In Vitro Effect of Activated Recombinant Factor VII (rFVIIa) on Coagulation Properties of Human Blood at Hypothermic Temperatures

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Background: Recombinant activated factor VII (rFVIIa) is currently administered off-label to control diffuse coagulopathic bleeding of patients with traumatic injuries. These patients are often cold, acidic, and coagulopathic upon arrival and each responds differently to rFVIIa therapy. This study investigated the effects of hypothermia on clotting and the potential benefit of rFVIIa administration on blood coagulation at different hypothermic temperatures.

Method: Citrated blood samples were collected from eight healthy volunteers (20–45 years old) and incubated at 37°C, 34°C, 31°C, and 28°C for 30 minutes. rFVIIa (1.26 μg/mL equivalent to 90 μg/kg in vivo dose) or vehicle solution (saline) was added to each blood sample, incubated (10 minutes), and analyzed at the respective temperatures by standard coagulation tests and thrombelastography.

Results: The clot reaction time of blood samples, measured as prothrombin time, activated partial thromboplastin time, and R time (thrombelastography analysis), was significantly prolonged at 31°C or below compared with at 37°C. The clot formation rate (α angle, maximum clotting velocity [Vmax]), clot strength (maximum amplitude) was decreased at all cold temperatures. Maximum clot strength (maximum amplitude) was only affected (reduced) at 28°C. Addition of rFVIIa shortened the prothrombin time, activated partial thromboplastin time, and R times at every temperature, surpassing the normal (37°C) temperature values in 31°C and 34°C cold samples. Similarly, clot formation rate parameters (clotting time, α angle, Vmax) were also improved by rFVIIa addition and normothermic values were restored in 31°C and 34°C cold blood samples. rFVIIa did not affect maximum amplitude at any temperature.

Conclusions: Mild to moderate hypothermia delayed the initial clot reaction and reduced clot formation rate without affecting ultimate clot strength. FVIIa effectively compensated for the adverse effects of hypothermia except in severe cases. These results suggest that administration of FVIIa should be beneficial in enhancing hemostasis in hypothermic trauma patients without the need for prior correction of the patient’s body temperature.

Key Words: Hypothermia, Recombinant factor VIIa, Coagulation, Thrombelastography (TEG), Human blood.


Trauma with hypothermia has been shown to have significantly worse prognosis than either trauma or hypothermia alone.7–9 Although isolated hypothermia (32°C) incidents led to 23% mortality, hypothermia in conjunction with severe trauma (Injury Severity Score, [ISS] ≥25) was associated with 100% mortality in patients with core temperature below 32°C.8,9 This critical temperature was identified as an ominous predictor of mortality independent of the ISS, presence of shock, or the volume of fluid resuscitation. The mortality of hypothermic patients with comparable ISS, shock, and fluid resuscitation was significantly higher than that of those who remained warm.8,9

A major risk factor of hypothermia is inducing coagulopathy with abnormal bleeding. Hypothermia can cause coagulopathy over a range of temperatures in a temperature-dependent fashion.8 Several mechanisms for the effect of hypothermia have been proposed, including reduced platelet function, decreased activity of coagulation enzymes, and activation of fibrinolysis; however, the exact mechanism continues to be debated.10

Recombinant activated factor VII (rFVIIa) has been considered as a “universal hemostatic agent”.11 It was originally developed for the treatment of bleeding complications in

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U ncontrollable bleeding accounts for 39% of trauma-related deaths and is the leading cause of potentially preventable death in patients with major trauma.1,2 A major contributing factor to bleeding-related mortality is coagulopathy, particularly when it is associated with metabolic acidosis and hypothermia, often referred to as the “lethal triad”.3–5 Substantial increases in mortality of trauma patients have been associated with profound hypothermia. Patients with admission temperatures less than 35°C had significantly greater mortality (25.5% vs. 3.0%) than patients with temperatures ≥35°C.6
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hemophiliacs patients with inhibitors against factors VIII or IX,12 and has been approved by the Food and Drug Administration for these indications. Successful treatment with this drug has been widely reported in hemophiliacs as well as other patients with pre-existing hemostatic defects (i.e., factor VIIa deficiency, acquired von Willebrand disease, uremia, and liver disease) for controlling acute bleeding episodes during surgical procedures.13,14 The off-label use of rFVIIa in a bleeding trauma patient, without preexisting hemostatic disorders, was first described in 1999.15 Since then, the emergency use of rFVIIa as an adjunct therapy for surgical or trauma patients with massive hemorrhage and coagulopathy has become an important new treatment option.16–25 It has provided a safe and effective means for control of severe bleeding when all other measures have failed. In a double-blinded, randomized controlled trial, rFVIIa treatment reduced transfusion requirements and the incidence of multiple organ failure and acute respiratory distress syndrome in severe blunt-trauma patients without increasing thromboembolic adverse effects.26 The treatment, however, did not change the patients’ mortality at either 48 hours or at 30 days after treatment.26 In a recent clinical study, it was reported that rFVIIa may be able to improve early survival of massively bleeding trauma patients.27 It is also suggested that the correction of acidosis and thrombocytopenia may be important prerequisites for rFVIIa efficacy.27 The high cost associated with this treatment and its questionable life-saving effect in trauma patients support the need for continued research to identify better conditions for improving the efficacy of this drug. The purpose of this study was to investigate the effects of hypothermia on coagulation parameters of normal human blood and determine the efficacy of rFVIIa to enhance coagulation at different hypothermic temperatures.

PATIENTS AND METHODS
Blood Sampling and Treatment
The Institutional Review Board of the US Army Institute of Surgical Research and Brooke Army Medical Center approved this study. After obtaining informed consent, blood was collected from eight adult healthy volunteers, (four men and four nonpregnant women), 20 years to 45 years old. None of the participants had received medication with acetylsalicylic acid or any other nonsteroidal anti-inflammatory drugs for the 7 days before blood sampling. A smooth cubital venipuncture was performed using 21-gauge needle and blood (~65 mL) was collected into citrated vacutainer tubes (0.109 mol/L = 3.2% sodium citrate), mixing one part of citrate with nine parts of blood. The first tube aspirated was discarded to minimize tissue thromboplastin contamination. Blood from the remaining tubes was pooled in a 250 mL Nalgene PETG bottle (Fisher Scientific, Pittsburgh, PA) and incubated at 37°C for 15 minutes. After initial equilibration, the blood was aliquoted (6.5 mL) into small tubes and placed in temperature-controlled water baths at 37°C, 34°C, 31°C, or 28°C for an additional 30 minutes. Next, blood specimens were treated with rFVIIa or vehicle. rFVIIa (1.2 mg kit, NovoSeven, Novo Nordisk, Denmark) was reconstituted in sterile water (2.2 mL) and 13.6 μL of the solution was added to each 6.5 mL blood sample. Normal saline (13.6 μL) was added to control blood. The final concentration of rFVIIa in blood was 1.26 μg/mL, equivalent to a bolus dose of 90 μg/kg in patients. The calculated concentration was based on the assumptions that rFVIIa will distribute evenly in 5 L circulating blood volume of an average patient with 70 kg body weight. The blood samples were kept at their normal or hypothermic temperatures for an additional 10 minutes and then assayed for coagulation function by the standard clinical tests and by thrombelastograph (TEG) analysis at their respective temperatures.

Coagulation Tests
After treatment, portions of blood samples were centrifuged at high speed, and the plasma collected for the standard clotting assays. Prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen concentration were measured in plasma using automated BCS Coagulation Analyzer (Dade Behring, Marburg, Germany). All of the tests were performed at the corresponding experimental temperatures (28°C–37°C) in duplicate. For PT measurement, the clotting was induced by the addition of recombinant human tissue factor and synthetic phospholipids (Innovin, Dade Behring, Marburg, Germany) to the plasma in a 2:1 volume ratio. For aPTT measurement, purified soy phosphatides in ellagic acid (Actin FS Activated PTT Reagent, Dade Behring, Marburg, Germany) was added to plasma samples in a 1:1 volume ratio and clotting time was measured. Fibrinogen in plasma was also determined based on a clotting assay in which a large excess of bovine thrombin (50 IU/mL) was added to plasma in a 2:1 volume ratio, and the clotting time was measured. The fibrinogen concentration, which largely depends on this clotting time, was estimated based on a standard curve generated by the machine. Calcium was added to overcome the citrate anticoagulant according to standard practice.

TEG Analysis
Coagulation of all blood samples was tested with a TEG Hemostasis Analyzer 5000 (Hemoscope, Niles, IL). The accurate measurement by the TEG machines was confirmed daily using quality control standards obtained from Hemoscope. Before testing, the machines were set at the selected experimental temperatures of the blood samples. Disposable cups and pins were then loaded and allowed to equilibrate to the selected temperatures for at least 15 minutes. Next, 10 μL Innovin (diluted 1:500 with saline), 20 μL of 0.2 mol/L CaCl2, and 4.3 μL of Corn Trypsin Inhibitor (19 μg/mL, Hematologic Technologies Inc., Essex Junction, VT), a contact activation inhibitor, were added to each cup and allowed to equilibrate for an additional 5 minutes. To start the reaction, 336 μL of citrated blood was added to each cup and measurement
started. Blood samples were tested in triplicate and TEG measurements continued until 30 minutes after maximum amplitude (MA) was reached. The following variables were measured for each sample at the experimental temperatures: reaction time \((R, \text{ min})\), the time that the initial fibrin formation is detected and signaled by a 2 mm rise of amplitude; clotting time \((K, \text{ min})\), indicates the speed of clot formation and is the time from the beginning of clotting, \(R\) time, until a clot with a fixed firmness is formed, 20 mm amplitude rise; angle \((\alpha \text{ degree})\), represents the kinetics of clot development; and MA (mm, measures the maximum strength or firmness of the developed clot). The velocity of clot formation was also calculated as the first derivative of the TEG tracings and maximum clotting velocity \((V_{\text{max}}, \text{ mm/min})\) and time to reach \(V_{\text{max}}\) \((t-V_{\text{max}}, \text{ min})\) were determined for each sample as described previously. Fibrinolysis was measured as percent clot lysis at 30 minutes after MA was reached. This is measured based on reduction of the area under the TEG tracing between the two time points.

**Statistical Analysis**

All statistical analyses were performed using the statistical program SAS, version 8.1 (SAS Institute Inc. 1999, Cary, NC). Data were examined for heterogeneity of variance and non-normality. These conditions were not detected in the data. The coagulation measurements such as PT, aPTT, fibrinogen and TEG parameters were first compared among temperatures within each treatment group using a mixed model of analysis of variance, allowing for temperatures as fixed effects and replicate subject as a random effect. If there was a significant temperature effect, Dunnett’s test was used to compare the normal (37°C) temperature with hypothermic temperatures. The parameters were then compared between the two treatment groups (rFVIIa vs. saline) at each temperature using a paired \(t\) test. Data are expressed as means ± SEM in the graphs. Statistical significance was assigned at a greater than 95% confidence level \((p < 0.05)\).

**RESULTS**

**Plasma Clotting Assays**

PT, which examines the in vivo (extrinsic) coagulation process induced by tissue factor was significantly prolonged in cold blood samples measured at 31°C and 28°C compared with 37°C (Fig. 1, top). The addition of rFVIIa to blood accelerated the initial clotting reaction and shortened the PT at every tested temperature. As a result, the PT of all rFVIIa treated samples was shorter than the value of untreated controls at 37°C (Fig. 1, top).

aPTT, a measurement of contact activation clotting (intrinsic) pathway, was more sensitive to low temperatures than PT (Fig. 1, bottom). The aPTT was prolonged at each hypothermic temperature in a temperature-dependent manner. The addition of rFVIIa to blood samples accelerated the clotting process and reduced aPTT at every temperature tested. The shortening of aPTT with rFVIIa compensated for the cold temperature effects measured in 31°C and 34°C blood samples (no difference compared with 37°C controls).

Fibrinogen measurement was the least affected by cold temperature. This assay is also based on a clotting time measurement but it does not depend on endogenous thrombin generation. The fibrinogen measurement was reduced (15%) only at the lowest temperature (28°C) compared with 37°C. rFVIIa addition had no significant effect on these measurements at any experimental temperature.

**TEG Measurements**

Typical TEG traces and velocity graphs of analysis of blood collected from a single subject at different temperatures are shown in Figure 2. Each tracing represents the average data obtained from triplicate tests. As seen in the figures, the cold temperatures adversely affected all aspects of clot formation except the clot strength. The detailed analysis of the TEG data, including the effect of rFVIIa at different temperatures, is as follows:

The initial clotting reaction time \((R)\) was prolonged significantly (~1–2 minutes) at 31°C and 28°C compared with 37°C (Fig. 3). The addition of rFVIIa sped up the initial
reaction and decreased the $R$ times by $\sim 2$ minutes in all the samples, irrespective of their temperatures. The clot propagation was more affected by the low temperatures than the initial reaction time. These changes were reflected as longer clotting time ($K$) and smaller clotting rate ($\alpha$ angle) of cold blood samples compared with normothermic controls (Fig. 4). Again, the addition of rFVIIa was effective at every temperature, reducing the clotting times by 0.6 minutes to 0.9 minutes and increasing the clotting rate by $\sim 8^\circ$ at every temperature tested. The calculated clotting velocity values ($V_{\text{max}}$ and $t\cdot V_{\text{max}}$) also indicated slower clotting kinetics under hypothermic conditions (Fig. 5). The decrease of $V_{\text{max}}$ and increase of $t\cdot V_{\text{max}}$ were significant at 31°C and 28°C compared with normal temperature. The addition of rFVIIa resulted in an increase in $V_{\text{max}}$ and a decrease in $t\cdot V_{\text{max}}$ in all of the samples at every temperature tested. The improvement achieved by adding rFVIIa compensated or surpassed the changes caused by hypothermic temperatures.

The MA, which represents clot strength and depends on fibrinogen and platelet interaction, was the least affected by low temperatures (Fig. 6). A significant decrease in MA was measured only at the lowest temperature (28°C) in both untreated and rFVIIa-treated samples. The addition of rFVIIa had no effect on clot strength at any temperature. With regard to fibrinolysis, only a small fraction ($\sim 0.2\%$) of the clot lysed 30 minute post-MA (LY30) at 37°C. This reaction was also affected by cold temperatures and reduced to 0% at 31°C and

**Fig. 2.** Typical TEG traces (left) and calculated velocity profiles (right) of blood from a subject at different temperatures. Each TEG trace represents the average of triplicate measurement of a blood sample at specific temperature. Velocity graphs were constructed from the triplicate TEG data measured at each temperature.

**Fig. 3.** Initial clotting reaction time ($R$) of saline or rFVIIa added human blood samples at normal or hypothermic temperatures. $R$-time was prolonged significantly at 31°C and 28°C, compared with 37°C, and was shortened by addition of rFVIIa at each specific temperature. See Figure 1 for notation of significance.

**Fig. 4.** Clot formation time ($K$) (top) and clotting rate ($\alpha$ angle) (bottom) of saline or rFVIIa added human blood samples at normal or hypothermic temperatures. The $K$-time increased and $\alpha$ angle decreased progressively with the decreasing of temperatures. rFVIIa addition shorten $K$ time and fasten the clotting rate at each specific temperature. See Figure 1 for notation of significance.
Hypothermia occurs when the body core temperature falls below 35°C (95°F). It has profound effects on every system of the body including reducing metabolism and oxygen demand, which may be beneficial, and decreasing the rate of temperature-dependent enzymatic function. For example, it is well recognized that, in general, for every 10°C decrease in temperature the activity of an enzyme is reduced by approximately 50%. This study investigated the effects of mild (34°C), moderate (31°C) and severe (28°C) hypothermia on the coagulation of human blood and the potential ability of rFVIIa to reverse the coagulopathy and restore normal clotting function in hypothermic blood. The low temperatures affected the kinetics of clot formation by slowing (1) the cascade reactions of coagulation factors leading up to the formation of initial fibrin strands, which was detected as prolongation of PT, aPTT, and R-time in TEG analysis; and (2) the build up and cross linking of fibrin (propagation of clot), as determined by K time and clotting velocity measurements (α angle and Vmax) by TEG analysis. The observed effects were temperature-dependent and proportional to the severity of hypothermia. These findings were consistent with other studies in which blood samples were collected from hypothermic patients and analyzed by TEG at corrected temperatures. The prolongation of PT and aPTT of normal blood when measured at hypothermic temperatures has been reported elsewhere. In clinics, these laboratory tests are usually performed on plasma samples after warming to 37°C, which can falsely produce normal values and underestimate coagulopathy in hypothermic patients.

Our TEG data showed that clot strength (MA), which represents the contribution of platelets and fibrinogen and their interaction to form a hemostatic clot, was not influenced by moderate hypothermia. The fibrinogen measurements estimated indirectly by adding excessive amounts of thrombin to plasma samples were also unaffected by hypothermia. These results collectively suggest that the moderate hypothermic temperatures slow down clot formation by decreasing enzymatic activity and reducing the thrombin generation rate without affecting the final contribution of platelets or fibrinogen to form the clot. The velocity profiles of hypothermic blood (Fig. 4), which display patterns similar to endogenous thrombin potential of the samples, also suggested temperature-dependent decreases in thrombin generation. This mechanism is supported by an in vitro study which has shown that the monocyte tissue factor activities, measured as factor Xa generation, as well as platelet thrombin generation were reduced by 38% and 26%, respectively, at 33°C.

Watts et al. reported significant alterations in both enzymatic clotting reaction and platelet function (MA) of blood collected from hypothermic trauma patients with temperature below 34°C. The TEG analysis also detected an acute hypercoagulability in blood of patients whose temperatures were ≥34°C. Other studies found no significant change in MA of blood collected from hypothermic patients, consistent with the present in vitro findings. The differing results reported among studies may be explained based on the difference in the sources of blood samples. The data reported by Watts et al. were obtained from blood samples collected from hypothermic patients with significant traumatic injuries.
(ISS ≥ 9) that may have adversely affected fibrinogen status and platelet function. The TEG analysis of blood collected from animals with moderate hypothermia also showed no change in the clot strength as compared with blood from normothermic animals.40,41

In the present study, the in vitro addition of rFVIIa to the samples at a concentration considered to be the equivalent to a 90 μg/kg in vivo dose corrected the coagulopathic effect of mild and moderate hypothermia (34°C and 31°C), but had less effect on severely hypothermic blood (28°C). rFVIIa shortened PT and aPTT of hypothermic samples and brought these clotting times to the level of normothermic blood but had no effect on fibrinogen measurements. This lack of effect can be explained by the fact that fibrinogen determination does not directly measure the concentration of the fibrinogen molecules in plasma but rather estimates the protein level of thrombin (an activated factor) is added. Thus, fibrinogen determination is not influenced by hypothermia because the clotting reaction bypasses the slowed coagulation process and delayed thrombin generation caused by hypothermia. rFVIIa addition would also have no effect on fibrinogen determination by this technique because by adding exogenous thrombin in excess, the need for FVIIa activity to propagate coagulation and enhance thrombin generation is totally circumvented.

To analyze the TEG findings, a summary of the changes in TEG parameters induced by hypothermia and the improvements achieved by the addition of rFVIIa as compared with the values of untreated blood at normal temperature are presented in Table 1. The effect of rFVIIa was temperature-dependent, improving both R-time and Vmax with greater effect on R-time. rFVIIa was most effective when added to mildly hypothermic blood; it not only corrected for the hypothermia effect but produced a clotting rate that surpassed the normothermic blood by ~25%. The increased coagulation rate observed here, may suggest the risk of thrombosis in patients treated with rFVIIa. However, rFVIIa is not indicated for treating hypothermic patient, it is rather targeted to the patients with significant coagulopathy and associated hypothermia and acidosis who are unresponsive to standard therapy. In these situations, the drug reverses the coagulopathy and restores normal clotting function without increasing the risk of thrombosis.24,25,42,45

With regard to contribution of platelet and fibrinogen in forming strong clot, rFVIIa did not have any impact even when it was added to normothermic blood. The effect of rFVIIa on fibrinolysis was also insignificant. The lack of effect of rFVIIa on clot strength (MA) was also reported in another in vitro study44 and in an in vivo study28 in which swine were injected with increasing doses of rFVIIa (180–720 μg/kg) and their blood was analyzed by TEG. These observations appear to be inconsistent with the reported effect of rFVIIa on increasing hemostatic strength of clot in an uncontrolled arterial hemorrhage model.45 In that study, pre-treatment of pigs with rFVIIa increased the clot strength and the pressure at which rebleeding occurred after fluid resuscitation.45 The addition of rFVIIa, factor X, and prothrombin to washed platelets and red cell media (plasma free blood) was also shown to increase platelet adhesion and activation on collagen- and fibrinogen-coated surfaces.46 These findings suggest that rFVIIa-mediated thrombin generation may enhance the hemostatic strength of clot and its attachment to injured tissues. However, this hemostatic improvement cannot be measured by the TEG analysis of blood that evaluates only the cohesive strength and not the adhesive strength of clot to the tissues.

The mechanism by which therapeutic doses of rFVIIa induce initial thrombin generation is still a matter of debate.47,48 According to a cell-based model, rFVIIa at pharmacological concentrations binds to activated platelets, exposing negatively charged phospholipids, and activates factor X independently from tissue factor.49 This pathway of rFVIIa activity was not affected by the reduced temperature (33°C), suggesting rFVIIa should be effective in enhancing hemostasis in hypothermic patients.50 Indeed, the effectiveness of rFVIIa to correct coagulopathy and stop bleeding in trauma patients with hypothermia was reported in the guidelines for the use of rFVIIa, and elsewhere.51,52 Experimental studies also showed that administration of rFVIIa in hemodiluted pigs improved coagulation parameters and reduced bleeding from a severe liver injury in the presence of hypothermia.53,54

<p>| Table 1 Changes in TEG Parameters of Hypothermic Blood Samples With or Without rFVIIa Compared With Those of Untreated Blood at Normal Temperature (37°C) |
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<td>28</td>
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*NS, not significant, defined as p > 0.1.
However, the decreases in the bleeding in these studies and the subsequent investigation with a greater number of animals did not result in a reduction in mortality rate.

The limitation of this study is that the rFVIIa effect was assessed in an isolated in vitro condition on blood collected from healthy volunteers, apart from many other influencing factors (e.g., tissue injury, hemorrhage, and acidosis) that may affect rFVIIa responses in trauma patients. On the other hand, this isolation is also the strength of this in vitro study that allows exploring the direct effect of hypothermia and defining the effectiveness of rFVIIa under different hypothermic conditions apart from other confounding factors that makes the interpretation of the findings very difficult.

In summary, hypothermia at 34°C, 31°C, and 28°C affected blood coagulation function by delaying the initial reaction process and decreasing the clot formation rate without impacting the final clot strength. Hypothermia alone, therefore, can be the cause of increased bleeding in trauma patients who may have normal clotting values (PT and aPTT measured at 37°C). The addition of rFVIIa to hypothermic blood compensated for the decreases and restored or increased the clotting reaction process above normothermic values in the cold blood samples. These data suggest that administration of rFVIIa should be beneficial in promoting hemostasis in hypothermic trauma patients without the need to correct the patient’s body temperature before the treatment.

REFERENCES


