Bleeding and the New Anticoagulants

Strategies and Concerns

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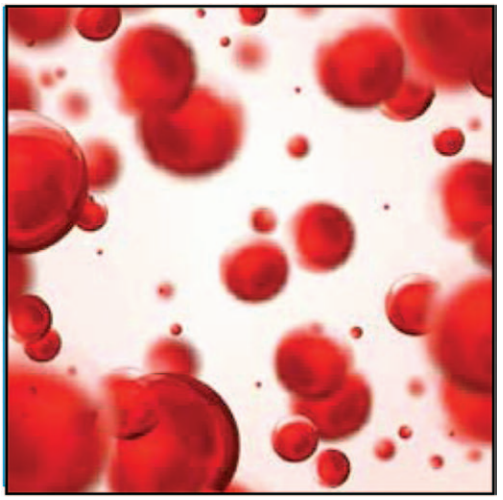
THE non–vitamin K antagonist K oral anticoagulants (NOACs) represent a major step forward as compared with low-molecular-weight heparins and vitamin K antagonists.[1] Four active molecules are now available on the market. Only one direct thrombin inhibitor (anti-IIa agent) has been developed: dabigatran (Pradaxa®) from Boehringer-Ingelheim (Biberach, Germany). Three anti-Xa agents are now marketed: rivaroxaban (Xarelto®) from Bayer (Leverkusen, Germany) and Johnson & Johnson (New Brunswick, NJ), apixaban (Eliquis®) from the alliance BMS (Princeton, NJ) and Pfizer (New York, NY), and edoxaban (Lixiana®) from Daiichi-Sankyo (Parsippany, NJ).

Although efficacy of NOACs administration was reported in different clinical trials, several issues deserve our attention. Both pharmacokinetics and pharmacodynamics for these agents are associated with significant intra- and interindividual variabilities and a huge number of drug interactions, and elimination is significantly affected by renal function, which could be associated with significant variations in plasmatic concentrations and an increased bleeding risk.[2] Although new tests become more readily available for monitoring, which include the diluted thrombin time for dabigatran (Hemoclot®; Hyphen BioMed, Neuville sur-Oise, France), and specific anti-Xa assays for other direct Xa inhibitors, ranges that allow an optimal balance between effective anticoagulation and lower bleeding risk still need to be better defined for these agents.[3] Finally, although clinical trials are underway, no specific antidotes are yet available and leave prothrombin complex concentrates (PCCs) as the principal therapeutic option. These hemostatic agents have been tested with conflicting results in different animal models[4,5] and healthy volunteers,[6] but efficacy has only been reported in a few bleeding cases.[7]



"When life-threatening bleeding occurs with any anticoagulant, a multimodal approach should be considered with hemodynamic and hemostatic resuscitation."

In this issue of Anesthesiology, the article by Hoffman *et al.*[8] adds new potential data. Using their cell-based model of thrombin generation, the authors have compared the efficacy of recombinant factor VIIa and a four-factor PCC in the presence of dabigatran. While enhancing the rate of thrombin generation and peak thrombin level mainly with PCC, the authors have observed a good correlation with hemostasis *in vivo* in a mouse saphenous vein bleeding model. Effects of PCC have been seen *in vitro* at both therapeutic and markedly supratherapeutic dabigatran levels, while beneficial effects of recombinant factor VIIa decreased as the dabigatran level increased.

This interesting scientific study performed by a well-known group in the field of the fundamental mechanisms of hemostasis represents a potential approach until a specific reversal agent becomes available for dabigatran with an already approved, widely available nonspecific procoagulation agent. However, even if the results herein are supportive regarding the efficacy of four-factor PCC, it has to be emphasized that the major part of these experiments has been performed *in vitro* and that their "modest dose" of dabigatran mimics a 472 ng/ml concentration in humans, which already represents a very high plasma concentration that is several-fold greater than a therapeutic level.[9,10] Furthermore, the *in vivo* data uses a mouse saphenous bleeding model that is difficult to extrapolate to humans, and the safety of this potential prothrombotic coagulation factor approach cannot be addressed based on their animal model.

Several important issues regarding dabigatran should be considered. Dabigatran has a very low bioavailability (~6%), has a long terminal half-life (13 to 17 h in healthy volunteers), and is eliminated *via* the kidney (80%). The published pivotal trials in venous thromboembolism prophylaxis[11] and

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Corresponding article on page 353.

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treatment, or the Randomized Evaluation of Long-Term Anticoagulation Therapy trial in atrial fibrillation patients,[12] have demonstrated a high level of efficacy and safety. Nevertheless, real-life settings sometimes differ, and following major bleeding events in patients treated with this agent, numerous regulatory agencies have issued alerts or caution for the need for monitoring renal function.[13] Dosing is also different in Europe and in the United States, with different dosing strategies. In addition, a recent controversy dealing with the potential benefits of a biological monitoring to optimize the safety and efficacy of dabigatran has reemerged.[14] However, it is important to realize that all anticoagulants can cause bleeding, and their relative risk *versus* benefits should be considered. When life-threatening bleeding occurs with any anticoagulant, a multimodal approach should be considered with hemodynamic and hemostatic resuscitation.[3]

In summary, all of the NOACs do represent important therapeutic approaches for our patients. Indeed, we need well-performed experimental studies as Hoffman *et al.*'s,[8] to try to find a scientific rationale for a nonspecific reversal in patients treated with NOACs and scheduled for an emergent procedure or for bleeding patients. However, specific monitoring tests are increasingly becoming clinically available to help better manage patients with all the different NOACs. Although a specific antidote is not yet available for all of the agents (both anti-IIa and anti-Xa), studies are underway with idarucizumab, a monoclonal antibody fragment for dabigatran,* and with andexanet, a Xa reversal agent.†

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Competing Interests

Dr. Samama received speaker fees from the Bayer (Leverkusen, Germany), Bristol-Myers Squibb (BMS, Princeton, New Jersey), Boehringer-Ingelheim (Biberach, Germany), CSL Behring (King of Prussia, Pennsylvania), Daiichi-Sankyo (Parsippany, New Jersey), LFB (Les Ullis, France), Octapharma (Lachen, Switzerland), and Pfizer (New York, New York). He is the member of the advisory committees for Bayer, BMS, Boehringer-Ingelheim, Daiichi-Sankyo, Pfizer, Portola (San Francisco, California), and Roche (Basel, Switzerland). He is the primary investigator for Bayer, BMS, Boehringer-Ingelheim, and LFB. Dr. Levy is the member of the research steering committees for CSL Behring, Boehringer-Ingelheim, Johnson & Johnson (New Brunswick, New Jersey), Marathon (Northbrook, Illinois), and Portola.

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* Available at: http://clinicaltrials.gov/ct2/show/NCT02104947?term=dabigatran+bleeding&rank=3. Accessed October 5, 2014.

† Available at: http://clinicaltrials.gov/ct2/show/NCT02220725?term=portola&rank=1. Accessed October 5, 2014.

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Reversal of Dabigatran Effects in Models of Thrombin Generation and Hemostasis by Factor VIIa and Prothrombin Complex Concentrate

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ABSTRACT

Background: The oral thrombin inhibitor dabigatran has the drawbacks that it does not have a validated antidote. Data from animal studies and plasma coagulation assays suggest that prothrombin complex concentrate (PCC) or recombinant factor VIIa (FVIIa) might reverse dabigatran anticoagulation.

Methods: Cellular elements make a significant contribution to hemostasis. Our goals were to (1) test the hypothesis that both FVIIa and a 4-factor PCC improve parameters of thrombin generation in the presence of dabigatran in a cell-based model; and (2) determine whether results in a cell-based model correlate with hemostasis *in vivo*.

Results: PCC reversed dabigatran effects on the rate, peak, and total amount of thrombin but did not shorten the lag (n = 6 experiments in triplicate). By contrast, FVIIa shortened the lag, increased the rate and peak, but did not improve total thrombin (n = 6). Effects of PCC were seen at both therapeutic and markedly supratherapeutic dabigatran levels, whereas beneficial effects of FVIIa decreased as the dabigatran level increased. The PCC effect was reproduced by adding prothrombin, factor X, and factor IX. At therapeutic dabigatran levels, both PCC and FVIIa normalized hemostasis time in a mouse saphenous vein bleeding model.

Conclusions: A cell-based model reflects the effects on thrombin generation of clinically relevant levels of FVIIa and PCC in the presence of dabigatran. Enhancing the rate of thrombin generation and peak thrombin level appear to correlate best with hemostasis *in vivo*. The ineffectiveness of FVIIa at supratherapeutic dabigatran levels may explain conflicting reports of its efficacy in dabigatran reversal. **(ANESTHESIOLOGY 2015; 122:353-62)**

PRADAXA® (Boehringer Ingelheim, Biberach, Germany) is an oral anticoagulant that contains the prodrug, dabigatran etexilate.[1] It is metabolized to dabigatran by esterases in the liver and elsewhere. Dabigatran is a specific high-affinity thrombin inhibitor. In clinical trials, it had less risk of bleeding than vitamin K antagonists.[2] In the United States, it is approved as an antithrombotic to prevent stroke in patients with "nonvalvular" atrial fibrillation.

Dabigatran has a relatively short half-life (between 7 and 17 h) in patients with adequate renal function (creatinine clearance > 80 ml/min).[3,4] Thus, minor bleeding can be managed by withholding the drug until its effects are reversed by clearance. However, life-threatening bleeding or an urgently needed invasive procedure can be difficult to manage due to the lack of a validated antidote.[5]

A humanized antibody antidote for dabigatran has been tested in animal models.[6] It is in clinical trials[7] but is not yet commercially available. Thus, other reversal strategies have been attempted. Dabigatran can be removed by dialysis, but this strategy is rarely used because of the need for large bore vascular access. More commonly, nonactivated prothrombin complex concentrates (PCCs), recombinant factor VIIa (FVIIa), or activated PCC (FEIBA®, Baxter International, Deerfield, IL) have been administered in attempts to reverse its anticoagulant effects.[8–10] No clinical trials of the ability of

What We Already Know about This Topic

- Prothrombin complex concentrates and recombinant factor VIIa are used as procoagulant therapies to treat life-threatening bleeding with the new oral anticoagulation agents
- New target-specific reversal agents are being developed that will bind to either the oral direct thrombin inhibitors (dabigatran) or the oral direct Xa inhibitors

What This Article Tells Us That Is New

- Using a cell-based coagulation model, the ability of either prothrombin complex concentrate or recombinant factor VIIa to restore hemostasis in the presence of dabigatran depends on the dose of procoagulant used and the level of dabigatran present and may explain the inconsistency of effects in different models and when used off label for treating bleeding

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these agents to restore hemostasis in bleeding human subjects have been conducted. Thus, data in support of reversal strategies come from case studies, animal studies, and reversal of dabigatran effects on coagulation tests in blood from normal subjects.

The studies in animal models or by spiking drugs into human or animal blood have sometimes given contradictory results. This may be because of the different models used, including differences in species studied, and differences in the doses of dabigatran and reversal agents used. In the aggregate, the published studies suggest that the common laboratory coagulation tests do not accurately reflect the anticoagulant effects of dabigatran or the potential reversal of those effects by "antidotes."[11] It appears that thrombin generation assays correlate better with hemostatic function. However, these assays are not well standardized and not easy to use in a clinical setting.[12] In spite of these issues, the literature suggests that PCCs may be useful in managing bleeding associated with dabigatran anticoagulation (reviewed by Dickneite and Hoffman[11]). By contrast, the literature is very contradictory on the potential of FVIIa to reverse the effects of dabigatran.

The goals of the current study were to: 1) test the hypothesis that both FVIIa and a 4-factor PCC improve parameters of thrombin generation in a cell-based model in the presence of dabigatran, and 2) correlate the results in this model with hemostatic effects in a new animal model that is sensitive to the effects of anticoagulants and is less variable than previous models.[13,14] Pursuit of these goals may help clarify the utility of a thrombin generation assay as a surrogate test for hemostasis in this setting. It may also clarify which parameters of thrombin generation are most predictive of hemostatic function.

Materials and Methods

Materials

The four-factor PCC (Beriplex® or Kcentra®, CSL-Behring, King of Prussia, PA) and rFVIIa (NovoSeven®, Novo Nordisk A/S, Bagsvaerd, Denmark) were reconstituted as directed by the manufacturers and stored frozen in aliquots at −80°C until use.

The active form of the anticoagulant in Pradaxa®, dabigatran, was purchased from Selleck Chemicals (Houston, TX).

Prothrombin and factor IX (FIX) were purified as described.[15] Factor X (FX) was purchased from Haematologic Technologies (Essex Junction, VT). All zymogen coagulation factors were treated with an inhibitor mixture (tosyl-lysyl chloromethyl ketone, tosylphenyl chloromethyl ketone, phenylmethyl sulphonyl fluoride, Phe-Pro-Arg chloromethyl ketone, and dansyl Glu-Gly-Arg chloromethyl ketone) for 1 h, and then repurified on HiTrap Q using calcium chloride elution.

Dabigatran stock solution was prepared in dimethyl sulfoxide. The activity of the dabigatran as a thrombin inhibitor was verified as follows. A high concentration of thrombin (30 nM) was added to various concentrations of dabigatran. Samples of the mixtures were added to a weak thrombin chromogenic substrate (methoxycarbonyl-D-cyclohexylalanyl-Glycyl-Arginine-*p*-nitroanilide, Pentapharm, Basel, Switzerland) and the remaining activity measured. Dabigatran concentration was determined from the intercept of the residual activity *versus* concentration of dabigatran plot. We found the Ki values to be in the low nanomolar range, as reported in the literature.[4]

Cells

Written informed consent was obtained before the collection of human blood samples used in these studies under a protocol approved by the Institutional Review Board at the University of North Carolina, Chapel Hill, North Carolina. Monocytes were isolated from freshly drawn human blood as previously described using Accuprep lymphocyte separation media (Accurate Chemicals, Westbury, NY).[16] Monocytes were plated at different densities in serum-free media and then incubated with lipopolysaccharide (500 ng/ml in the serum free media) for 1 h to induce expression of tissue factor (TF). Monocytes were then incubated overnight. Before use, test wells were assayed for tissue factor expression by monitoring factor Xa generation when FVIIa (1 nM) and FX (135 nM) were added to the wells. The density of monocytes that corresponded to 1 pM TF activity was used in thrombin generation experiments.

Platelet-rich plasma (PRP) was prepared from blood drawn into citrate anticoagulant by centrifugation (150*g*, 15 min) within 1 h of collection. The platelet count was measured on a Z1 Series Coulter Counter (Beckman Coulter, Fullerton, CA) and standardized by dilution with autologous platelet-free plasma to a final concentration of 150,000/μl.

Modified Calibrated Automated Thrombogram Method

Fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC) and thrombin calibrator (α2-macroglobulin/thrombin) were purchased from Diagnostica Stago (Parsippany, NJ). The CAT® assay was performed as previously described,[17] with the following modifications: cultured monocytes, as described in the "Cells" section, were used as a TF source; and PRP was used as the source of (unactivated) platelets.

Dabigatran was added to PRP at concentrations selected to simulate the peak therapeutic dose (0.4 μM, 189 ng/ml), modest (1 μM, 472 ng/ml) and extreme (2 μM, 944 ng/ml) supratherapeutic levels. PCC or FVIIa was added to PRP at concentrations indicated in the different experiments. The concentrations of PCC used in both the human PRP experiments *in vitro* and the mouse bleeding experiments *in vivo* were based on the doses of this product used in human patients. The doses of PCC needed to reverse warfarin anticoagulation in mice are comparable to those required in humans.[18] This suggests that basing dosing for mice on human doses is reasonable. However, the dosing of FVIIa in mice is quite different than in humans. Hemophilic mice

require many times higher doses of FVIIa (either human[19,20] or mouse)[21] than do hemophilic humans to exert a hemostatic effect. While human hemophilia patients usually require 90–270 μg/kg FVIIa for hemostasis, a mouse model of severe injury, such as the tail clip model, can require 1 mg/kg FVIIa or more.

In some experiments, purified prothrombin, FX, and FIX were added to the CAT® assays at concentrations equal to those present in the specific PCC used in our experiments (Beriplex/K-Centra). PCCs are labeled based on their FIX content. Thus, 1 IU/ml of the PCC contains 1 IU/ml of FIX. For every 1 IU of FIX, it also contains 1.07 IU prothrombin and 1.43 IU/ml FX.[22] Additions to the PRP were made immediately before initiation of thrombin generation by adding the PRP to TF-bearing monocytes, then adding the calcium and fluorogenic substrate reagent. All assays were performed in triplicate. We recalculated parameters of thrombin generation from the raw fluorescence data because the proprietary software sometimes gave implausible results.

Mouse Saphenous Vein Bleeding Model

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. C57BL6/J mice were purchased from Charles Rivers Laboratories (Wilmington, MA). The *in vivo* assessment of bleeding was carried out as previously described.[13,14,23] This saphenous vein bleeding model performs extremely well for the assessment of abnormalities of coagulation, including the effects of anticoagulants.[24]

Mice were randomly assigned to receive buffer (n = 6), dabigatran alone (n = 9), or dabigatran plus 25 or 50 U/kg of PCC or 124, 270, or 540 μg/kg of FVIIa (n = 4 in each group). On any given day, at least one control and one dabigatran-treated mouse were tested, along with two or three dabigatran plus reversal agent–treated mice. These numbers were chosen based on our previous experience and the desire to use the minimum number of animals necessary to detect statistically significant differences between treatments. All experiments were performed by one animal technician, who was not blinded as to treatment. Mice were anesthetized with isoflurane throughout all procedures. The hair on the ventral side of both hind limbs was removed, and the animal placed supine on a THM 100 Temperature & electrocardiogram Monitoring Board (Indus Instruments, Houston, TX). The paws were gently restrained by the hook-and-loop closures attached to the electrocardiogram pads and/or by a loop of polyethylene tubing attached to the board. The skin overlying both the left and right ventral hind limbs was incised, a length of the saphenous neurovascular bundle exposed and covered with normal saline to prevent drying of the tissues. To allow for injection of pharmacologic agents, the left saphenous vein was cannulated under a SZX12 dissecting microscope (Olympus, Tokyo, Japan) using a catheter constructed of pulled PE-10 tubing (Braintree Scientific, Braintree, MA) with a 3.0-mil cleaning wire (Hamilton, Reno, NV) placed into the lumen as a stylet. Dabigatran injections (15 μg/kg) were followed 5 min later by injection of reversal agent, then 5 min later by initiation of the saphenous vein incision.

Hemostasis was assessed by determination of serial bleeding times from a saphenous vein incision. The right saphenous vein was transected by piercing through and through with a 23-gauge needle (BD Biosciences, San Jose, CA). A longitudinal incision was made in the distal portion of the vessel using Student Vannas Spring Scissors (Fine Science Tools, Foster City, CA) by inserting one blade into the vessel and then cutting. Blood was gently wicked away with a Kimwipe (Kimberly-Clark, Roswell, GA) until hemostasis occurred. The clot was then disrupted using a 30-gauge needle, and blood wicked away until hemostasis occurred again. Clot disruption was repeated after every incidence of hemostasis until 30 min after the initial 23-gauge needle injury. Two parameters were measured: (1) the number of times that hemostasis occurs in a 30-min period, and (2) the time required for each hemostasis event. The hemostasis times for each individual mouse were averaged to obtain a single value for each mouse.

At the conclusion of each hemostasis experiment, blood was withdrawn from the inferior vena cava of each mouse to use for measuring dabigatran concentrations; nine parts blood were mixed with one part of 0.109 M citrate. All animals were killed at the end of the procedure. All injury, clot disruption, and hemostasis time points were recorded on the Lab Chart software (ADInstruments, Colorado Springs, CO).

Dabigatran concentrations were determined using a modified thrombin time. One part of mouse plasma as the source of dabigatran was mixed with one part of normal pooled human plasma as a standardized source of fibrinogen. Clotting was initiated by addition of one volume of plasma to an equal volume of 1 U/ml thrombin. Clotting times were converted to dabigatran concentration from a standard curve generated using known concentrations of dabigatran.

Statistical Analysis

Data are shown as the mean ± SD, unless otherwise indicated. Statistical analyses were performed using Prism version 6 software (GraphPad Software, San Diego, CA). A P value of less than 0.05 was considered statistically significant. The effects of multiple levels of dabigatran, each in the presence of different levels of reversal agent, on parameters of thrombin generation were assessed by two-way repeated-measures ANOVA. Statistical significance between treatments was assessed with a two-tail t-statistic using the Bonferroni correction for multiple comparisons. Differences between parameters of thrombin generation in groups to which different combinations of coagulation factors were added were analyzed by one-way repeated-measures ANOVA and Tukey procedure for multiple comparisons. The statistical significance of effects of reversal agents on hemostasis times in the mouse bleeding model was assessed by one-way ANOVA

with Tukey procedure for multiple comparisons. For clarity of presentation, not all significant differences are shown.

Results

Effects of Dabigatran in the Cell-based CAT® Model

Dabigatran in the cell-based CAT® progressively prolonged the lag before the onset of thrombin generation. This effect is generally similar to reports in the literature using a standard CAT® assay system. Representative data showing the thrombin generation curves from a single experiment are shown in figure 1. Mean effects from six separate experiments using blood from different donors are shown in figure 2.

At a level similar to peak concentrations attained during therapeutic anticoagulation (0.4 μM or 189 ng/ml), dabigatran nearly doubled the lag before the onset of thrombin generation (to 178.3 ± 63.8% of control; $P < 0.05$) and decreased the area under the curve (AUC) to 71.0 ± 3.6% ($P < 0.05$) and the peak thrombin level to 81.7 ± 6.7% ($P > 0.05$). At this concentration, dabigatran did not reduce the rate of thrombin generation. At supratherapeutic concentrations, dabigatran progressively prolonged the lag and reduced the peak, AUC, and rate of thrombin generation.

Effect of PCC and FVIIa on Thrombin Generation in the Presence of Dabigatran

Addition of PCC does not improve the prolonged lag time before the onset of thrombin generation (fig. 3A). However, it can normalize the rate (fig. 3B), peak (fig. 3C), and area under the thrombin generation curve (AUC, fig. 3D) in the cell-based CAT® model at all levels of dabigatran tested. At therapeutic levels of dabigatran, this four-factor PCC at 1 U/ml (equivalent to a dose of 50 U/kg *in vivo*) increased the peak thrombin level attained and the area under the thrombin generation curve (AUC) to supranormal levels.

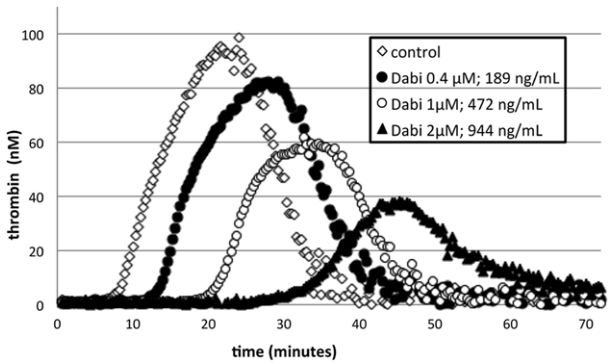

Fig. 1. Effects of dabigatran on thrombin generation curves in a cell-based CAT® model of thrombin generation. This figure shows representative thrombin generation curve data from one experiment. Increasing concentrations of dabigatran (Dabi) progressively prolongs the lag before the onset of thrombin generation as well as depressing the peak, rate of thrombin generation, and total amount of active thrombin in the model system. The CAT® assay is a product of Diagnostica Stago (Parsippany, NJ).

By contrast, FVIIa significantly shortens the lag (fig. 3E) although not back to control level. It also increases the rate (fig. 3F) and peak (fig. 3G). The improvement in rate and peak thrombin is abolished at the 2 μM (high overdose) level of dabigatran. FVIIa did not affect the AUC at any concentration of dabigatran (fig. 3H).

PCC at a level equivalent to 50 U/ml *in vivo* was able to return the rate, peak, and AUC parameters to normal or supranormal levels at all concentrations of dabigatran tested, up to a severe overdose level. The effects of FVIIa varied much more with the level of dabigatran present. At a therapeutic level of dabigatran, FVIIa boosted the rate and peak thrombin above control values. At a modest overdose level, FVIIa returned these parameters to control levels, but at a level comparable to a severe overdose of dabigatran, FVIIa had little or no effect on thrombin generation.

PCC and FVIIa Affect Different Parameters of Thrombin Generation

The effect of different concentrations of PCC and FVIIa on the lag and AUC are shown in figure 4. These data show that PCC has no effect on the lag (fig. 4A), but FVIIa shortens the lag in a dose-dependent manner (fig. 4B). Conversely, PCC has a dose-dependent effect on the AUC (fig. 4C), whereas FVIIa has no effect on this parameter (fig. 4D). Note that even though FVIIa shortens the lag, it does not return it to normal. PCC returns the AUC to a level not significantly different from normal at the highest concentration tested, 1 U/ml (equivalent to an *in vivo* dose of 50 U/kg).

The Effect of FVIIa on Parameters of Thrombin Generation Depends on the Level of Dabigatran Present

At a concentration of 1 μM dabigatran (equivalent to a moderately supratherapeutic level), both FVIIa and PCC had dose-dependent effects to increase the peak thrombin level attained (fig. 5A). Both agents were able to restore this parameter to normal or even supranormal levels under these conditions. By contrast, at 2 μM dabigatran (severely supratherapeutic level), PCC was still able to improve the peak thrombin to a level not significantly different from control at 1 U/ml. However, at this higher level of dabigatran, FVIIa had no significant effect. The pattern of FVIIa and PCC effects on the rate of thrombin generation was identical to that for the peak (fig. 5B). Thus, the ability of FVIIa to reverse the effects of dabigatran on thrombin generation is highly dependent on the level of dabigatran present.

Which Components of PCC Are Needed to Enhance Thrombin Generation in the Presence of Dabigatran?

Our group has extensively investigated the effects of coagulation factor levels on thrombin generation in a cell-based model system.[25,26] PCC contains significant amounts of prothrombin—about 1 U/ml of prothrombin for every 1 U/ml of FIX.[22] The ability of PCC to increase the peak and total thrombin generated appeared similar to the effects of

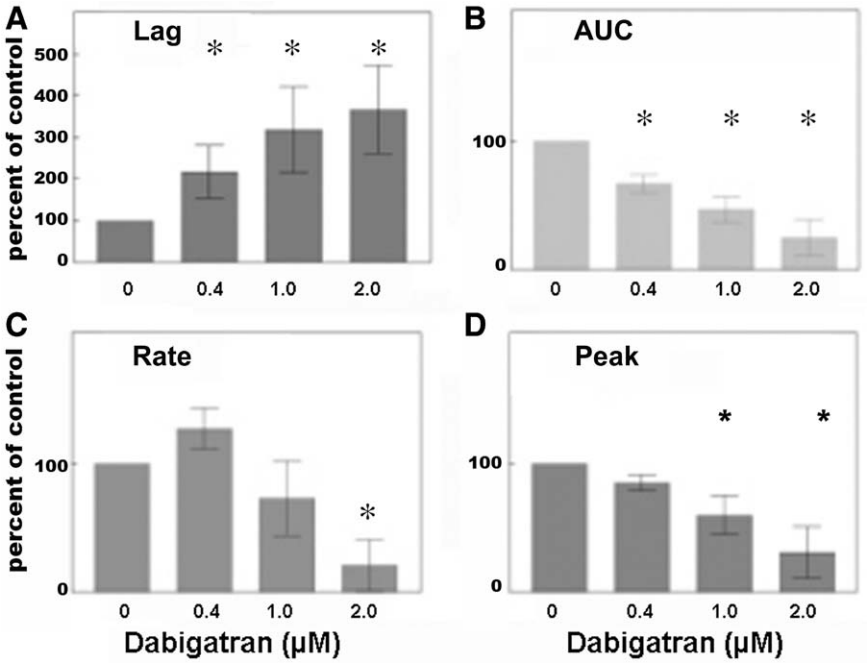

Fig. 2. Effects of dabigatran on parameters of thrombin generation in the modified CAT® assay. At a therapeutic level (0.4 μM), the major effect of dabigatran was on the lag (*A*) and area under the curve (AUC) (*B*), whereas at supratherapeutic levels (1 or 2 μM), dabigatran affected the rate of thrombin generation (*C*) and peak level (*D*), as well. The graphs show the mean ± SD of data from six experiments with different blood donors. Results are expressed as a percent of the control value in the absence of dabigatran in the same experiment. *$P < 0.05$ compared to control in a one-way repeated-measures ANOVA with Tukey method for multiple comparisons. The CAT® assay is a product of Diagnostica Stago (Parsippany, NJ).

adding prothrombin (FII) in the cell-based model. Thus, we hypothesized that the prothrombin content of PCC was the principle component responsible for its prohemostatic activity in the presence of dabigatran. However, the addition of prothrombin at a level equal to that provided by the complete PCC did not reproduce the effects of PCC on thrombin generation, although it did increase the AUC from 52.0 ± 9.8% to 83.3 ± 4.7% of control ($P < 0.05$). Neither did prothrombin plus FX reproduce the effects of PCC on the peak or AUC. Addition of prothrombin plus FX plus FIX did increase the peak thrombin to a level (140 ± 6.0% of control) that was not significantly different from the effect of PCC (146.0 ± 2.6%). However, prothrombin plus FX plus FIX did not reproduce the effect of PCC on the AUC (101.3 ± 18.5% *vs.* 123.0 ± 22.1% in the presence of PCC) (fig. 6, A and B). Thus, although the prothrombin level makes a significant contribution to the amount of thrombin generated (AUC), all components of the PCC are apparently required for its full hemostatic effect.

Reversal of Dabigatran Anticoagulation in a Mouse Saphenous Vein Bleeding Model

In the mouse bleeding model, dabigatran significantly prolonged the time to hemostasis to about twice that measured in control mice. The drug was administered at 15 μg/kg, which produced an average plasma level of 65 ± 18 ng/ml at the end of the hemostasis time experiment. PCC shortened the hemostasis time, with the 50 U/kg dose restoring the hemostasis time to a level that was not significantly different from baseline (fig. 7A). However, 25 U/kg did not have a significant effect on the hemostasis time. Thus, while we did not test very many different doses of PCC in this model, it appears that the dose–response relationship between PCC dose and an effect on hemostasis is quite steep. At least in this model, a dose of 50 U/kg is required for a beneficial hemostatic effect of PCC in the presence of dabigatran.

FVIIa also shortened the time to hemostasis, with the 270 μg/kg dose significantly reducing the bleed time, although a 540 μg/kg dose was needed to restore hemostasis to the control level (fig. 7B).

Discussion

This work tests the ability of PCC and FVIIa to act as "reversal agents" to counteract the anticoagulant effects of dabigatran. Neither PCC nor FVIIa truly reverses the effects of dabigatran, that is, they are not direct antidotes. This is different than when a PCC is used to reverse the effect of a vitamin K antagonist. In that case, the PCC preparation is restoring the levels of vitamin K–dependent factors that are reduced by the drug. By analogy to the terminology used in hemophilia, the use of either FVIIa or PCC to reverse dabigatran would be a type of "bypassing" therapy. In other words, the mechanisms of their effects are not to restore something that is deficient as a result of the drug, but rather to enhance thrombin generation in an attempt to circumvent or overcome the effects of dabigatran.

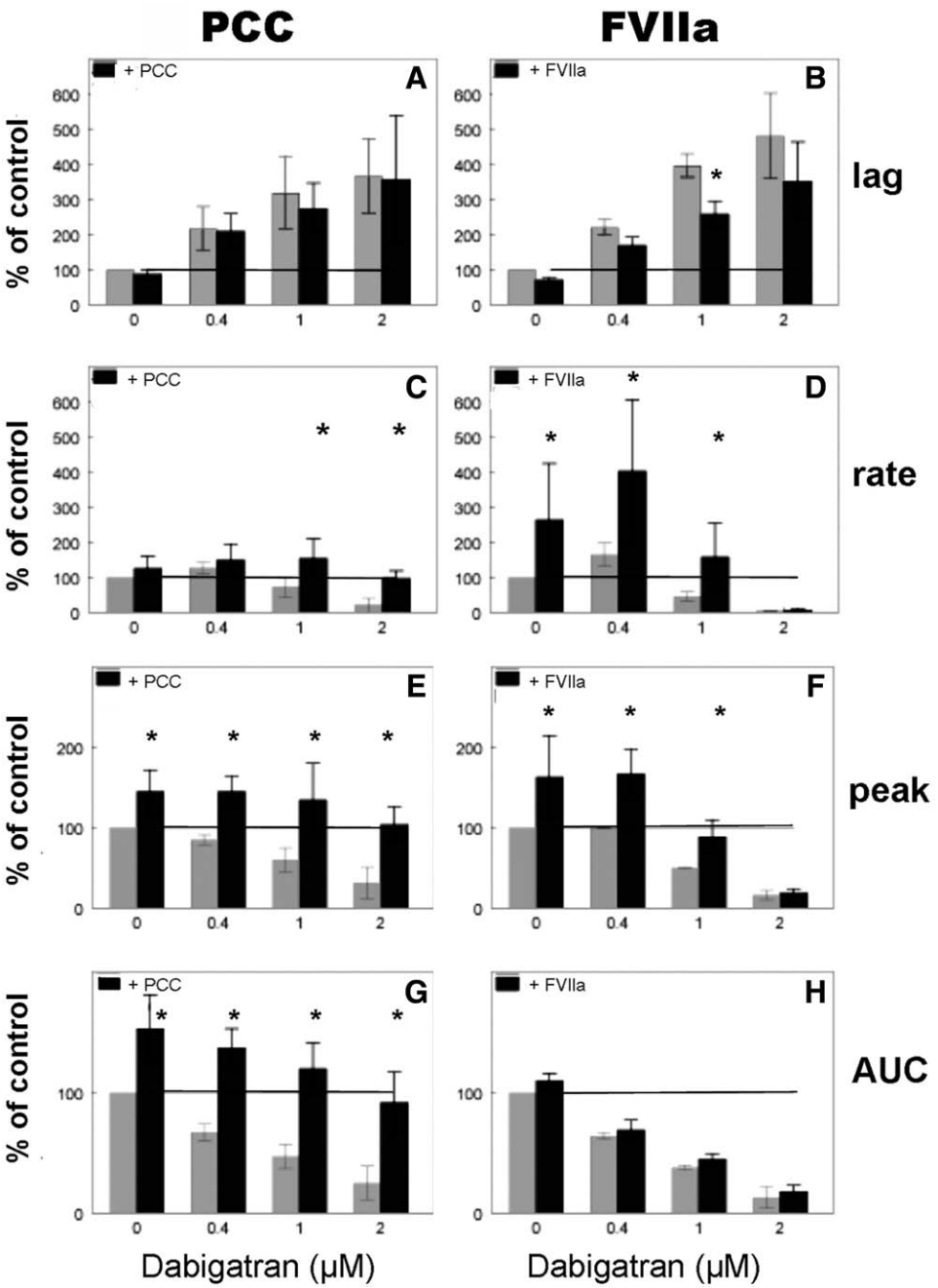

Fig. 3. Effect of prothrombin complex concentrate (PCC) and recombinant factor VIIa (FVIIa) on parameters of thrombin generation in the presence of different levels of dabigatran in a cell-based CAT® assay. Results are shown for lag time (*A* for PCC and *B* for FVIIa), rate of thrombin generation (*C* for PCC and *D* for FVIIa), peak thrombin concentration achieved (*E* for PCC and *F* for FVIIa), and total active thrombin generated (area under the curve [AUC]; *G* for PCC and *H* for FVIIa). Results are expressed as a percent of the value for that parameter in the absence of any reversal agent. The control value (100%) is indicated by the horizontal bar in each panel. The *gray bars* in each panel indicate the values in the absence of reversal agent, and the *black bars* indicate the values in the presence of 1 U/ml PCC (*A*, *C*, *E*, and *G*) or 100 nM FVIIa (*B*, *D*, *F*, and *H*). *$P < 0.05$ compared to no added PCC or FVIIa by two-way repeated-measures ANOVA with Bonferroni correction for multiple comparisons. Data are the mean ± SD of eight experiments. The CAT® assay is a product of Diagnostica Stago (Parsippany, NJ).

The common coagulation assays, prothrombin time, activated partial thromboplastin time, and thrombin time, perform well when used to monitor coagulation factor deficiency and replacement therapy. The utility of these assays has also been empirically defined in certain specific settings, such as warfarin anticoagulation and heparin anticoagulation. However, they do not truly reflect the hemostatic status of the patient. Thrombin generation assays have been reported to correlate better with the effects of anticoagulants.[27] Thus, it should not be surprising that the common

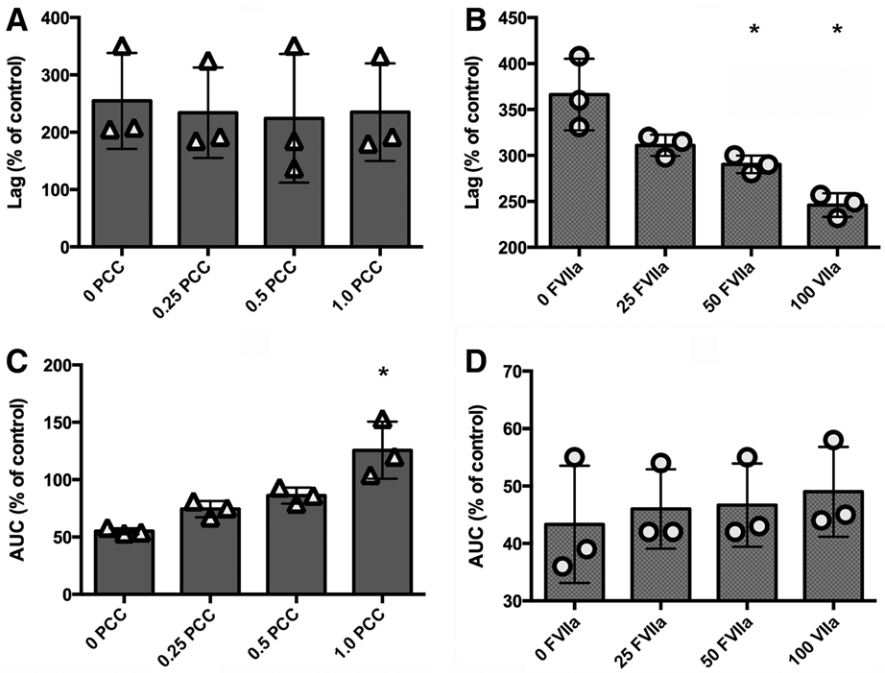

Fig. 4. Dose–response relationship of prothrombin complex concentrate (PCC) and recombinant factor VIIa (FVIIa) on the lag and area under the curve (AUC) of thrombin generation in the presence of 1 μM dabigatran. The *top* of each bar represents the mean (± SD) of three experiments using cells and plasma from different donors. The *symbols* represent the mean value of triplicate wells in each of the experiments: *triangles* for the experiments with addition of PCC and *circles* for experiments with addition of FVIIa. PCC (at 0.25, 0.5, and 1.0 IU/ml) had no significant effect on the lag at any concentration tested (*A*). FVIIa (at 25, 50, and 100 nM) shortened the lag in a dose-dependent manner (*B*), which reached statistical significance at 50 and 100 nM. Conversely, PCC increased the AUC in a dose-dependent manner (*C*), which reached statistical significance at 1 IU/ml. FVIIa had no effect on AUC at any concentration tested (*D*). *$P < 0.05$ compared to no added PCC or FVIIa using two-way repeated-measures ANOVA with Bonferroni correction for multiple comparisons.

assays are not very useful in reflecting the hemostatic status of patients receiving the novel oral anticoagulants. At this time, a review of the literature suggests that thrombin generation assays correlate best with the hemostatic status of a patient or experimental animal on dabigatran.[11]

We found that dabigatran progressively lengthens the lag before the onset of thrombin generation and also reduces the total and peak levels of active thrombin in a cell-based CAT® assay system. This is similar, although not identical to published work using thrombin generation assays employing platelet poor plasma, with phospholipid vesicles in place of platelets.[28] The delay in the lag due to dabigatran is more pronounced in our assays with platelets. This is likely because the phospholipid vesicles do not require "activation," as do platelets, before expressing their procoagulant activity. Thus, it is not surprising that our results for dabigatran effects are quite similar to those recently reported by others who also used PRP.[29]

We draw the following conclusions from this work:

- In a cell-based thrombin generation assay, PCC (at clinically relevant doses) can increase the peak and total amount of thrombin generated to normal or supranormal levels at all concentrations of dabigatran tested, from therapeutic to severe overdose. However, PCC does not improve the lag time.
- The FII, FX, and FIX content of this four-factor PCC (Kcentra® or Beriplex®) is sufficient to normalize parameters of thrombin generation. However, the maximal effect on total thrombin generation (AUC) requires the complete PCC preparation.
- FVIIa improves the lag time, rate of thrombin generation, and peak level reached but does not increase the total amount of thrombin generated (AUC).
- FVIIa only has a beneficial effect on parameters of thrombin generation if the dabigatran level is not too high (not > about 1 μM or 480 ng/ml in this model).
- While neither FVIIa nor PCC normalizes all of the parameters of thrombin generation in the presence of dabigatran, each can normalize hemostasis in a mouse bleeding model in the presence of a therapeutic level of dabigatran.
- There is an extremely steep dose–response relationship between the dose of PCC or FVIIa administered and shortening of the hemostasis time in the mice given a therapeutic level of dabigatran. This may indicate a threshold effect in that little, if any, benefit is seen unless the concentration of the "reversal" agent is above a critical threshold. Based on our studies and the previously published literature, this appears to be about 50 U/kg for the particular PCC we used (Kcentra® or Beriplex®). Dosing of FVIIa in mice cannot be extrapolated to humans.

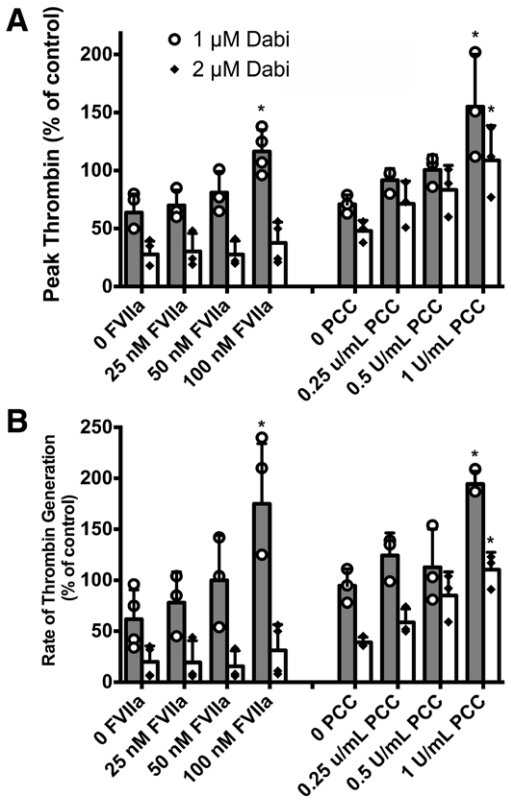

Fig. 5. The effect of prothrombin complex concentrate (PCC) and recombinant factor VIIa (FVIIa) on the peak and rate of thrombin generation depends on the level of dabigatran (Dabi) present. This figure shows the effects of different concentrations of added FVIIa or PCC (as indicated by the labels for the *X*-axis) in the presence of 1.0 μM (*open circles* superimposed on *gray bars*) or 2.0 μM dabigatran (*black diamonds* superimposed on *white bars*). The data for the peak thrombin generation are shown in (*A*) and for the rate of thrombin generation in (*B*). The bars are the means (± SD) of four separate experiments using cells and plasma from different donors. The *symbols* represent the mean of three triplicate values in each individual experiment. The data are expressed as a percent of the control value (no dabigatran, PCC, or FVIIa added) in the same experiment. **P* < 0.05 compared to control using two-way repeated-measures ANOVA with Bonferroni correction for multiple comparisons.

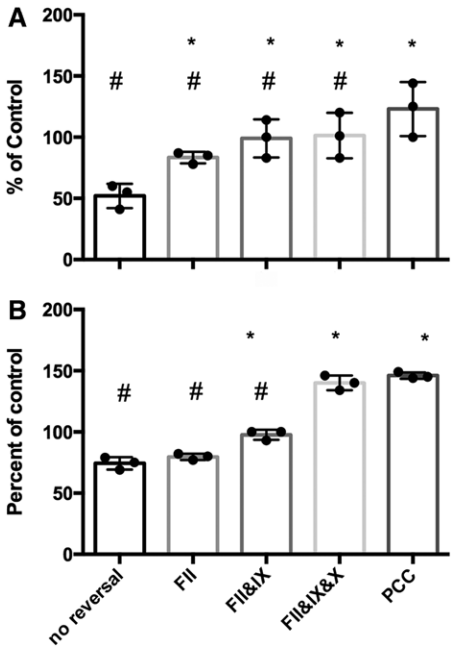

Fig. 6. Effect of components of prothrombin complex concentrate (PCC) on the peak level of thrombin generation and area under the curve (AUC) of thrombin in the presence of 1 μM dabigatran. Purified prothrombin (FII), prothrombin and FX (FII&X), prothrombin and FX and FIX (FII&X&IX), or 1 U/ml PCC (PCC) were added to platelet-rich plasma assayed in the cell-based CAT® in the presence of 1 μM dabigatran. The purified coagulation factors were added at levels equal to the levels of these factors present in the PCC. *Bars* represent the mean (± SD) of three experiments. The *black circles* represent the mean values of triplicate wells for each of the experiments. Results are expressed as a percent of the control (no additions) in the same experiment. The results in the presence of 1 μM dabigatran, but no added coagulation factors, are indicated as "no reversal." Data for the AUC are shown in *A* and peak thrombin in *B*. *Value significantly different from "no reversal"; #value significantly different from dabigatran + 1 U/ml PCC (PCC) by repeated-measures ANOVA with Tukey multiple comparisons test. Thus, the AUC (*A*) was significantly greater in the presence of PCC than any of the combinations of added factors. However, the peak thrombin (*B*) in the presence of dabigatran plus factors II/X/IX was not significantly different from dabigatran plus PCC.

Thus, although tests of thrombin generation show a correlation with dabigatran effects and reversal of those effects, the thrombin generation curve does not have to be restored to "normal" in order for hemostatic function to be restored. We hypothesize that some combination of the initial rate of thrombin generation and the peak thrombin level attained will correlate best with hemostasis *in vivo*.

The effects of dabigatran in this model were generally similar to those reported for a conventional CAT® assay in which the tissue factor initiator is incorporated in phospholipid vesicles, and phospholipid is usually used in place of platelets as the surface for large-scale thrombin generation. However, there do appear to be some modest differences between our results and those reported previously.

Marlu *et al.*[8] tested the effects of PCCs and FVIIa on the anticoagulant activity of dabigatran using thrombin generation tests. Healthy subjects ingested a single dose of dabigatran (150 mg). Blood samples were collected just before drug administration and 2 h thereafter. Reversal of anticoagulation was tested by adding potential reversal agents to the stored plasma *in vitro*. They found that dabigatran affects the kinetics of thrombin generation with prolonged lag time and time to peak. However, they did not see as dramatic a prolongation of the lag time as we do. We think this is because a significant portion of the lag is due to the time required for platelet activation. When phospholipid vesicles are substituted for platelets, this component of the lag is not reflected

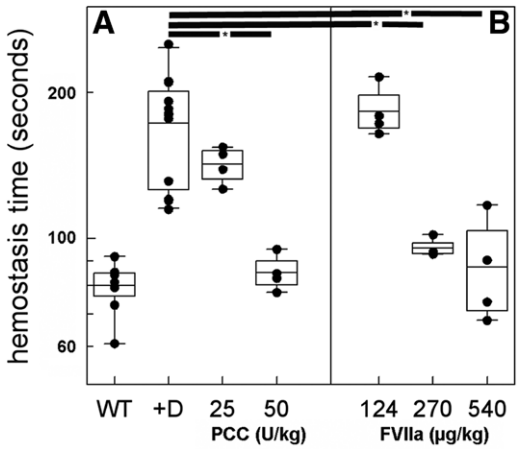

Fig. 7. Effect of prothrombin complex concentrate (PCC) and recombinant factor VIIa (FVIIa) on hemostasis time in a mouse saphenous vein bleeding model. (*A*) The hemostasis time of wild-type (WT) mice given no active pharmacologic agents (n = 6), mice given 15 μg/kg dabigatran IV (+D, n = 9), and mice given dabigatran plus 25 or 50 U/kg PCC (n = 4 in each group). (*B*) The hemostasis time of mice given 15 μg/kg dabigatran plus 124, 270, or 540 μg/kg FVIIa (n = 4 in each group). Each *dot* represents the mean hemostasis time from one mouse. The *bottom* and *top* of the "box and whiskers" plot are the first and third quartiles of the hemostasis times, the *line* within the box is the median of all mice in the treatment group, and the "*whiskers*" indicate the upper and lower extreme values of the data. The doses of 50 U/kg PCC and 270 and 540 μg/kg FVIIa returned the hemostasis time to a level that was significantly different from mice given dabigatran alone and not significantly different from control mice. *$P < 0.05$ by one-way ANOVA with Tukey procedure for repeated comparisons.

in the result. They found that FVIIa could restore the lag to normal. We similarly found that FVIIa could shorten the lag, although not restore it to normal. However, they did not use any assessment of hemostasis to determine whether the parameters of thrombin generation correlated with reversal of anticoagulation.

Pragst *et al.*[9] studied reversal of dabigatran effects in a rabbit model of bleeding from a kidney incision. They used a dose of dabigatran of 0.4 mg/kg, which resulted in a peak level of 786 ng/ml. This high dose of dabigatran was noted to depress the peak level of thrombin in a CAT® assay to about 20% of normal. This is approaching the highest concentration of dabigatran that we tested in the thrombin generation assays (2 μM = 944 ng/ml). As in our study, a four-factor PCC could restore the peak thrombin level to normal or even supernormal levels, even in the presence of this extremely high dabigatran level. Other parameters of thrombin generation were not reported in the study by Pragst *et al.*[9] However, 20 U/kg of PCC restored the peak thrombin level to normal, but a much higher dose, 50 U/kg, was required to reduce bleeding to normal. Thus, while supporting the efficacy of PCC for reversal of dabigatran effects, normalization of the peak level of thrombin attained in the conventional CAT® assay was not tightly correlated with normalization of hemostasis in the rabbit kidney bleeding model.

Zhou *et al.*[10] also showed a beneficial effect of PCC on dabigatran-associated bleeding in a mouse intracerebral hemorrhage model. However, they administered up to 4 mg/kg dabigatran, resulting in peak plasma levels of around 1,200 ng/ml. In this model, with such high dabigatran levels, 100 U/kg PCC reduced hemorrhage, but an extremely high dose of FVIIa (8 mg/kg) had no beneficial effect. This result is consistent with our prediction that FVIIa would be ineffective in restoring hemostasis at such a high concentration of dabigatran.

We believe that our results can explain some of the inconsistencies in the results of previously published *in vitro* and *in vivo* studies. The ability of either PCC or FVIIa to restore hemostasis in the presence of dabigatran depends on the dose of reversal agent used and the level of dabigatran present. The effects of FVIIa are much more dependent on dabigatran level than are the effects of PCCs. This may account for the reason that the majority of animal studies (though not all) conclude that PCCs can at least partially correct the hemostatic abnormality due to dabigatran, whereas the majority of studies on FVIIa (though not all) conclude that FVIIa is only marginally effective or not effective in reversing the effects of dabigatran. We think the bulk of data is most consistent with the hypothesis that PCC at a dose of 50 U/kg can exert a hemostatic effect over a wide range of dabigatran levels, whereas a dose of FVIIa similar to that used in hemophilia (90–270) can exert a hemostatic effect at therapeutic dabigatran levels, but not if the dabigatran level is extremely increased.

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Competing Interests

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