Effects of recombinant factor VIIa on platelet function and clot structure in blood with deficient prothrombin conversion

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Summary
While recombinant factor VIIa (rFVIIa) shows promise as a broad-spectrum hemostatic agent, questions remain regarding the most appropriate dose and the best way to monitor its effects. In this study we tested the sensitivity of a thrombin dependent platelet assay, platelet contractile force, to the effects of rFVIIa in normal, factor-deficient, and inhibitor-containing blood samples. Dose dependent effects of rFVIIa on platelet contractile force (PCF) and clot elastic modulus (CEM) were measured in all blood samples.

rFVIIa minimally affected PCF and CEM in normal blood clotted with thrombin or batroxobin. While rFVIIa minimally altered PCF and CEM in factor VIII (FVIII) deficient blood clotted with thrombin, rFVIIa increased PCF and CEM and shortened the lag phase in a dose dependent manner in batroxobin-induced clots.

Key words
Recombinant FVIIa, hemophilia, platelet contractile force, clot elastic modulus, thrombin generation

Introduction
Recombinant factor VIIa (rFVIIa) holds promise as a broad-spectrum hemostatic agent (1-3). Currently approved in the United States for treatment of patients with high level factor VIII inhibitors, rFVIIa’s potential utilization in clinical settings such as trauma and surgery has raised questions regarding the most appropriate dosing regimen and the best marker or method for documenting its clinical response.

Some authors have recommended higher doses of rFVIIa to generate a thrombin “burst” that will lead to rapid formation of a more robust clot that is more resistant to subsequent fibrinolysis. The effects of rFVIIa in factor IX (FIX) deficient blood mirrored the effects seen in FVIII deficient samples. Whether clotted with thrombin or batroxobin, baseline PCF and CEM were abnormally low in FVIII deficient samples containing FVIII inhibitors. In such samples, rFVIIa caused dose dependent improvement of PCF, CEM, and lag phases. In one patient with a spontaneous inhibitor, rFVIIa caused dose dependent increases in PCF and CEM in blood clotted with either enzyme. rFVIIa corrects the deficient thrombin generation seen in FVIII and FIX deficiency, and in blood containing FVIII inhibitors. As a consequence, platelet function is improved and clot structure is enhanced. Platelet contractile force and clot elastic modulus measurements are sensitive to the dose dependent effects of rFVIIa.
lysis (4, 5). Their arguments are bolstered by in vitro evidence that higher thrombin concentrations alter clot structure with resultant increased clot rigidity (6-14). Recommendations for laboratory monitoring have ranged from assays such as the prothrombin consumption time to measurement of clot structure via the thrombogram (15).

In this study, we measured the dose dependent effects of rFVIIa on the generation of platelet contractile force (PCF) and clot elastic modulus (CEM) in blood from patients with a variety of conditions known to delay prothrombin conversion. Since development of PCF is a thrombin dependent process, PCF production is delayed or reduced if thrombin is inhibited or its generation is delayed (16, 17). We hypothesized that PCF and CEM would be abnormal in blood obtained from patients with factor VIII deficiency and factor IX deficiency and from patients with inhibitors to factor VIII. We further hypothesized that rFVIIa would correct these abnormalities in a dose dependent fashion. The effects of rFVIIa in these abnormal samples were compared to the effects seen in blood from normal controls. The ability of rFVIIa to correct abnormal PCF and CEM was assessed.

### Materials and methods

#### Human subjects

This study was approved by the Institutional Review Board for Human Studies of Virginia Commonwealth University. Written, informed consent was obtained from all patient and control subjects. Ten adult males and two females were enrolled in this study. Of the twelve subjects enrolled, three were healthy volunteers with no known coagulation deficiencies, three patients had factor VIII deficiency, three patients had factor IX deficiency, two patients had factor VIII deficiency and factor VIII inhibitor, and one patient had a spontaneous factor VIII inhibitor (Table 1).

#### Sample preparation

Blood was collected via aseptic venipuncture into evacuated tubes containing 3.2% sodium citrate.

#### Materials

Batroxobin was purchased as Atroxin®, *Bothrops atrox* venom protein (Sigma Diagnostics, St. Louis, MO), dissolved in deionized water, and used without further modification. It was maintained on ice and used within 3 h of being reconstituted.

#### Table 1: Patient baseline laboratory characteristics.

<table>
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<th>Level (%)</th>
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<th>Platelet Count (platelets/μL)</th>
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* N/A= non-applicable
thrombin, greater than 90% alpha, was purchased as a lyophilized powder (Sigma Chemical Co., St. Louis, MO). The material, with a specific activity of 4300 NIH U/mL, was dissolved in water, diluted with 0.10 M NaCl to a final concentration of 20 U/mL, divided into 1 mL lots, and frozen at −90 °C. Thrombin was free of plasmin and plasminogen. Recombinant Factor VIIa (NovoSeven®) was generously provided by Novo Nordisk (Copenhagen, Denmark). Nanopure water was used in the preparation of all solutions.

All clots were formed using 700 µL of citrated whole blood. Increasing concentrations of rFVIIa were added to the samples prior to initiating clotting. The final concentrations of rFVIIa were determined based on the recommended dose of 90 µg/Kg. Assuming a weight of 70 Kg and blood volume of approximately 5,000 mL, this dose should yield a peak concentration approaching 1.25 µg rFVIIa/mL of blood. The final concentrations of rFVIIa in this study were 1.024 µg/mL (≈ 80% recommended dose), 2.048 µg/mL (≈ 160%), 4.096 µg/mL (≈ 320%), 8.00 µg/mL (≈ 380%). Clotting was initiated at time zero by adding CaCl₂ and either thrombin or batroxobin. Unless otherwise stated, final clotting conditions included: CaCl₂ 10 mM, batroxobin 0.21 µg/mL or thrombin 1 NIH Unit/mL, pH 7.4, ionic strength 0.15M and a final volume of 0.750 mL. Force development was measured for 1200 sec and recorded in kilo dynes. Clot elastic modulus was measured concurrently and reported in kilo dynes per cm².

**Platelet contractile force development during clot retraction**

The Hemodyne® Hemostasis Analyzer (Hemodyne, Inc., Richmond, VA) (Fig. 1) measures forces generated by platelets within a clot formed between a temperature-controlled cup and parallel upper surface (10, 18). Before clotting, the upper surface is centered above the cup and lowered into the blood sample. As the clot forms, it attaches to the upper and lower surfaces. As soon as a fibrin network is formed, platelets within the network pull fibrin strands inward transmitting force through the network to the surfaces to which the clot is adherent. PCF measurement is accomplished utilizing a displacement transducer coupled to the upper surface. As platelets contract, the transducer produces an electrical output proportional to the amount of force generated. Normal values for PCF at 1200 sec after batroxobin addition have been established (10, 19). For healthy volunteers (n = 25), force development is 7.82 ± 1.34 Kdynes (mean ± Standard Deviation).

**Measurement of clot elastic modulus**

CEM can be obtained simultaneously with PCF. The ratio of applied force (stress) to measured displacement (strain) is used to calculate the elastic modulus:

$$\text{CEM} = \frac{\text{stress}}{\text{strain}}$$

Where stress equals the applied force divided by the area of application, and strain is the degree of shape change induced by the applied force. In the present case, the strain induced by the applied force is measured as the change in clot thickness, which is the same as the change in the gap between the two surfaces. Strain is recorded as the ratio of the change in gap distance (d₁) to the original gap distance (d₀). Because the clot is a cylinder of radius (r) and length d₀:

$$\text{CEM} = \frac{(F_{\text{applied}}/r^2)}{(d_1/d_0)}$$

The distance moved (d₁) is measured directly by the displacement transducer.

For healthy volunteers (n = 25), clot elastic modulus is 18.84 ± 5.65 Kdynes/cm² (mean ± SD).

Lag phases for PCF and CEM development were measured from the kinetic curves as the point at which a line drawn tan-

![Figure 1: Schematic of device used to measure platelet contractile force. Clots are formed between two parallel surfaces. A transducer connected to the upper surface detects retraction forces. Elastic modulus is measured by intermittently stressing the clot by pressing on the upper surface with a known force.](image-url)
entially from the flat early portion of the curve intersected a line drawn tangentially from the initial up swing in the measured parameter. The lag phase range for healthy volunteers (n = 25) is 227 to 557 sec.

All measurements were done in duplicate and are presented as the mean ± standard error of the mean (which is the same as the sample range). The upper and lower extent of the sample bar depicts the actual data points.

Results

Effect of rFVIIa on PCF and CEM in normal whole blood

Just prior to the addition of calcium (10mM final) and a clotting agent (thrombin 1 NIH U/ml or batroxobin 0.21 µg/ml), increasing concentrations of rFVIIa were added to 700 µl of citrated (3.2%), normal whole blood. The kinetics of PCF and CEM development were followed for 1200 sec. Final rFVIIa concentrations were 0, 1.024, 2.048, 4.096, and 8.000 µg/mL of plasma. Blood from three normal controls were studied. The results are recorded in table 2 and illustrated in figure 2. As can be seen in table 2, rFVIIa minimally affected PCF in blood clotted with thrombin. CEM in those same clots increased by less than 20% as rFVIIa was increased from 1.024 to 8.000 µg/mL. Results in clots formed with batroxobin were similar with minimal effects of rFVIIa on PCF and minimal increase in CEM (<20%). The values for CEM and PCF at 800 seconds were the same for clots formed with batroxobin and with thrombin. Figure 2A demonstrates the kinetic results of blood from one normal control clotted with batroxobin in the presence of increasing concentrations of rFVIIa. There was an initial lag phase when the samples were clotted with batroxobin; however, final PCF values were unchanged. In figure 2b the effect of rFVIIa on PCF, CEM, and lag phase for clots formed by addition of batroxobin to normal blood are summarized. RFVIIa did not affect lag phase or PCF and only affected CEM at the highest dose tested (8.00 µg/ml).

Effect of rFVIIa on PCF and CEM in whole blood from patients with factor VIII deficiency

In measurements identical to those performed for normal volunteers, increasing concentrations of rFVIIa (0, 1.024, 2.048, 4.096 and 8.000 µg/mL final) were added to 700 µl of citrated (3.2%) whole blood from three patients with documented factor VIII deficiency. These results are recorded in table 2 and illustrated in figure 4. The effects of rFVIIa in whole blood clots formed from factor VIII deficient blood were strikingly different from those seen in normals. While the effects on PCF and CEM were minimal in thrombin-induced clots, the effects in batroxobin-induced clots were marked. PCF increased by 340%,

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<th>Thrombin CEM</th>
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<td>Baseline + 8.0 µg/mL rFVIIa</td>
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Table 2: Effect of rVIIa on platelet contractile force and clot elastic modulus in whole blood clots formed by the addition of exogenous thrombin.
Recombinant factor VIIa and thrombin generation

**Figure 2:** (A) Effect of increasing concentrations of rFVIIa on the kinetics of force development in normal whole blood clotted with batroxobin. Clotting enzyme and 10mM calcium chloride were added at time zero. rFVIIa concentrations are as indicated in the legend. (B) Effects of increasing concentrations of rFVIIa on CEM and PCF values 1200 seconds after the addition of batroxobin and 10 mM calcium to normal citrated blood. Bottom panel shows the effect of rFVIIa on the lag phase prior to the first development of force. All data were run in duplicate and the error bars represent the standard error of the mean. Data are for three normal controls as indicated in the legend of the top panel.

**Figure 3:** (A) Effect of increasing concentrations of rFVIIa on the kinetics of force development in whole blood from a patient with factor VIII deficiency. Samples were clotted with batroxobin. Clotting enzyme and 10mM calcium chloride were added at time zero. rFVIIa concentrations are as indicated in the legend. (B) Effects of increasing concentrations of rFVIIa on CEM and PCF values 1200 sec after the addition of batroxobin and 10 mM calcium to citrated blood obtained from patients with known factor VIII deficiency. Bottom panel shows the effect of rFVIIa on the lag phase prior to the first development of force. All data were run in duplicate and the error bars represent the standard error of the mean. Data are for three factor VIII deficient patients as indicated in the legend of the top panel.
Figure 4: (A) Effect of increasing concentrations of rFVIIa on the kinetics of force development in whole blood from a patient with factor IX deficiency. Samples were clotted with batroxobin. Clotting enzyme and 10mM calcium chloride were added at time zero. RFVIIa concentrations are as indicated in the legend. (B) Effects of increasing concentrations of rFVIIa on CEM and PCF values 1200 seconds after the addition of batroxobin and 10 mM calcium to citrated blood obtained from patients with known factor IX deficiency. Bottom panel shows the effect of rFVIIa on the lag phase prior to the first development of force. All data were run in duplicate and the error bars represent the standard error of the mean. Data are for three factor IX deficient patients as indicated in the legend of the top panel.

Effect of rFVIIa on PCF and CEM in whole blood from patients with factor IX deficiency

In measurements identical to those performed for normal volunteers, increasing concentrations of rFVIIa (0, 1.024, 2.048, 4.096, and 8.000 µg/mL final) were added to 700 µl of citrated (3.2%) whole blood from three patients with documented factor IX deficiency. The results are recorded in table 2 and illustrated in figure 4. The effects of rFVIIa in factor IX deficient blood were remarkably similar to the results seen in factor VIII deficient blood. PCF increased by 700%, 750%, and 1,080% in the three patients. These increases were accompanied by a remarkable shortening of the lag phase for PCF in batroxobin-induced clots. The patient with mild factor IX deficiency had a greater PCF at baseline. Figure 4B summarizes the effects of rFVIIa on PCF, CEM, and lag phase in these patients. As opposed to normal controls, patients with factor IX deficiency show dose dependent effects of rFVIIa on all of these parameters.

Effect of rFVIIa on PCF and CEM in whole blood from patients with factor VIII deficiency and documented factor VIII inhibitor and in one patient with a spontaneous factor VIII inhibitor

In measurements identical to those performed for normal volunteers, increasing concentrations of rFVIIa (0, 1.024, 2.048, 4.096, and 8.000 µg/mL final) were added to 700 µl of citrated whole blood from two factor VIII deficient patients with documented factor VIII inhibitors. One patient who developed a spontaneous factor VIII inhibitor was studied in an identical manner. The results are recorded in table 2 and illustrated in figures 5 and 6. Patients with factor VIII inhibitors, whether spontaneous or acquired in hemophilia, demonstrated altered responses to rFVIIa. In these patients, even when the blood was clotted with thrombin, PCF and CEM increased with rFVIIa. The degree of increase varied from patient to patient. PCF in the absence of rFVIIa in these thrombin-induced clots did not correlate with factor VIII inhibitor titer. Responses to rFVIIa in batroxobin-induced clots more closely mirrored those seen in factor VIII and factor
Figure 5: (A) Effect of increasing concentrations of rFVIIa on the kinetics of force development in whole blood from a patient with factor VIII deficiency and a factor VIII inhibitor. Samples were clotted with batroxobin. Clotting enzyme and 10mM calcium chloride were added at time zero. RFVIIa concentrations are as indicated in the legend. (B) Effects of increasing concentrations of rFVIIa on CEM and PCF values 1200 seconds after the addition of batroxobin and 10 mM calcium to citrated blood obtained from patients with known factor VIII deficiency and a factor VIII inhibitor. Bottom panel shows the effect of rFVIIa on the lag phase prior to the first development of force. All data were run in duplicate and the error bars represent the standard error of the mean. Data are for two factor VIII deficient patients with factor VIII inhibitors as indicated in the legend of the top panel.

Figure 6: (A) Effect of increasing concentrations of rFVIIa on the kinetics of force development in whole blood from a patient with a spontaneous factor VIII inhibitor. Samples were clotted with batroxobin. Clotting enzyme and 10mM calcium chloride were added at time zero. RFVIIa concentrations are as indicated in the legend. (B) Effects of increasing concentrations of rFVIIa on CEM and PCF values 1200 seconds after the addition of batroxobin and 10 mM calcium to citrated blood obtained from a patient with a high titer factor VIII inhibitor. Bottom panel shows the effect of rFVIIa on the lag phase prior to the first development of force. All data were run in duplicate and the error bars represent the standard error of the mean.
IX deficient blood. As can be appreciated in figure 5A, the presence of an inhibitor virtually eliminated PCF development induced by batroxobin in a hemophilia. The dose dependent effects of rFVIIa on PCF, CEM, and lag phase in hemophilia with an inhibitor can be appreciated in figure 5B. One of the patients showed no response to the initial dose of NovoSeven® (1.024 µg/mL). In a patient with a spontaneous factor VIII inhibitor, the effects of rFVIIa are dramatic in both batroxobin and thrombin induced clots. PCF increased by 85% in batroxobin-induced clots and by 200% in thrombin-induced clots (Fig. 6A and Table 2). The increase in PCF in the batroxobin-induced clots was accompanied by a remarkable shortening of the lag phase. Figure 6B summarizes the effects of rFVIIa on PCF, CEM, and lag phase in this patient. As opposed to normal controls, the patient with a spontaneous factor VIII inhibitor showed dose dependent effects of rFVIIa on all of these parameters.

**Discussion**

**Utility of the batroxobin based PCF assay as a measure of thrombin generation in whole blood**

Most clinical assays measure clotting times (PT, PTT, TCT, etc.) in the absence of cells or platelet function (platelet aggregation, platelet adhesion, aperture closure, etc.) in the absence of clotting. The PCF assay utilised in this investigation allows the measurement of platelet function during clot formation. Since PCF is thrombin dependent, clotting with a combination of batroxobin and calcium allows assessment of thrombin generation. Batroxobin causes rapid clot formation. Addition of calcium allows reversal of citrate induced anticoagulation and subsequent generation of thrombin on cellular surfaces. Once a small amount of thrombin forms, multiple positive feedback loops cause an explosion of thrombin activity. Since batroxobin has already produced a fibrin network, PCF can be measured from its onset and serves as a marker of thrombin generation. Similar reptilase clot retraction assays have proven useful in evaluating effects of agents on platelet function (20, 21).

**Consequences of deficient thrombin generation**

*In vitro* studies indicate the speed of clot formation effects how long the patient bleeds and the quality of the clot. Specifically, the type and quality of fibrin network are influenced by thrombin concentration (6). High thrombin concentrations are associated with rapid more structurally-robust clot formation. Such clots are composed of smaller diameter fibers (6, 7), have smaller pores (6, 8), are more rigid (9, 10), and are more resistant to degradation (11-14). Increased thrombin-activatable fibrinolysis inhibitor (TAFI) activity could also enhance clot stability.

A significant consequence of decreased PCF is reduced clot elastic modulus (rigidity). The forces generated by platelets during clot retraction put the fibrin network under stress and lead to aligned fibrin fibers. These changes increase clot rigidity and may play a significant role in increasing clot resistance to fibrinolysis (22).

**Effects of rVIIa in normal and thrombin deficient plasma**

rVIIa had minimal effects on platelet function and clot structure in normal blood clotted with either batroxobin or thrombin. The number of normal subjects tested in this study was limited, and therefore significant variability in the generation of thrombin may be noted in a larger population of normals. The lag phase (approx. 5 min.) prior to the onset of PCF development in this study agrees with the time to thrombin burst noted in model systems (23-26).

Blood from factor VIII and factor IX deficient patients demonstrated abnormally low PCF and CEM when clotted with batroxobin, but appeared to be normal when clotted with thrombin. Deficient thrombin generation in factor VIII or factor IX deficient blood results in delayed clot formation and inadequate platelet function. When exogenous thrombin is used to clot the sample, platelet function and clot structures are normal. Thus, the abnormalities in platelet function and structure noted when clots are formed with batroxobin, are not due to inherent platelet abnormalities, but rather are a direct reflection of deficient thrombin generation. rVIIa corrects deficient thrombin generation in factor VIII and IX deficient blood. The dose dependence of rVIIa effects varied somewhat between patients. The majority of patients achieved most of their benefit at a final concentration of 5.12 µg/ml.

PCF and CEM in factor VIII deficient patients with inhibitors was more abnormal than in factor VIII deficient blood. Blood from the two inhibitor patients displayed abnormal PCF and CEM when clotted with batroxobin and thrombin. This unexpected result remains unexplained. Addition of thrombin to the blood should obviate the need for plasma factors. This appears to be the case in normals and factor deficient patients. How the presence of an inhibitor alters this response is not clear, but this finding may imply direct anti-platelet effects of some of these antibodies that are independent of their effects on thrombin generation.

The response to rVIIa in the two acquired inhibitor patients is similar to that seen with factor deficient patients. However, the recommended dose of rVIIa was insufficient to correct PCF in one of the patients. In addition, one of these was the only patient where high concentrations of rVIIa resulted in “supranormal” PCF and CEM values. This patient had high fibrinogen levels, advanced arteriosclerosis, and underlying malignancy. High fibrinogen (>400 mg/dl) is known to increase PCF and CEM (27), and patients with atherosclerosis have been shown to have elevated baseline PCF values (28, 29). Whether these high PCF and CEM values represent increased thrombotic risk in this patient is currently unknown. In the absence of his inhibitor, he would clearly be considered to be at thrombotic risk.
Conclusion

In summary, PCF and CEM measurements are sensitive to the dose dependent effects of rVIIa. RVIIa has minimal effects on platelet function and clot structure in normal blood. RVIIa corrects deficient thrombin generation, improves platelet function and enhances clot structure in patients with factor VIII deficiency, factor IX deficiency and factor VIII inhibitors. Response to rVIIa varies between individuals.

Limitations of the study: The current investigation was completely in-vitro in nature and does not assure that similar changes will be seen in patients undergoing treatment. Recently, samples were obtained from one De Novo spontaneous factor VIII inhibitor patient presenting with acute soft tissue bleeding and a factor VII deficient patient being treated for an elbow hemorrhosis. Analysis revealed shortening of the lag phase and enhanced PCF and CEM after rFVIIa infusions (abstracts submitted for presentation at NHF and ASH). Ex-vivo patient monitoring studies are in preparation and an additional in-vitro study of the effects of other clotting concentrations on lag phase, PCF and CEM are under way.

Acknowledgements

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References