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Laboratory Investigation

LABORATORY INVESTIGATION

Factor IX from prothrombin complex concentrate augments low dose tissue factor-triggered thrombin generation: an *in vitro* study

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Abstract

Background: Prothrombin complex concentrate (PCC) is increasingly used to correct acquired coagulopathy in trauma and surgery. Dosing of PCC is guided by the prothrombin time, which only reflects the onset of thrombin generation, but does not account for variations in intrinsic pathway coagulation factors, including factor IX (FIX). We hypothesised that FIX contained in PCC could strongly influence thrombin generation patterns.

Methods: Pooled normal, FIX-deficient, and warfarinised plasma were used to analyse the effects of FIX contained in PCC. PCC was evaluated at final concentrations of 0.2 and 0.4 IU ml^{-1} in FIX-deficient and normal plasma, and at 0.6 IU ml^{-1} in warfarinised plasma with elevated FVIII (1.5 IU ml^{-1}), 40% dilution with saline, or both. The effects on thrombin generation were assessed by measuring both procoagulant and inhibitory segments.

Results: FIX-deficient plasma had lower peak thrombin generation [30.6 (20.5–35.8) nM *vs* 130.2 (107–168) nM] and endogenous thrombin potential [472 (391–532) nM *vs* 1096 (958–1190) nM] than normal plasma. PCC addition resulted in significant increases of peak thrombin generation [81.8 (37.3–98.3) nM] and endogenous thrombin potential [808 (472–842) nM] in FIX-deficient plasma. The combination of FVIII and PCC resulted in greater increases relative to each agent alone, restoring normal thrombin generation. After 40% dilution, adding PCC, FVIII, or both, to FIX-deficient plasma increased peak thrombin generation, and prolonged the inhibitory phase of the endogenous thrombin potential.

Conclusions: FIX derived from PCC strongly enhances tissue factor-triggered thrombin generation in the presence of elevated FVIII activity. Haemodilution further enhances procoagulant effects of FIX and FVIII by slowing down inhibition of procoagulant enzymes. Dosing of PCC per prothrombin time may underestimate PCC's procoagulant potential because it does not account for intrinsic tenase or antithrombin activity.

Keywords: blood coagulation factors; coagulants; haemostasis; thrombin

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Editor's key points

- Prothrombin complex concentrate (PCC) dosing is usually guided by assessment of prothrombin time results.
- The contribution of individual coagulation factors, such as factor IX (FIX) and FVIII is, however, masked by a high tissue factor concentration in this test.
- Under low tissue factor concentrations, this study shows that FIX, together with FVIII, influences PCC-induced thrombin generation, which could have clinical relevance in warfarinised patients.
- These findings support development of a tissue factor-activated coagulation test that reflects overall thrombin generation.

Management of coagulopathy can be a major challenge during complex cardiovascular surgery and after severe trauma.[1,2] Plasma-derived and recombinant factor concentrates are increasingly important therapeutic options in these settings.[2–5] Prothrombin complex concentrate (PCC) is a lyophilised coagulation factor mixture containing prothrombin [factor II (FII)], FVII, FIX, and FX, which has been recently approved for acute warfarin reversal in the USA.[3,5] The dosing of PCC is based on the amount of FIX per body weight (IU kg^{-1}), and therapeutic responses are determined by correction of prothrombin time (PT) or international normalised ratio (INR).[3,5] However, tissue factor-triggered PT/INR are unaffected by FIX, and it is possible that procoagulant activity of PCC is not fully appreciated in these results. While PT and activated partial thromboplastin time (aPTT) reflect the onset of fibrin gel formation, the propagation phase of thrombin generation, where thrombin mediates the feedback-activation of multiple factors, cannot be detected by either test. The thrombin generation assay is more sensitive than aPTT to the changes in thrombin generation because of antithrombin (AT)-mediated inhibitions of activated FX (FXa) and thrombin.[6,7] FVIII is a crucial cofactor of FIX in the formation of tenase (FIXa–FVIIIa), which propagates FX activation and subsequent thrombin generation through the intrinsic pathway. It is important to consider the contribution of intrinsic tenase to thrombin generation because elevated FVIII or FIX concentrations have been associated with prothrombotic tendency under certain conditions including advanced age[8] and presence of a ventricular assist device.[9] It was hypothesised that the interaction between FVIII and PCC-derived FIX plays a pivotal role in augmenting thrombin generation in dilutional coagulopathy. The aim of the present study is to quantify the PCC-mediated augmentation of intrinsic tenase and thrombin generation in normal and FIX-deficient plasma samples with and without haemodilution.

Methods

All parts of the study were conducted under institutional review board approval at the University of Maryland, Baltimore, MD, USA. Normal pooled plasma, FIX deficient (haemophilia B) plasma, and warfarin-treated plasma derived from healthy volunteers or de-identified clinical patients were obtained from George King Biomedical, Overland Park, KS, USA. The range of the INR in warfarin treated plasma was 2.3–2.9 (median, 2.6). All plasma samples were stored at −80°C, and then warmed to 37°C in a water bath immediately before analysis. Plasma samples underwent no more than one freeze-thaw cycle.

Thrombin generation assays were performed using a calibrated automated thrombography technique as originally described by Hemker and colleagues.[10] Briefly, 20 μl of tissue factor activator (final concentration, 2 pM) was added, followed by 80 μl of plasma to the wells of a microtitre plate (Thermo Labsystems, Franklin, MA, USA). Tissue factor concentrations of 1–2 pM are suitable for the evaluation of FIX activity, FVIII activity, or both.[11–13] The plate was incubated for 10 min at 37°C. A mixture of 20 μl (in total) of fluorogenic substrate/$CaCl_2$ (Diagnostica Stago, Parsippany, NJ, USA) and HEPTEM reagent (TEM Innovations, Munich, Germany) was then automatically dispensed into each well to start the reaction. The latter was added to neutralise the *in vitro* effects of heparin contained in commercial PCCs.[14] A thrombin calibrator with a known, constant thrombin-like activity was used in parallel to eliminate the signal differences due the light absorption characteristics of different plasmas, inner filter effects, and non-linearity of the emission signal. Calibrator wells were plated in an identical manner to other wells, with 20 μl of thrombin calibrator in place of the tissue factor activator. The thrombin generation reaction was monitored using a microplate fluorometer (Fluoroskan Ascent, Labsystems, Vantaa, Finland) set at 390 nm excitation and 460 nm emission wavelengths. Fluorescence was recorded every 20 s for 90 min. The acquired data were automatically processed by the Thrombinoscope software (Thrombinoscope, Stago, Maastricht, The Netherlands) to calculate thrombin generation parameters. The following parameters were evaluated: time to initiate thrombin generation (lag time, min), peak thrombin (nM), and the area under the curve [endogenous thrombin potential (ETP); nM×min]. ETP was subsequently separated into two segments (Excel® 2011, Microsoft, Seattle, WA, USA) as described previously,[15] representing the procoagulant phase (ETP*p*) before the peak of thrombin generation, and inhibitory phase (ETP*i*) after the peak when AT-mediated inhibitions of FXa and thrombin take place. Briefly, this was calculated by integrating the area under the curve up to the peak to give ETP*p*, and then subtracting that from the total to give ETP*i*.

Contributions of intrinsic tenase to thrombin generation

To investigate the contributions of intrinsic tenase to thrombin generation, FIX-deficient and warfarin-treated plasma samples were evaluated with exogenous FIX derived from PCC (Octaplex®; Octapharma AG, Lachen, Switzerland). Final concentrations of FIX were 0.2 and 0.4 IU ml^{-1} in FIX-deficient plasma, and 0.6 IU ml^{-1} in warfarin plasma samples, which is the therapeutic concentration for warfarin reversal.[15,16] The respective concentration represents a 20, 40, and 60% increase in FIX concentration. The interactions of FVIII and PCC-derived FIX were assessed by adding FVIII concentrate (Wilate®; Octapharma AG) at a final concentration of 1.5 IU ml^{-1}, corresponding to a 150% increase in FVIII concentration. This FVIII concentrate contains both FVIII and von Willebrand factor at a ratio of approximately 1:1.

Thrombin generation patterns under moderate (40%) haemodilution were modelled by mixing each plasma sample with saline (6:4 vol/vol). These conditions were selected to match typical patterns of haemodilution observed after moderately complex non-cardiac and cardiac operations.[17,18] The 40% dilution lowers procoagulant factor concentrations, but it also affects AT activity, modulating therapeutic responses to PCC.[15,19] For all experiments, phosphate buffered saline was added to control samples to account for the added volume of factor