Monitoring recombinant factor VIIa treatment: efficacy depends on high levels of fibrinogen in a model of severe dilutional coagulopathy


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Abstract

OBJECTIVES: Recombinant activated factor VII (rFVIIa) is increasingly being given to treat massive bleeding. However, there is no clear guidance on which patients are suitable for treatment and how the effects of treatment should be monitored. The aim of this in vitro study was to assess the coagulation status of severely hemodiluted blood samples before and after treatment with therapeutic doses of rFVIIa and/or fibrinogen with 2 viscoelastic point-of-care coagulation analyzers: ROTEM (Pentapharm GmbH, Munich, Germany) and Sonoclot (Sienco Inc, Arvada, CO). DESIGN: Laboratory study.

SETTING: Research coagulation laboratory. PARTICIPANTS: Ten healthy male volunteers without hereditary or acquired coagulation disorders. INTERVENTIONS: Blood samples were obtained. After severe hemodilution with albumin 5%, therapeutic doses of rFVIIa and/or fibrinogen were added, and the coagulation status was assessed with new 1:1,000 diluted tissue factor-activated tests from ROTEM and Sonoclot. MEASUREMENTS AND MAIN RESULTS: The administration of therapeutic doses of rFVIIa to hemodiluted samples shortened the initiation phase of coagulation only. Isolated fibrinogen administration to physiologic levels improved both the initiation of coagulation as well as clot formation and strength. Combined fibrinogen and rFVIIa administration further improved both effects.

CONCLUSIONS: ROTEM and Sonoclot were able to monitor the effects of rFVIIa and fibrinogen administration with 1:1,000 diluted tissue factor-activated tests. The efficacy of rFVIIa was largely dependent on the presence of high levels of fibrinogen in reversing this severe dilutional coagulopathy.
Monitoring recombinant factor VIIa treatment: Efficacy depends on high levels of fibrinogen in a model of severe dilutional coagulopathy

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ABSTRACT

Objectives: Recombinant activated factor VII (rFVIIa) is increasingly being given to treat massive bleeding. However, there is no clear guidance on which patients are suitable for treatment and how the effects of treatment should be monitored. The aim of this in vitro study was to assess the coagulation status of severely hemodiluted blood samples before and after treatment with therapeutic doses of rFVIIa and or fibrinogen with two viscoelastic point of care coagulation analyzers, ROTEM and Sonoclot.

Design: Laboratory study.

Setting: Research coagulation laboratory.

Participants: 10 healthy male volunteers without hereditary or acquired coagulation disorders.

Interventions: Blood samples were obtained. After severe hemodilution with albumin 5% therapeutic doses of rFVIIa and / or fibrinogen were added and the coagulation status assessed with new 1:1,000 diluted tissue factor activated tests from ROTEM and Sonoclot.

Measurements and Main Results: Administration of therapeutic doses of rFVIIa to hemodiluted samples shortened the initiation phase of coagulation only. Isolated fibrinogen administration to physiological levels improved both the initiation of coagulation as well as clot formation and strength. Combined fibrinogen and rFVIIa administration further improved both effects.

Conclusions: ROTEM and Sonoclot were able to monitor the effects of rFVIIa and fibrinogen administration with the new 1:1,000 diluted tissue factor activated tests
Furthermore, efficacy of rFVIIa was largely dependent on the presence of high levels of fibrinogen in reversing this severe dilutional coagulopathy.

**Key words**

Recombinant activated factor VII, fibrinogen concentrate, hemodilution, dilutional coagulopathy, thrombelastography, thrombelastometry, ROTEM, Sonoclot Analyzer.
INTRODUCTION

Recombinant activated factor VII (rFVIIa) is increasingly being given on a compassionate basis to control severe bleeding\(^1\) and has been shown to result in a significant reduction in red blood cell transfusion in severe blunt trauma.\(^2\) Although consensus guidelines for use of rFVIIa have been published recently,\(^3,4\) a paucity of data from randomized controlled trials limit the strength and scope of these recommendations. No specific method is currently available to indicate the need for rFVIIa or to monitor its efficacy. Therefore, the administration of rFVIIa in patient with massive hemorrhage still depends largely on clinical judgment.\(^5\) Viscoelastic techniques like thrombelastography/thrombelastometry or Sonoclot analysis appear to be highly desirable and have shown some promising results.\(^6-8\) Coagulation is assessed in whole blood allowing coagulation factors to interact with red cells and platelets. rFVIIa increases thrombin formation by tissue factor dependent and – independent activation of factor X on the surface of platelets.\(^9\) To study effects of rFVIIa, blood samples need to be activated with minute amounts of tissue factor for best sensitivity.\(^10,11\) Presence of high amounts of tissue factor may directly activate the coagulation cascade (bypassing agent) and effects of rFVIIa may no longer be monitored.\(^11\)

Modern practice of coagulation management is based on rapid diagnosis of the patient’s coagulation status, specific component therapy, and monitoring of the pro-coagulant treatment.\(^12\) Measuring clotting factors during progressive hemodilution showed that fibrinogen is the first factor to become critically low.\(^13,14\) Fibrinogen is a key coagulation factor (substrate to form a clot) and isolated fibrinogen substitution in severe
models of dilutional coagulopathy resulted in a significantly improved clot strength and reduced blood loss.\textsuperscript{15-18}

The aim of this \textit{in vitro} study was to determine the efficacy of rFVIIa in a model of severe dilutional coagulopathy by two different viscoelastic point of care (POC) coagulation analyzers (ROTEM system, Pentapharm GmbH, Munich, Germany and the Sonoclot Analyzer, Sienco Inc. Arvada, CO, USA). Our hypothesis was that rFVIIa may only reverse severe dilutional coagulopathy in presence of sufficient fibrinogen levels.

**METHODS**

With institutional approval blood was withdrawn from 10 healthy volunteers. Exclusion criteria were hereditary or acquired coagulation disorders including pharmacologically induced coagulopathies i.e., treatment with any anticoagulants or antiplatelet drugs 14 days before blood withdrawal.

\textit{Blood Sampling}

Fifty millilitres of venous blood were sampled after application of minimum stasis from the antecubital fossa \textit{via} a 21-gauge needle. The blood was filled into citrated tubes (sodium citrate, 0.109 mol/l; BD Vacutainer, Becton Dickinson) and the initial tube was discarded before collection of the test tubes to avoid tissue factor contamination. An extra tube (EDTA; BD Vacutainer, Becton Dickinson) was obtained for whole blood count and platelet measurement.

\textit{Hemodilution}

In order to mimic a scenario of maximum dilution of clotting factors but maintaining a minimally required hemoglobin concentration and platelet count, we processed our
samples to obtain platelet-rich plasma (PRP; centrifugation at 80 g for 8 min). PRP was carefully collected and the remaining sample re-centrifuged (400 g for 3 min) to further concentrate the red blood cells (RBC). The supernatant (remaining plasma and buffy coat) was discarded and the RBC washed twice with 0.9% normal saline. Then, PRP was diluted 1:20 with albumin 5% (ZLB Behring, Berne, Switzerland; sodium citrate, 0.109 mol/l was added to the albumin solution prior to the dilution of PRP) and washed RBC were added to obtain a final targeted hematocrit of 20%. Albumin 5% was chosen in this hemodilution model because it has been shown previously to have the least adverse effect on hemostasis compared to other colloids.19,20

Treatment of hemodiluted blood samples with rFVIIa and fibrinogen

The reconstituted blood samples were treated with a therapeutic dose of rFVIIa (Novo Nordisk, Copenhagen, Denmark; final concentration 1.4 μg/ml) corresponding to a clinical dose of 100 μg/kg bodyweight. Furthermore, diluted blood samples were treated with fibrinogen (Calbiochem, San Diego, CA; final targeted concentration 2 g/l) alone or combined with rFVIIa. Because the maximum effect of rFVIIa was seen in blood samples treated with fibrinogen, a dose response of rFVIIa was then performed in these blood samples.

Coagulation Analysis

Whole blood coagulation analyses were done with the rotation thrombelastometry ROTEM (Pentapharm GmbH, Munich, Germany) and the Sonoclot Coagulation and Platelet Function Analyzer (Sienco Inc. Arvada, CO, USA). Both technologies have been described in detail elsewhere.21,22 Briefly, the measurements are based on the detection
of viscoelastic changes of a blood sample after activation with different coagulation activators (1:1,000 diluted tissue factor was used for this study).

ROTEM measures and graphically displays the time until initial fibrin formation (clotting time, CT: denoted as clot initiation), the kinetics of fibrin formation and clot development (alpha angle, $\alpha$: denoted as clot propagation) and the ultimate clot strength and stability (maximum clot firmness, MCF).\textsuperscript{16,23-25} In order to study thrombus generation, modified ROTEM parameters (based on the original tracing) have been introduced recently: maximum velocity of clot formation (maximum rate of thrombus generation, MaxVel), time to reach MaxVel (time to maximum thrombus generation, $t$-MaxVel) and total thrombus generation (area under the curve, AUC).\textsuperscript{11,23} These parameters are supposed to be more sensitive to rFVIIa than standard ROTEM parameters.\textsuperscript{26} The Sonoclot Analyzer provides information on the entire hemostasis process both in a qualitative graph, known as Sonoclot Signature and as quantitative results: the activated clotting time (ACT), the clot rate (CR) and the platelet function. The ACT is the time from the activation of the sample until the beginning of a fibrin formation. Besides providing information on the initiation phase of coagulation, the Sonoclot Analyzer also measures the kinetics of fibrin formation and clot development, expressed as CR (i.e., the maximum slope of the Sonoclot Signature during initial fibrin polymerization and clot development). Furthermore, the Sonoclot Analyzer analyses the timing and quality of the clot retraction and calculates the function of the platelets accordingly.\textsuperscript{25}

All analyses were performed at 37°C in duplicate using 1:1,000 diluted tissue factor activated tests provided by the manufacturer (for ROTEM: tif-TEM, Pentapharm GmbH, Munich, Germany; for Sonoclot: microPT, Sienco Inc., Arvada, CO, USA). Re-
calcification of blood samples was done by adding 20 µl of 0.25 M CaCl₂ to the test cuvettes containing 20 µl of coagulation activator. Subsequently, 330 µl of blood specimen was dispensed with a pipette, mixed and analyzed.

Routine coagulation tests included platelet count, prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen concentration determined by Clauss method.

Data analysis, Statistics

StatView for Windows version 5.01 (SAS Institute Inc, Cary, NC) and SPSS for Windows Release 12.0.2 (SPSS Inc, Chicago, IL) were used to perform the statistical analyses. ANOVA with post-hoc Bonferroni-Dunn correction was done to compare the different conditions of the blood samples (p value adjusted to 5 [effect of rFVIIa and fibrinogen on hemodiluted blood samples] and 4 comparisons [dose response of rFVIIa], respectively). Test variability was calculated as the % difference between duplicate measurements in relation to the mean of duplicate measurements. Results are given as mean±SD, p<0.05 was considered significant.

RESULTS

Venous blood was obtained from 10 male donors (age = 35.5±7.5 years). They were non-smokers, had no history of abnormal bleeding and presented without a coagulation disorder or anemia (Table 1).

In-vitro hemodilution

Diluting blood samples according to our model resulted in a final hematocrit of 19% on average and significantly impaired standard coagulation values (Table 1). ROTEM
and Sonoclot variables reflected the severe hemodilution: ROTEM's CT and t-MaxVel significantly increased, whereas all other variables (angle $\alpha$, MCF, MaxVel and AUC) significantly decreased. The same effect was observed for the Sonoclot variables ACT and CR (Figure 1,2).

Effect of rFVIIa and fibrinogen on hemodiluted blood samples

Administration of therapeutic doses of rFVIIa to hemodiluted samples significantly shortened PT (-31±11 sec), ROTEM's CT (-180±90 sec) and Sonoclot's ACT (-88±180 sec). However, no effects on ROTEM's angle $\alpha$, MCF and Sonoclot's CR were observed (Figure 1,2).

Single administration of fibrinogen to diluted samples resulted in a final concentration of fibrinogen of 2.0±0.3 g/l as determined by the Clauss method. Compared to hemodiluted blood samples, fibrinogen substitution decreased PT significantly by -12±7 sec. Furthermore, all ROTEM and Sonoclot variables improved significantly (Figure 1,2): There was a reduction of CT, t-MaxVel and ACT values combined with an increase of angle $\alpha$, MCF, MaxVel, AUC and CR values.

Combined application of rFVIIa and fibrinogen further reduced PT significantly (-15±9 sec). ROTEM's CT and t-MaxVel significantly decreased. Angle $\alpha$, MCF, MaxVel and AUC significantly increased. A similar effect was observed for the Sonoclot variables ACT and CR (Figure 1,2).

Dose response of rFVIIa

Because administration of fibrinogen was necessary for rFVIIa to show an effect in our dilutional model, a dose response in samples containing fibrinogen was performed. ROTEM's CT and Sonoclot's ACT improved equally in all doses studied, even at the
lowest dose. ROTEM’s angle $\alpha$, MCF and Sonoclot’s CR showed comparable results at half of the recommended dose compared to the recommended dose, but the pro-coagulant effect was lost at a quarter of the recommended dose. Again, the new ROTEM variables did follow the trend of the CT, angle $\alpha$ and MA (Table 2).

**Test variability**

Overall ROTEM test variability was 14±14% for CT, 9±11% for angle $\alpha$, 10±12% for MCF, 13±14% for $t$-MaxVel, 9±13% for MaxVel and 10±16% for AUC.. For the Sonoclot Analyzer test variability was 9±18% for ACT and 10±18% for CR.

**DISCUSSION**

The present study investigated the effects of administration of rFVIIa and fibrinogen in an *in vitro* hemodilution model. The ROTEM and Sonoclot Analyzer were able to monitor the pro-coagulant effects of rFVIIa and fibrinogen administration with diluted tissue factor activated tests, tif-TEM and MicroPT. Interestingly, rFVIIa required high concentrations of fibrinogen in order to improve the severe coagulopathy.

In the initial period of fluid resuscitation of patients with massive hemorrhage, large amounts of crystalloids, colloids, and universal 0 type RBC are typically infused. Testing and preparing of blood coagulation products (e.g., fresh frozen plasma, cryoprecipitate, and platelets) is time consuming. Therefore, a severe dilutional coagulopathy may develop in the early period of massive transfusion because these coagulation products may not be readily available. Our dilutional model aimed at reproducing an extreme dilutional coagulopathy but keeping minimal hemoglobin and platelet levels (Table 1). In this model of severe hemodilution, rFVIIa only improved the initiation phase of coagulation (PT; CT; ACT). By contrast, isolated fibrinogen administration improved not
only the initiation of coagulation also formation and strength of the developing clot (α-angle, MCF; CR). Combined application of rFVIIa and fibrinogen further improved the coagulation status of the sample significantly.

There is a growing body of evidence suggesting that aggressive fibrinogen substitution may significantly reverse dilutional coagulopathy, thereby reducing blood loss after severe injury. Substitution of fibrinogen in our dilutional model up to 2 g/l significantly improved the coagulation status of the blood samples. Fibrinogen is the substrate to form a clot thereby explaining its key role in coagulation. Furthermore, combining fibrinogen and prothrombin complex concentrate (factor II, VII, IX, X) administration has recently been shown to further improve dilutional coagulopathy resulting in normalization of coagulation parameters measured.

rFVIIa treatment is currently approved for patients with congenital or acquired hemophilia with antibodies to Factor VIII or IX (United States and Europe), factor VII deficiency and Glanzmanns thrombasthenia (Europe). However, VIIa is increasingly used in off-label indications to control severe bleeding. Because rFVIIa is a very potent pro-hemostatic agent, there is concern that rFVIIa may cause thromboembolic adverse events resulting in serious morbidity and mortality. Therefore, the risks and benefits have to be carefully outweighed before each single administration. In cardiac surgery for example, there is little evidence to suggest a prophylactic use of rFVIIa. However, in patients experiencing refractory postoperative hemorrhage, the use of rFVIIa seems promising and relatively safe although definitive evidence from randomized controlled trials is lacking. Doses of rFVIIa ranging from 20-200 μg/kg have been given with varying success and 90-120 μg/kg of rFVIIa is generally considered the recommended initial dose. Our results in severe hemodilution suggest that half of the
recommended dose (~50 μg/kg, final concentration of 0.7 μg/ml) has similar effect as compared to the full dose. Further decreasing the administered dose however resulted in a loss of the pro-coagulant effect of rFVIIa.

Our data are in accordance with previously published reports showing that isolated rFVIIa administration has failed to correct coagulopathy in an in vitro model of hemodilution. Furthermore, rFVIIa treatment has shown minimal effects on blood loss after major hemorrhage in animals with severe dilutional coagulopathy. The concept of rFVIIa is to activate coagulation at sites of tissue factor exposure. The resulting thrombin burst then leads to the formation of a fibrin clot, provided sufficient fibrinogen levels and platelets are present. In order to get the maximum effect of rFVIIa, consensus guidelines recommend first that the bleeding source should be controlled as best as possible and second that patients be optimized prior to administration of rFVIIa targeting a hematocrit >24%, fibrinogen >0.5-1 g/l, platelets >50-100,000 x 10⁹ /l and pH ≥7.2. In our dilutional model however, rFVIIa improved the coagulation status significantly even with lower platelets (32,000 x 10⁹ /l) if prior fibrinogen substitution was performed.

ROTEM and the Sonoclot Analyzer are both viscoelastic POC coagulation analyzers. They measure the entire clotting process starting with fibrin formation and continue through to clot retraction and lysis at the bedside with minimal time delays. Furthermore, physiological clot development is depicted as a result of whole blood analysis of the coagulation status. ROTEM measures the shear elasticity of the blood sample. The rotation of the pin begins to be impaired after fibrin-platelet bonding has linked the cup and pin together. The strength of these fibrin-platelet bonds affects the magnitude of the pin motion. Thus, the output is directly related to the strength of the formed clot. The Sonoclot Analyzer on the other hand uses a different approach. A plastic probe is
immersed and oscillates vertically in the sample. The output is sensitive to viscosity and monitors viscosity changes that occur during initiation of coagulation and clot development.

Viscoelastic POC analyzers have been used to evaluate the effects of hemostatic agents, such as rFVIIa.6-8 In these studies, thrombelastography/thrombelastometry seems to be a promising methods for characterization of phenotypic variance amongst patients as well as in detection of the individual response to rFVIIa prior and following rFVIIa administration. Presence of high amounts of tissue factor may directly activate the coagulation cascade (bypassing agent) and effects of rFVIIa may no longer be monitored. Therefore, activation with minute amounts of tissue factor has been recommended to visualize the particular signature in different coagulopathies, and particularly to demonstrate the rFVIIa response.11

Recently, modified parameters of the thrombelastograph/thrombelastometry have been introduced based on the first derivative of the original tracing and been shown to correlate closely with thrombin formation.11,23 These parameters, time to maximum thrombus generation (t-MaxVel) maximum rate of thrombus generation (MaxVel), and total thrombus generation (area under the curve, AUC) are supposed to be more sensitive to rFVIIa treatment than standard thrombelastography/thrombelastometry parameters.26 However, these modified parameters did not better perform in monitoring rFVIIa administration in the present study compared to ROTEM’s conventional α-angle and MCF as well as Sonoclot’s CR.

Interpretation of the data presented has to be within the limitations of an in vitro investigation with cautious extensions of the findings into clinical application only. In vitro coagulation studies may not necessarily reflect in vivo conditions because the effects of
tissue damage, endothelial injury, and physiological reactions to correct coagulation are missing *in vitro*. Furthermore, the study was performed with citrated blood samples of healthy male volunteers only. Additionally, we chose to study severe hemodilution with albumin 5% to have minimal effects on coagulation other than dilutional. It is likely that *in vitro* hemodilution with other colloids or crystalloids will show different results. Nevertheless, the present study provides some mechanistic insights into how rFVIIa is dependent on sufficient concentration of fibrinogen and investigates for the first time the two POC coagulation analyzers ROTEM and Sonoclot in this indication.

In summary, administration of rFVIIa alone resulted in our *in vitro* model of severe hemodilution in a shortened initiation phase of coagulation as detected by ROTEM and Sonoclot. Isolated fibrinogen administration to physiological concentrations improved both the initiation of coagulation as well as clot formation and strength. Combined fibrinogen and rFVIIa administration further improved both effects significantly. These data suggest that fibrinogen is a key component in the coagulation process and that adequate levels of fibrinogen should be present before considering the administration of rFVIIa. However, further studies *in vivo* are required to confirm these findings.
REFERENCES


FIGURE LEGENDS

Figure 1. Effects of recombinant factor VIIa (rFVIIa) and or fibrinogen substitution on ROTEM and Sonoclot variables. The two phases of coagulation, initiation of coagulation (1A) and clot formation, strength and stability (1B) are analyzed by the ROTEM and Sonoclot Analyzer. CT = clotting time, ACT = activated clotting time, $\alpha =$ angle alpha, MCF = maximum clot formation, CR = clot rate.

#, p<0.05: effect of modified hemodilution; §, p<0.05: effect of therapeutic doses of rFVIIa; °, p<0.05: effect of fibrinogen administration; *, p<0.05: CT vs. ACT.

Figure 2. Effects of recombinant factor VIIa (rFVIIa) and fibrinogen on additional ROTEM variables. $t$-MaxVel = time to reach MaxVel (time to maximum thrombus generation), MaxVel = maximum velocity of clot formation (maximum rate of thrombus generation), AUC = area under the curve (total thrombus generation).

#, p<0.05: effect of modified hemodilution; §, p<0.05: effect of therapeutic doses of rFVIIa; °, p<0.05: effect of fibrinogen administration.
### Table 1: Hematocrit and standard coagulation variables before and after hemodilution.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Hemodilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT %</td>
<td>42±3</td>
<td>19±2</td>
</tr>
<tr>
<td>PT sec</td>
<td>10±1</td>
<td>74±20</td>
</tr>
<tr>
<td>aPTT sec</td>
<td>42±5</td>
<td>&gt;180</td>
</tr>
<tr>
<td>Platelets $10^3$ mm$^{-3}$</td>
<td>178±62</td>
<td>29±14</td>
</tr>
<tr>
<td>Fibrinogen g l$^{-1}$</td>
<td>2.31±0.47</td>
<td>0.15±0.21</td>
</tr>
</tbody>
</table>

HCT = Hematocrit, PT = Prothrombin time, aPTT = Activated partial thromboplastin time
FIGURE 1

A

sec

1200

1000

800

600

400

200

CT

ROTEM

ACT

Sonoclot

Baseline

Dilution

Dilution + rFVIIa

Dilution + Fibrinogen

Dilution + Fibrinogen + rFVIIa

B

α

60

50

40

30

20

10

0

Baseline

Dilution

Dilution + rFVIIa

Dilution + Fibrinogen

Dilution + Fibrinogen + rFVIIa

angle α

MCF

CR

mm

ROTEN

Sonoclot
FIGURE 3

A

\[ \text{ % } \]

\[ \begin{array}{c}
\text{Dilution + Fibrinogen} \\
\text{+0.35} \\
\text{+0.7} \\
\text{+1.4}
\end{array} \]

\[ \begin{array}{c}
\text{CT} \\
\text{ACT}
\end{array} \]

B

\[ \text{ % } \]

\[ \begin{array}{c}
\text{Dilution + Fibrinogen} \\
\text{+0.35} \\
\text{+0.7} \\
\text{+1.4}
\end{array} \]

\[ \begin{array}{c}
\text{angle \( \alpha \)} \\
\text{MCF} \\
\text{CR}
\end{array} \]
FIGURE 4