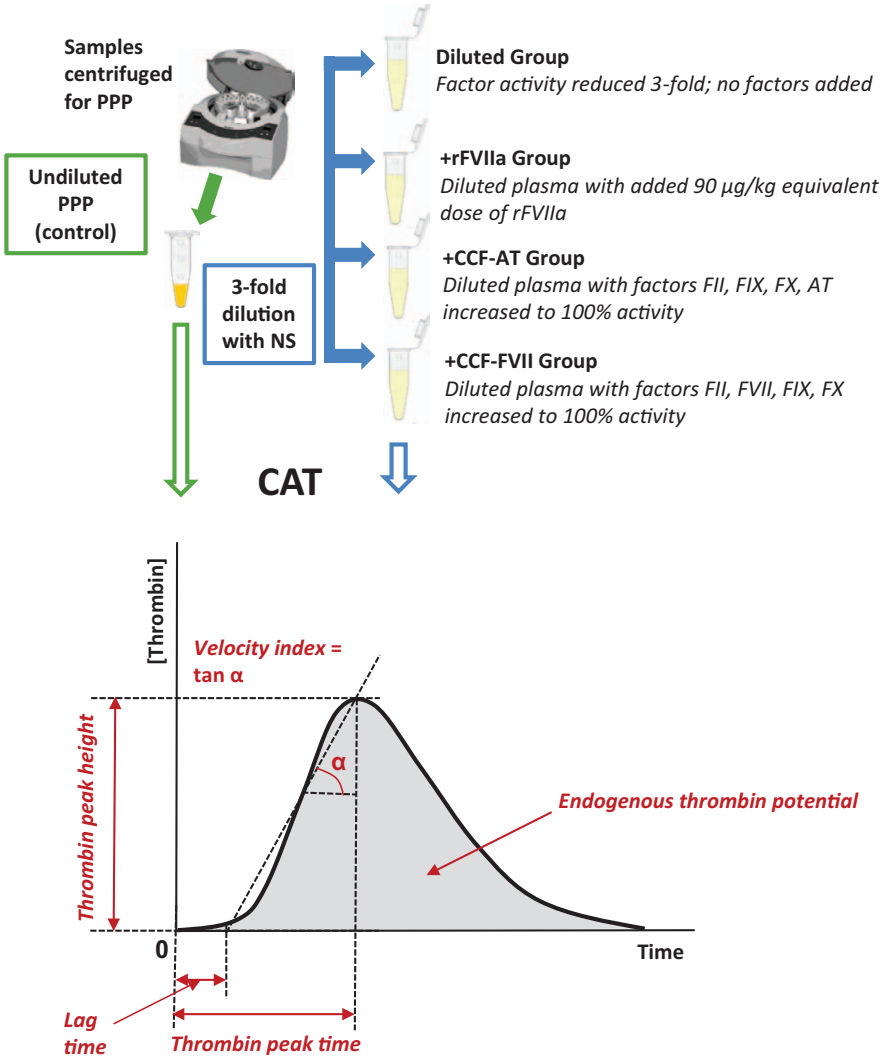

Figure 1. Experimental design. Blood samples from each subject were used to obtain (undiluted) platelet-poor plasma (PPP). From the undiluted plasma sample for a given subject, the following samples were additionally prepared: a plasma sample diluted 3-fold with normal saline (NS), a diluted plasma sample with added 40 nM recombinant factor VIIa (rFVIIa), a diluted plasma sample with added combination of coagulation factors (consisting of FII, FIX, FX, and antithrombin [AT]; CCF-AT), and a diluted plasma sample with added CCF-FVII (a combination of the coagulation factors FII, FVII, FIX, and FX). In each of the samples for each of the subjects, thrombin generation was measured using Calibrated Automated Thrombogram (CAT), and the Thrombinoscope software was used to process the raw thrombin generation data and extract thrombin generation parameters: lag time, thrombin peak time, and the velocity index (the timing parameters), as well as thrombin peak height and endogenous thrombin potential (the amount parameters).

and samples supplemented with clotting factors. All coagulation protein concentrations were measured (except tissue factor pathway inhibitor [TFPI]) with a Diagnostica Stago Compact analyzer (Diagnostica Stago, Parsippany, NJ). Coagulation factor activities were determined using 1-stage clotting assays in individual factor-deficient plasmas according to activated partial thromboplastin time (aPTT) for FVIII and FIX and prothrombin time (PT) for FII, FV, FVII, and FX. AT level measurements were performed using thrombin-based amidolytic assay and a synthetic chromogenic substrate. TFPI analyses were performed using a Diagnostica Stago Asserachrom Total TFPI ELISA kit. Although only a fraction of total TFPI displays functional activity, total TFPI levels were used as a proxy for the levels of functional TFPI.[31]

Thrombin Generation Measurement

A commercially available Calibrated Automated Thrombogram (CAT)[32] system was used for the assessment of TG. All supplies and reagents were from Diagnostica Stago and were used according to manufacturer's directions. Briefly, to each well of a 96-well microtiter plate, 80 µL of undiluted PPP, diluted PPP, or diluted PPP with supplemented factors was added followed by 20 µL of tissue factor (TF)-based activator that also contained phospholipids (final TF and phospholipid concentration in each well, 5 pM and 4 µM, respectively). The progress of the TG reaction (run at 37°C) was continuously monitored for 60 to 90 minutes with a fluorescence reader (Fluoroscan, Thermo Labsystems, Franklin, MA) set to 390/460-nm excitation/emission wavelengths (the reader measures the fluorescence resulting from the cleavage of a fluorogenic thrombin substrate, ZGGR-AMC, by the generated thrombin). A dedicated software program (Thrombinoscope, Diagnostica Stago) was used to record the experiment and to calculate the TG parameters. These parameters were as follows: lag time (LT, also referred to as clotting time), time to the thrombin peak (ttP; also referred to as thrombin peak time), velocity index (VI, also referred to as maximum rate of TG), thrombin peak height (PH), and endogenous thrombin potential (also referred to as the area under the thrombin curve). The definitions of these parameters are shown in Figure 1. LT characterizes the length of the initiation phase of TG and is often defined as the time to 10 nM thrombin.[28] The Thrombinoscope software used in this study calculates LT as the time to 1/6 of thrombin PH (Peter Giesen, personal communication). Maximum rate of TG is defined as

the maximum derivative (ie, maximum slope) of the thrombin curve.[28] The Thrombinoscope software approximates this value by calculating VI = PH/(ttP – LT) (Paul Riley, personal communication).

Statistical Analyses

Our previous work and the work of others suggested that a sample size of 6 is required to achieve a statistical significance of $\alpha \leq 0.05$ and power $\beta \geq 0.80$ during in vitro dilution and blood clotting experiments.[12,13] In this study, we selected a sample size of 10 to go beyond this necessary level of power in view of our original experimental design involving clotting factor supplementation. The Jarque-Bera test was used for normality testing.[33] If the test failed to reject the normality hypothesis at a significance level of 0.05, then the differences between samples were assessed using the paired *t* test; otherwise, they were assessed using the Wilcoxon signed-rank test. The Bonferroni correction was used in the case of multiple comparisons.

Computational Modeling

Our kinetic model of TG is an extension of the updated version[28,34] of the Hockin-Mann model[10] and has the same default values for the kinetic parameters (ie, the rate constants of the biochemical reactions in the TG network) and for the initial conditions (ie, initial concentrations of the coagulation factors). The model is a system of coupled, nonlinear, ordinary differential equations that represent the mass action kinetics of the interactions between clotting factors in inactive and active forms, their cofactors, and the anticoagulants AT and TFPI. Each equation corresponds to 1 biochemical species; the model comprises 42 equations and has 52 kinetic parameters. The concentration of free thrombin is the main model output. In addition to the reactions and default parameter values represented in the Hockin-Mann model,[28,34] our model contains reactions that are necessary to model TG in the CAT assay, as shown in the recent study by Kremers et al[35] (Table 1). Although the model currently simulates TG occurring at 37°C with a pH of 7.4, it can be extended using our recently developed strategies to simulate TG under the conditions of hypothermia[36] and acidosis.[37]

The model was implemented in the SimBiology toolbox of the MATLAB software suite (MathWorks, Natick, MA). All computations were performed in MATLAB R2014a. The reactions in Table 1 were incorporated into the model using the graphical user interface (GUI) provided with the SimBiology toolbox; this GUI was also used for our original Hockin-Mann model implementation.[28] The equations and parameters entered via the GUI were automatically converted into ordinary differential equations, which were then numerically solved as previously described.[28]

The parameters inputted to generate model predictions for each subject were the subject-specific initial clotting factor concentrations.[21,38] We used the subject-specific relative levels of the clotting factors from our measurements (summarized in the "Dilution and Clotting Factor Supplementation of the Plasma Samples" subsection of the Results section) and then multiplied them by the known average normal factor concentrations to obtain the subject-specific absolute concentration values. (Because TFPI was measured in absolute units, we used those subject-specific measurements directly [after converting to molar concentrations].) The initial concentrations of other proteins in the model were either set to 0 (for proteins and complexes absent in quiescent plasma) or set to their known or assumed values (Table 1). Three-fold dilution was represented as a 3-fold reduction in the initial protein concentrations, and clotting factor supplementation was represented by using the undiluted initial concentration values for the supplemented CCF-AT and CCF-FVII proteins (or, for the rFVIIa supplementation, the diluted FVIIa value with added 40 nM rFVIIa; rFVIIa in the model was represented by the same variable as native FVIIa).

Table 1. Biochemical Reactions Absent in the Original Hockin-Mann Kinetic Model[10,34] of Thrombin Generation and Present in Our Thrombin Generation Model

Reaction	Reaction definition	Default kinetic parameter value	Source
1	FIIa + FS → FIIa:FS	1×10^8 $(M \cdot s)^{-1}$	Assumed fast
2	FIIa:FS → FIIa + FS	35,798 s^{-1}	Calculated from Ref. 35
3	FIIa:FS → FIIa + FS_conv	2 s^{-1}	Ref. 35
4	FIIa + α2M → FIIa:α2M	736 $(M \cdot s)^{-1}$	Ref. 35
5	FIIa:α2M + FS → FIIa:α2M:FS	1×10^8 $(M \cdot s)^{-1}$	Assumed fast
6	FIIa:α2M:FS → FIIa:α2M + FS	35,798 s^{-1}	Calculated from Ref. 35
7	FIIa:α2M:FS → FIIa:α2M + FS_conv	2 s^{-1}	Ref. 35
8	FIIa + Serpins → FIIa:Serpins	1000 $(M \cdot s)^{-1}$	This study

The default values of the kinetic parameters are the values of these parameters before subject-specific model training.

Here, coagulation factor (F)IIa designates free thrombin. The symbol ":" denotes complex formation. The main added model variables representing (bio)chemical species are as follows (with their initial concentrations in undiluted plasma in parentheses): FS (417 μM) is the fluorogenic substrate ZGGR-AMC, FS_conv (0 μM) is the enzymatically converted fluorogenic substrate, and α2M (3.03 μM) is α_2-macroglobulin.[35] "Serpins" (10 μM) is a model variable that represents the action of (possibly numerous) plasma serpins that cannot be easily identified and quantified.[35]

To make accurate predictions, the model's kinetic parameters needed to be adjusted using experimental data (ie, fitted to the data). These data were the TG trajectory data described in the "Thrombin Generation Trajectories" subsection of the Results section. The entire modeling process (leave-one-out cross-validation) can be briefly described as follows. First, a subject was selected, and the training data (namely, the TG data for undiluted plasma from the remaining 9 subjects) were used to "train" the model, as described in more detail below. Then, the model's initial conditions were defined to reflect the individual clotting factor composition of the selected subject. The model's initial conditions were then modified to reflect the considered dilution/supplementation scenarios, and the model was solved to simulate TG for those scenarios. Finally, to validate the model, these simulation results were compared with the experimentally measured TG trajectories for the selected subject. This procedure was performed for each subject in the subject group.

Model training for a selected subject consisted of alternating model fitting steps and prediction steps. The prediction steps were introduced to test the model's prediction

accuracy and thereby avoid overfitting. For the fitting steps, we used the subject-specific coagulation factor level data and TG data from (randomly chosen) 6 of the remaining 9 subjects, and for the prediction steps, we used the coagulation factor level data and TG data from the other 3 of the remaining 9 subjects. This choice of subjects was performed once at the beginning of the training process.

To initiate the first fitting step, we randomized the model's kinetic parameters to obtain 2000 random parameter sets. The parameter set that resulted in the best fit between the model's thrombin output and the TG trajectories (in undiluted plasma) for the 6 subjects was chosen as the best initial parameter set for model training. Throughout the entire model training process, the model's kinetic parameters were allowed to deviate by ≤3-fold from the default parameter set. The quality of fit for the fitting steps or prediction accuracy for the prediction steps was defined as the root-mean-square error between the model simulated and experimentally measured thrombin trajectory points for a given subject, summed over all the subjects in the respective data set.

Model fitting was performed using the MATLAB function FMINCON implementing the interior point constrained optimization algorithm. To use the algorithm in the presence of kinetic parameters differing by orders of magnitude, the parameter values were log-transformed before being passed to FMINCON. During a fitting step, the model was fitted to the data from the 6 subjects by fine tuning the model's kinetic parameters. The fitting step terminated when the number of function evaluations in the fitting procedure exceeded 200. Then the prediction accuracy of the fitted model was tested by comparing its subject-specific TG predictions with the undiluted plasma TG data from the 3 subjects. If the comparison result satisfied the GL2 stopping criterion (defined and used for model training in neural network research[39]), the model training process was terminated. If the criterion was not met, the kinetic parameter set obtained as the result of model fitting became the initial parameter set for the next model fitting step.

RESULTS

Subject Group Characteristics

Plasma samples for each of the 10 subjects were analyzed. The blood counts for the samples were as follows (mean ± SD, range): white blood cell count (5.41 ± 1.24, 4.1–7.4 × 10^3 1/μL); hemoglobin (12.92 ± 1.03, 11.0–13.9 g/dL), hematocrit (39.29 ± 2.80, 33.5%–41.9%), and platelets (209.70 ± 37.71, 133–262 × 10^3 1/μL). All the measured values were within normal ranges.

Dilution and Clotting Factor Supplementation of the Plasma Samples

The measurement results for the procoagulant and anticoagulant proteins are shown in Figures 2 and 3, respectively. As expected, 3-fold dilution of the plasma samples resulted in a decrease in the measured coagulation factor levels. Likewise, the postsupplementation protein levels were close to their predilution levels. The measured levels of anticoagulants were near the expected values for the considered scenarios (Figure 3). However, for all the procoagulant proteins, rFVIIa supplementation resulted in increased protein levels (Figure 2); this effect was particularly extreme in the case of FVII (3686% FVII activity; Figure 2C). Such bias was not entirely unexpected and is consistent with the 1-stage assay design,[40] whose results may be skewed when the test plasma contains a supraphysiologic concentrations of a clotting factor.

Thrombin Generation Trajectories

The TG trajectory data for the 5 considered dilution/supplementation scenarios are summarized in Figure 4, and the individual subject trajectories are shown in Figures S1 and S2 (Supplemental Digital Content, http://links.lww.com/AA/B432). All the trajectories had the expected 1-peak shape that allowed the automatic calculation of the TG parameters defined in Figure 1. In the case of CCF-FVII supplementation, the right-hand "tails" of the thrombin curves were characterized by high variability for certain subjects (eg, Figure S2, A and B), which occurred apparently because of an increased noise-to-signal ratio resulting from fluorogenic substrate depletion caused by the strong CCF-FVII-triggered TG in the samples.

Direct visual inspection of the summarized thrombin trajectories (Figure 4) and the thrombin trajectories for individual subjects (Figures S1 and S2) allowed us to identify patterns that held for each subject despite the intersubject variability in the data set. Specifically, dilution always reduced the height of the TG peak but did not increase the ttP. Moreover, rFVIIa supplementation visibly accelerated the onset of TG in diluted plasma but it did not considerably change the PH. Supplementation with rFVIIa resulted in the earliest onset of TG (in some subjects, closely matched by that in CCF-FVII-supplemented plasma). Finally, among the 5 considered dilution/supplementation scenarios, the largest TG peak resulted from CCF-FVII supplementation and the lowest peak was either the one in the diluted plasma or the rFVIIa-supplemented plasma. In several subjects, CCF-AT supplementation resulted in a nearly normal TG trajectory (Figures S1, D and F, and S2, B and D). In contrast, the rFVIIa and CCF-FVII supplementations did not result in accurate thrombin trajectory normalization in any of the 10 subjects (Figures S1 and S2). The ability of CCF-AT, and the inability of both rFVIIa and CCF-FVII, to restore normal TG in diluted plasma is clearly demonstrated by the summarized TG data (Figure 4).

Standard Coagulation Tests and Thrombin Generation Parameters

In our experiments, PT was prolonged by dilution (Figure 5A). Supplementation with rFVIIa shortened PT to a near-normal level, whereas CCF-AT and CCF-FVII supplementation resulted in prolonged PT (compared with undiluted plasma). The PT after CCF-AT supplementation was somewhat prolonged compared with those after CCF-FVII supplementation, which is consistent with the anticoagulant action of AT present in CCF-AT. aPTT modulation was qualitatively similar to that of PT (Figure 5A).

Both PT and LT reflect the duration of the initiation phase of TF-triggered TG. However, in contrast to the dilution-induced prolongation of PT and aPTT (Figure 5A), no

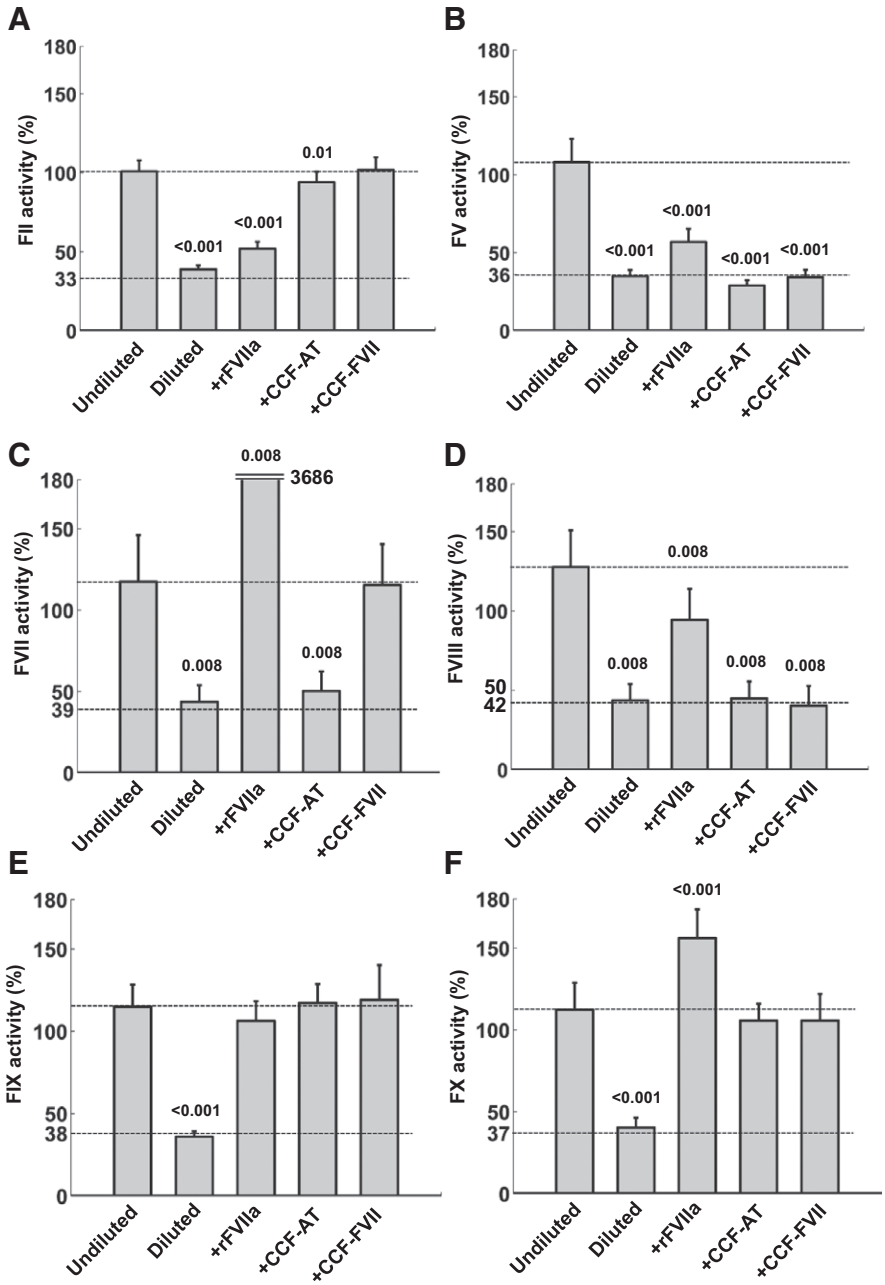

Figure 2. Coagulation factor levels for different dilution/supplementation scenarios. Shown are the results for coagulation factor (F)II (A), FV (B), FVII (C), FVIII (D), FIX (E), and FX (F). The shaded bars and error bars represent means and SDs, respectively, calculated for the subject group (n = 10). The x-axis labels represent the 5 dilution/supplementation scenarios considered in this study: undiluted plasma, diluted plasma, diluted plasma with supplemented rFVIIa (+rFVIIa), diluted plasma with supplemented CCF-AT (+CCF-AT), and diluted plasma with supplemented CCF-FVII (+CCF-FVII). A–F, The top horizontal dashed line marks the average factor level for undiluted plasma, and the bottom dashed line marks that value divided by 3 (ie, it reflects the expected 3-fold dilution). For the samples that showed significant differences from the corresponding undiluted-plasma samples ($P \leq 0.05$ or $P \approx 0.05$, Bonferroni-corrected with denominator 4), the actual P values are shown in the figure (as numbers above the bars). In the sample comparisons for FVII (*C*) and FVIII (*D*), the Wilcoxon test was used. AT = antithrombin; CCF = combination of the coagulation factors; rFVIIa = recombinant factor VIIa.

significant difference in LT was detected between undiluted and diluted plasma (P = 0.19, Figure 5B), which is consistent with our earlier in vitro dilution experiments.[12,13] Both the VI and the thrombin PH were decreased in diluted plasma (Figure 5, D and E).

Supplementation with rFVIIa noticeably shortened both LT and ttP in diluted plasma (Figure 5, B and C). Thrombin PH in the diluted plasma supplemented with rFVIIa, although, was similar to that in diluted plasma and significantly smaller than that in undiluted plasma (Figure 5E). These results were consistent with previous computational predictions.[21,28] As predicted,[38] dilution reduced the VI, but contrary to the prediction,[21] rFVIIa supplementation did not return this parameter to baseline (ie, to its value in undiluted plasma; Figure 5D). CCF-AT and CCF-FVII supplementation resulted in VI values considerably exceeding the baseline.

Previous simulations have suggested that CCF-FVII supplementation, in contrast to rFVIIa, should primarily affect those parameters reflecting peak TG, such as the thrombin PH and endogenous thrombin potential.[21] In our experiments, CCF-FVII supplementation not only resulted in higher-than-baseline values for both peak TG and endogenous thrombin potential, but also both these values were the largest among the 5 considered dilution/supplementation scenarios (Figure 5, E and F). The respective P values were $P < 0.001$ and $P < 0.055$ (the Wilcoxon test for the latter). These P values were Bonferroni corrected with denominator 4 because, in this analysis, it was sufficient to compare the samples for the CCF-FVII supplementation with each of the corresponding samples for the remaining 4 scenarios). Moreover, as predicted,[21] CCF-FVII supplementation was the only dilution/supplementation scenario that caused a

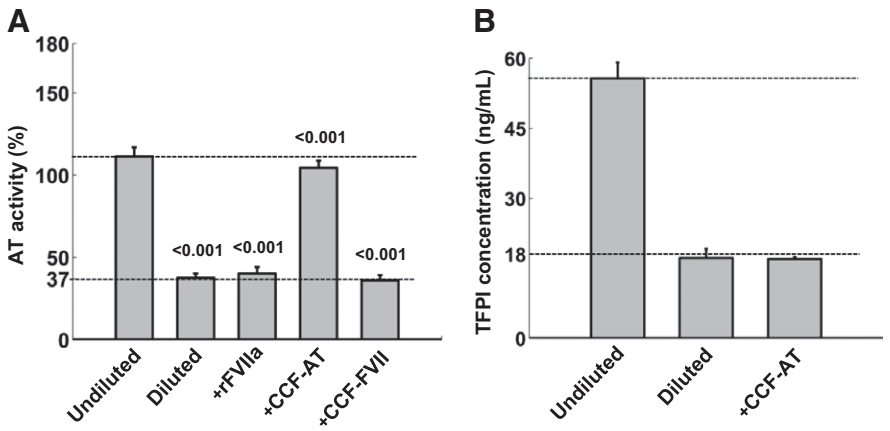

Figure 3. Natural anticoagulant levels for different dilution/supplementation scenarios. Shown are the results for antithrombin (AT, A) and tissue factor pathway inhibitor (TFPI, B). The gray bars and the error bars represent means and SDs, respectively (A, $n = 10$; B, $n = 3$). The x-axis labels represent all or some of the 5 dilution/supplementation scenarios tested: undiluted plasma, diluted plasma, diluted plasma with added rFVIIa (+rFVIIa), diluted plasma with added CCF-AT (+CCF-AT), and diluted plasma with added CCF-FVII (+CCF-FVII). A and B, The top horizontal dashed line marks the average factor level for undiluted plasma and the bottom dashed line marks that value divided by 3 (ie, it reflects the expected 3-fold dilution). The measurements for TFPI were performed for only 3 of 10 subjects and for 3 of 5 dilution/supplementation scenarios because of the limited amount of subject plasma available for experimentation. A and B, For the samples that showed statistically significant (ie, $P \leq 0.05$ or $P \approx 0.05$, Bonferroni-corrected with denominator 4 [A] or 2 [B]) differences from the corresponding undiluted-plasma samples, the actual P values are shown in the figure (as numbers above the bars). In the sample comparisons for TFPI (B), the Wilcoxon test was used. AT = antithrombin; CCF = combination of the coagulation factors.

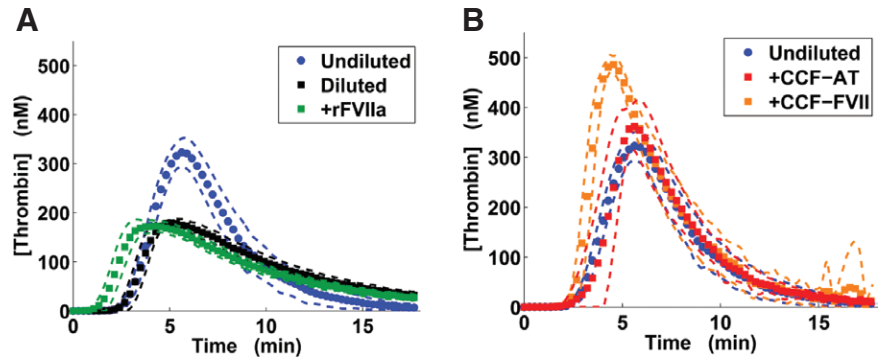

Figure 4. Thrombin generation under different dilution/supplementation scenarios. The data represent the 5 dilution/supplementation scenarios considered: undiluted plasma (A and B), diluted plasma (A), diluted plasma with added rFVIIa (+rFVIIa, A), diluted plasma with added CCF-AT (+CCF-AT, B), and diluted plasma with added CCF-FVII (+CCF-FVII, B). The round and square markers reflect the mean values of the individual thrombin generation curves averaged over the entire subject group ($n = 10$) for a given dilution/supplementation scenario. The dashed lines represent the mean ± 1 SD. The 2-subplot design was chosen to enhance visual data presentation, and the data for undiluted plasma were reproduced in both (A) and (B) for comparison purposes. The mean and SD data were computed from the individual thrombin generation trajectories shown in Figures S1 and S2. AT = antithrombin; CCF = combination of the coagulation factors; rFVIIa = recombinant factor VIIa.

significant difference in the endogenous thrombin potential compared with undiluted plasma (Figure 5F).

CCF-AT supplementation restored ttP to baseline level (Figure 5C) and yielded a thrombin PH value that was, on average, the closest to its predilution level (Figure 5E). Unexpectedly, however, CCF-AT supplementation resulted in a somewhat prolonged LT (Figure 5B). Supplementation with CCF-AT resulted in a VI value approximately 2-fold higher than the baseline value, whereas the value of this parameter in diluted plasma was approximately 2-fold lower than the baseline (Figure 5D). CCF-FVII supplementation resulted in the largest VI value across the 5 considered dilution/supplementation scenarios (Figure 5D; $P \leq 0.039$ [the Wilcoxon test], Bonferroni-corrected with denominator 4, similarly to the analysis described earlier). The above-normal levels of the VI (Figure 5D) and thrombin PH (Figure 5E) after both CCF-AT and CCF-FVII supplementations can be explained by noticing that dilution reduces the concentrations of all natural procoagulants and anticoagulants, whereas CCF-FVII replenishes none of the anticoagulants and CCF-AT replenishes only 1 of the anticoagulants (ie, AT). Indeed, the levels of TFPI, as well as those of other coagulation inhibitors,[35] were not restored in our clotting factor supplementation scenarios.

To test whether the effects of CCF-AT were different from those of rFVIIa and CCF-FVII, we compared the TG parameters for the CCF-AT supplementation with the corresponding samples for the rFVIIa and CCF-FVII supplementations (Figure 5, B–F). The differences between the effects of CCF-AT versus those of rFVIIa and CCF-FVII were significant for LT ($P < 0.001$ and $P = 0.005$, respectively), ttP ($P < 0.001$ and $P = 0.004$, respectively), VI ($P < 0.001$ and $P = 0.019$ [the Wilcoxon test for the latter], respectively), thrombin

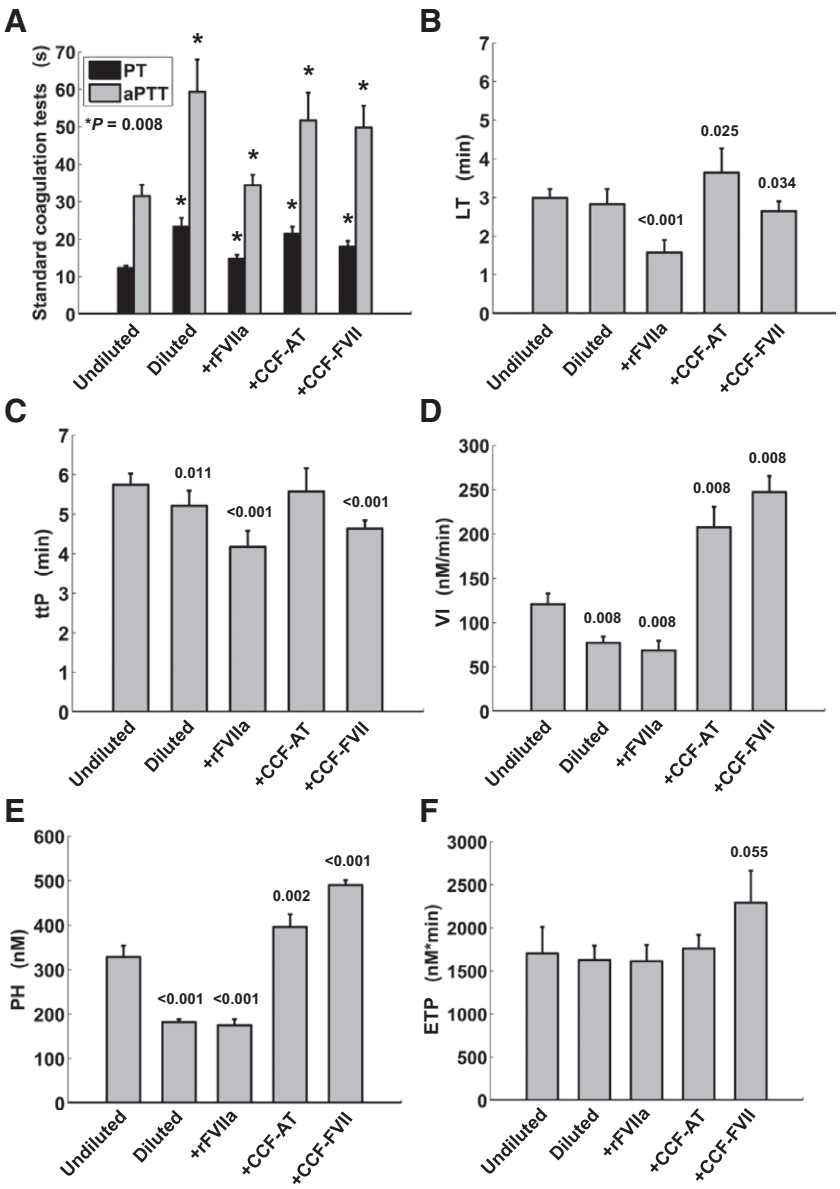

Figure 5. Standard coagulation tests and quantitative parameters of thrombin generation for different dilution/supplementation scenarios. A, The data for prothrombin time (PT) and activated partial thromboplastin time (aPTT). The data for lag time (B), time to thrombin peak (C), velocity index (D), thrombin peak height (E), and endogenous thrombin potential (F) are shown. The gray bars and the error bars represent means and SDs, respectively, calculated for the subject group (n = 10). For the samples that showed significant (ie, $P \leq 0.05$ or $P \approx 0.05$, Bonferroni-corrected with denominator 4) differences from the corresponding undiluted-plasma samples, the actual P values are shown in the figure (as asterisks in (A) or as numbers above the bars in other subplots). In the sample comparisons for PT and aPTT (A), velocity index (D), and endogenous thrombin potential (*E*), the Wilcoxon test was used. AT = antithrombin; CCF = combination of the coagulation factors; ETP = endogenous thrombin potential; LT = lag time; PH = peak height; rFVIIa = recombinant factor VIIa; ttP = time to the thrombin peak; VI = velocity index.

PH($P < 0.001$ for both comparisons), and endogenous thrombin potential ($P = 0.034$ and $P = 0.019$ [the Wilcoxon test for the latter], respectively). These P values were Bonferroni corrected with denominator 2 for each TG parameter analyzed independently. Similar comparisons for the corresponding PT and aPTT also indicated significant differences (all corresponding P values did not exceed 0.05, except the CCF-AT versus CCF-FVII comparison for aPTT).

Predictive Analysis of the Thrombin Trajectories Using the Extended Computational Model

As a result of 10 cross-validation rounds (1 round for each subject), we obtained 10 model variants with subject-specific initial conditions, which were somewhat different in their kinetic parameter values but were similar in their subject-specific predictive capacity. In each of the 10 model variants, the kinetic parameters reflected the training data from the 9 subjects who were used in model training (but not in model validation). To obtain 1 model that would contain information about all 10 subjects in our subject group, each kinetic parameter value was averaged across the 10 model variants, which resulted in an averaged-parameter model. This model was used with subject-specific initial conditions to generate thrombin trajectories for distinct subjects.

Model cross-validation results for the subject group are summarized in Figure 6; the modeling results for individual subjects are shown in Figures S3 to S12. The trained model accurately predicted TG kinetics in undiluted plasma (Figure 6A), and the predictions for rFVIIa and CCF-AT supplementation were also rather accurate (Figure 6, C and D, respectively). Less accurate were the predictions of TG in unsupplemented diluted plasma (Figure 6B). Yet, the model qualitatively predicted the thrombin PH decrease in diluted plasma (Figure 6, A and B), and the exaggerated PH and endogenous thrombin potential in diluted plasma supplemented with CCF-FVII (Figure 6, A and E). In the case of CCF-FVII supplementation, the model prediction for thrombin PH was quantitatively

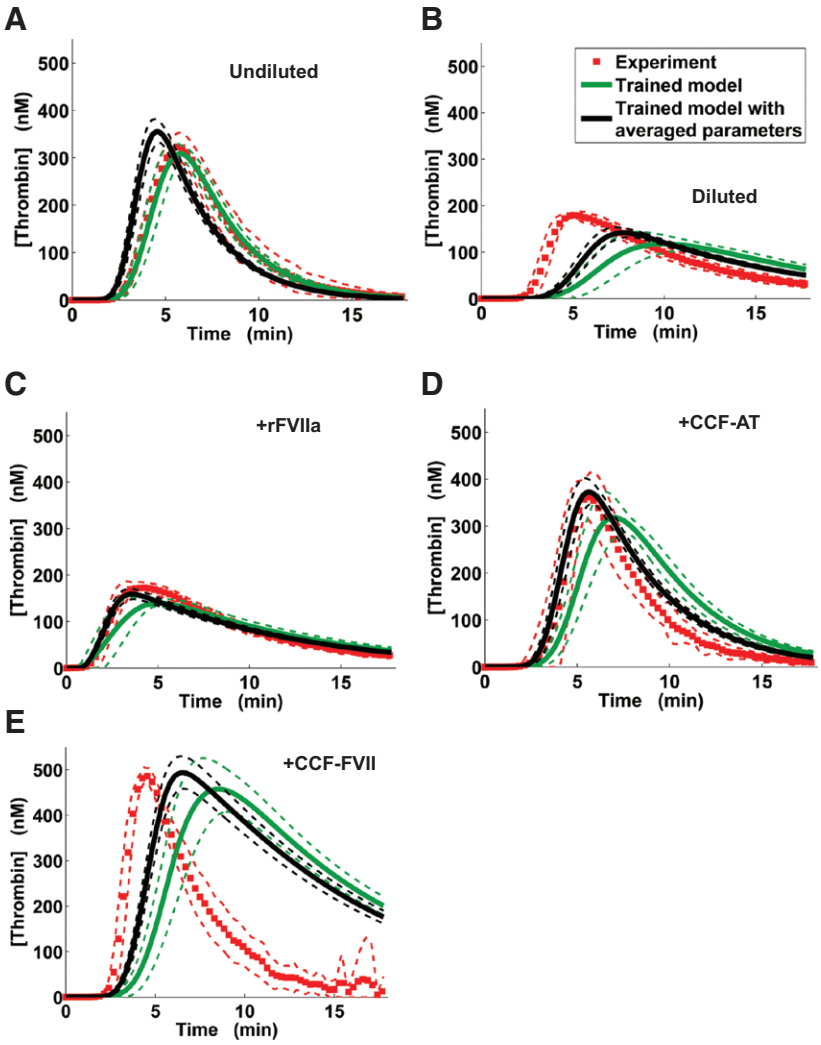

Figure 6. Thrombin generation experimental data and model predictions. Each of the subplots represents one of the considered dilution/supplementation scenarios: undiluted plasma (A), diluted plasma (B), diluted plasma with added rFVIIa (+rFVIIa, C), diluted plasma with added CCF-AT (+CCF-AT, D), and diluted plasma with added CCF-FVII (+CCF-FVII, E). The red markers represent experimental data reproduced from Figure 4 for the purpose of comparison with the computational model outputs. The green lines represent computational model variants trained by leaving 1 subject out and then used (with the initial conditions representing that subject) to compute thrombin generation trajectories for that subject. The black lines represent the averaged-parameter model outputs. The markers and solid lines show the mean values of the individual (experimentally measured and model-predicted, respectively) thrombin generation trajectories averaged over the entire subject group for each considered plasma dilution/supplementation scenario. The dashed lines show the mean ± 1 SD. The mean and SD data were computed from the individual thrombin generation trajectories shown in Figures S3 to S12. AT = antithrombin; CCF = combination of the coagulation factors; rFVIIa = recombinant factor VIIa.

accurate, and ttP was similar to that detected experimentally (Figure 6E). However, the decay of the thrombin trajectory after the peak was noticeably slower in the model-predicted thrombin trajectories than in the experimental data. For most scenarios, using the averaged-parameter model led to increased modeling accuracy (cf. green lines and black lines in Figure 6, C–E) but somewhat reduced the modeling accuracy for undiluted plasma (Figure 6A).

DISCUSSION

Trauma-induced coagulopathy is a significant cause of morbidity and mortality worldwide,[1,2] with limited therapeutic approaches. In our experiments, the constituent coagulation factor levels for the CCF-AT and CCF-FVII supplementations were restored to their predilution values, which is consistent with the factor levels detected after PCC administration in a porcine model of dilutional coagulopathy.[29,30] The CCF-AT supplementation scenario achieved a more balanced enhancement of TG compared with rFVIIa and CCF-FVII supplementation. This is because the latter 2 scenarios strongly accelerated the onset (especially, rFVIIa) and increased the abundance (CCF-FVII) of TG to suprabaseline levels.

Plasma dilution in vitro is only a simplified representation of some of the changes to the hemostatic proteome occurring in vivo during trauma-induced coagulopathy. Yet, blood coagulation analysis in diluted/supplemented plasma may be regarded as a first step toward the mechanistic understanding of coagulopathic conditions. Although different resuscitation fluids have distinct effects on TG,[41] we chose normal saline as an example of a resuscitation fluid in trauma[42] and priming solution in cardiac surgery.[43] Clinical studies have shown that, in human trauma, the degrees of coagulation factor depletion are both factor- and study-dependent.[3–5] Given this uncertainty, we chose an equal-degree dilution scenario, in which every coagulation factor was diluted to the same degree (ie, 3-fold).

Our results suggest that our dilution/supplementation procedure achieved the target coagulation factor levels in the subjects' plasma samples. However, measurement assays using PT-based and chromogenic methods depend on FXa generation, which is increased in rFVIIa-supplemented plasma.[44] As another example, a commercially available (from Abcam®, Cambridge, UK) immunofunctional/chromogenic assay for FVII can also be biased by the presence of FVIIa (Abcam scientific support, email communication). Thus, accurate clotting factor measurements in the presence of high rFVIIa levels appear to be a common problem for

various methods, and the 1-stage clotting assays should still be preferred in general situations.

In this study, we specifically focused on the effects of normalizing the levels of the major coagulation components found in PCCs rather than using the preformulated, commercially available products. This unique approach allowed us to independently control the concentration of supplemented AT. AT is the most abundant natural inhibitor of the blood coagulation proteases and may be regarded as the strongest natural anticoagulant.[10,45,46] Therefore, the design of the CCF-AT supplementation strategy indicates its ability to considerably strengthen both coagulation and anticoagulation processes in plasma, which may be more difficult to achieve with other natural TG inhibitors, such as TFPI and protein C. Most commercially available PCCs only include small amounts of AT (1%–5%) compared with higher concentrations of procoagulant factors.[47,48]

The possibility of thromboembolic complications is a major concern when procoagulants are used in traumatic and surgical coagulopathy. Indeed, rFVIIa is known to cause thromboembolic complications[49] (which may depend on the dose, patient age, and other factors[50]). Similar concerns are valid for PCCs, even though the information from clinical studies may not be sufficient for a definitive conclusion.[15,51] In our opinion, administration of AT together with commercially available PCCs is a promising approach, but the relative dosage and administration time for the 2 distinct components will require careful investigation. With properly balanced procoagulants and anticoagulants, both 3- and 4-factor PCCs could likely achieve the desired improved level of safety without sacrificing efficacy. However, 3-factor PCCs should probably be preferred because their activity lacks the uncertainty associated with the function of FVII. Indeed, although FVII is generally considered a procoagulant, it has been shown to inhibit TG in vitro,[52] which suggests that its overall contribution to blood coagulation may depend on the specific situation and be difficult to predict or control.

Our experimental approach has a number of limitations. First, our study relied on a widely used experimental model that measures TG in a static, cell-free in vitro system. Therefore, the effects of platelets, blood vessel endothelial cells, and blood flow on TG could not be captured in this study. Although the use of PPP (rather than platelet-rich plasma or whole blood) may be regarded as a limitation of this study, the CAT methodology for TG measurement is particularly well established for PPP, which allows for reliable data generation and direct comparisons with available literature. Second, the effects of thrombomodulin and protein C were not investigated. Third, because we focused solely on TG, other aspects of blood coagulation, such as fibrin generation and fibrinolysis, were not addressed. Indeed fibrinogen deficiency, which may affect fibrin accumulation, is likely to be the primary manifestation of trauma-induced coagulopathy. The final limitation stems from our use of an in vitro model of clotting factor dilution/depletion and supplementation scenarios that may not fully capture the characteristics of these processes in vivo. Nevertheless, the design and results of this study form a foundation for future investigations into the biological reactions taking place in vivo.

The results of this study indicate that the possible detrimental effects of rFVIIa and current PCC products may be caused by a distortion of TG kinetics caused by these treatment strategies; this distortion may be avoided if procoagulant components are combined with AT. Overall, our results suggest that if hemostatic balance is to be achieved, the therapeutic intervention strategy itself should be balanced. The notion of balance is a key concept in our investigation and can be defined as the presence of both procoagulant and anticoagulant components at comparable levels of abundance or activity in the therapeutic strategy. We believe that this concept should guide the search for optimized clotting factor compositions, and one approach to perform such searches is through computational modeling.

We devised a computational modeling strategy to predict TG in the diluted/supplemented subject plasma samples. The strategy involved subject-specific parameter tuning during model training. This was based on the use of TG data obtained from the subjects' plasma, an approach advocated in a critique of mathematical modeling in blood coagulation research.[53] It appears that to capture the specifics of the experimental protocol and to better reflect intersubject variability, such parameter tuning is necessary. Without it, the modeling accuracy can typically be only qualitative or semiquantitative at best.[23] The cross-validation predictions of TG for the undiluted plasma scenario were quantitatively accurate for most subjects (Figure 6 and Figures S3 to S12). The TG model prediction accuracy for the diluted and supplemented plasma was somewhat lower for certain subject/scenario combinations (particularly, for dilution and CCF-FVII supplementation), which warrants future efforts to improve both the mathematical model and training/validation algorithm.

One of our model's main limitations is its limited accuracy in certain situations. It is plausible that including TG data for diluted/supplemented plasma in the training data sets would further improve the model's accuracy in predicting TG under these conditions. Moreover, the limitations indicated for our experimental phase also apply to the computational modeling phase and suggest directions for model improvement. For example, our recently developed computational model[23] of fibrin formation and fibrinolysis may be combined with the subject-specific model training algorithm presented here and may be used to predict fibrin accumulation in blood samples from individual subjects.

The results of this study support the notion that mathematical modeling can facilitate hypothesis generation and thereby successfully guide experimental and clinical research. The presented modeling strategy can be applied as a "virtual test bed" to investigate subject-specific effects of coagulopathic conditions (including hypothermia[36] and acidosis[37]) and clotting factor supplementation on TG. Research objectives for future simulation-driven predictive analyses include understanding of the effects of current commercial PCC products under different coagulopathic conditions, the effects of plausible therapeutics, such as combinations of rFVIIa with natural anticoagulants, and the effects of intervention dosage and timing variations on the therapeutic outcomes.

Software Availability

The computer code implementing our mathematical model and the computational analyses is available from the authors on request.

Disclaimer

The opinions and assertions contained herein are private views of the authors and are not to be construed as official or as reflecting the views of the US Army or the US Department of Defense. This article has been approved for public release with unlimited distribution. ■

DISCLOSURES

Name: Alexander Y. Mitrophanov, PhD.
Contribution: This author helped design the research, perform the computational modeling, analyze the data, and write the manuscript.
Conflicts of Interest: Alexander Y. Mitrophanov declares no conflicts of interest.
Name: Fania Szlam, MMSc.
Contribution: This author helped design the study, carry out in vitro experiments, analyze the data, and prepare the manuscript.
Conflicts of Interest: Fania Szlam declares no conflicts of interest.
Name: Roman M. Sniecinski, MD.
Contribution: This author helped collect the data, analyze the data, and prepare the manuscript.
Conflicts of Interest: Roman M. Sniecinski reports grants and personal fees from Grifols and grants from Shire ViroPharma outside the submitted work.
Name: Jerrold H. Levy, MD.
Contribution: This author helped collect the data, analyze the data, and prepare the manuscript.
Conflicts of Interest: Jerrold H. Levy reports participation in the Steering Committees for CSL Behring, Boehringer-Ingelheim, Jansen, and Grifols during the conduct of the study.
Name: Jaques Reifman, PhD.
Contribution: This author helped design the study, analyze the data, and edit the manuscript.
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RECUSE NOTE

Dr. Roman M. Sniecinski is the Section Editor for Hemostasis for *Anesthesia & Analgesia*. This manuscript was handled by Dr. Charles W. Hogue, then Associate Editor-in-Chief and Section Editor for Cardiovascular Anesthesia, and Dr. Sniecinski was not involved in any way with the editorial process or decision.

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