Recombinant factor VIIa reduces bleeding after blunt liver injury in coagulopathic, hypofibrinogenaemic pigs


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Key points
- Off-label use of recombinant factor FVIIa (rFVIIa) has reportedly been used in coagulopathic-associated massive bleeding, but lack of fibrinogen may limit its effectiveness.
- Using a liver injury plus haemodilution model in pigs under conditions of low fibrinogen, rFVIIa was beneficial.
- Replacing fibrinogen before rFVIIa therapy was even better, with no thromboembolism.
- rFVIIa remains ‘off label’ for coagulopathy or massive bleeding.

Background. Recombinant factor VIIa (rFVIIa) has been successfully used in various clinical conditions to treat severe coagulopathy, but its efficacy may be affected by the underlying conditions. We therefore investigated the efficacy of rFVIIa treatment under conditions of hypofibrinogenaemia in a pig model of blunt liver injury.

Methods. Severe haemodilution was instigated in four groups of seven anaesthetized pigs. Before inflicting liver injury, animals were assigned to receive either 70 mg kg⁻¹ fibrinogen (fibrinogen group) or placebo (control group). Thirty seconds after injury, rFVIIa (180 μg kg⁻¹) (rFVIIa and fibrinogen+ rFVIIa groups) or vehicle (control and fibrinogen groups) was administered. Haemodynamic variables, coagulation parameters, and blood loss were monitored for 2 h. Histology was examined to evaluate the presence of thrombi and the consistency of liver injury.

Results. At the end of the observation period, total blood loss [median (range)] decreased in all intervention groups [fibrinogen: 1275 (1221–1439) ml, P=0.036; rFVIIa: 966 (923–1136) ml, P=0.008; fibrinogen+rFVIIa: 678 (475–756) ml, P=0.008] when compared with control animals [blood loss: 1752 (1735–2221) ml]. The mortality rate in the control group was 100%, whereas only 42% of fibrinogen-substituted animals died (P=0.023). All animals treated with rFVIIa or fibrinogen+rFVIIa (P<0.001) survived and no signs of thromboembolism were observed.

Conclusions. rFVIIa under conditions of hypofibrinogenaemia exhibited a positive impact on coagulation parameters and a reduction in blood loss. These effects were significantly improved after prior substitution with fibrinogen.

Keywords: haemorrhage; haemostasis; shock; trauma; thromboelastometry

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Haemorrhagic shock is the leading cause of death among trauma patients in the early phase after hospitalization. The underlying pathophysiology of trauma-induced coagulopathy is a result of multiple factors, including acidosis, hypothermia, anaemia, hyperfibrinolysis, and hypotension-induced inflammation and also consumption and dilution of coagulation factors. To protect trauma victims from exsanguination and to preserve the pool of coagulation factors, generally allogenic blood products are transfused. As the transfusion of allogenic blood is associated with adverse events, studies over the past several years have investigated the impact of using site-specific i.v. pro-coagulants to terminate uncontrollable haemorrhage and to reinstate haemostasis.

Recombinant factor VIIa (rFVIIa, NovoSeven, Novo Nordisk, Bagsværd, Denmark) was originally developed as a haemostatic agent for the use in haemophilic patients who developed inhibitors against factor VIII or IX and is approved for these indications. The first successful ‘off-label’ treatment of a nearly exsanguinated trauma patient with rFVIIa was reported in 1999. Since then, the potential of rFVIIa to terminate severe or life-threatening bleeding has been investigated in a number of experimental studies, randomized controlled trials (RCTs), and individual therapeutic trials in an array of clinical indications. Owing to the increase in ‘off-label’ use, international consensus recommendations have summarized the current evidence to guide therapy with rFVIIa. To optimize rFVIIa therapy, it is advised to aim for a target of haematocrit >24%, platelets >50 000 μl⁻¹, pH ≥7.2, and fibrinogen >50–100 mg dl⁻¹. However, rFVIIa is often used as a last resort to treat severe coagulopathy and massive bleeding in patients where other measures have failed. As fibrinogen is the first coagulation factor to reach critically low levels in experimental models of progressive haemodilution, sufficient fibrinogen...
concentrations might be a limiting factor for the effective treatment with rFVIIa.\textsuperscript{10, 11} At present, no in vivo studies have systematically investigated the efficacy of rFVIIa treatment under the condition of low concentrations of fibrinogen and after fibrinogen replacement. Therefore, this pilot study determined the efficacy of rFVIIa under conditions of hypofibrinogenaemia and after the prior substitution with fibrinogen in a model of blunt liver injury in coagulopathic pigs.

**Methods**

All experiments were performed in accordance with the German legislation governing animal studies and The Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1996).\textsuperscript{12} Official permission for this study was granted from the governmental animal care and use office (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany). Before surgery, male German landrace pigs from a disease-free barrier breeding facility were housed in ventilated rooms and allowed to acclimatize to their surroundings for a minimum of 5 days. Animals were fasted overnight before surgical procedure, with water allowed ad libitum.

Twenty-eight pigs, weighing 29–35 kg, were included in this RCT. For pre-medication, animals received an i.m. injection of 4 mg kg\textsuperscript{-1} azaperone. Anaesthesia was induced by i.v. injection of 3 mg kg\textsuperscript{-1} propofol followed by oral intubation. The animals’ lungs were ventilated with 40% oxygen at 20–26 bpm and a tidal volume of 10 ml kg\textsuperscript{-1} to keep the end-tidal partial carbon dioxide (\(P_{CO_2}\)) tension between 36 and 42 mm Hg. To prevent hypoxia, the inspiratory oxygen concentration was set to 100% during the time of haemodilution. Anaesthesia was maintained with isoflurane at a concentration of 1–1.2% and continuous infusion of fentanyl at a concentration of 3–4 \(\mu\)g kg\textsuperscript{-1} h\textsuperscript{-1}. The basic fluid requirement was met by continuous administration of Ringer’s lactate solution (RL) at a rate of 4 ml kg\textsuperscript{-1} h\textsuperscript{-1}.

After laparotomy, the infusion of RL was increased to 8 ml kg\textsuperscript{-1} h\textsuperscript{-1}. Temperature was maintained for the entire experiment between 36.5 and 37.0°C.

Vital signs were monitored by electrocardiography, tail pulse oximetry, temperature, and femoral arterial and central venous pressure using a standard anaesthesia monitor (AS/3, Datex Ohmeda, Helsinki, Finland).

**Surgical preparation and haemodilution**

The right femoral artery was cannulated with an 18 G catheter to collect blood samples and to measure continuous arterial pressure. To withdraw blood, an 8.5 Fr catheter was advanced in the left femoral vein. For volume substitution and insertion of a pulmonary artery catheter, two 8.5 Fr catheters were surgically implanted in the right and left jugular veins. After line placement, pancuronium (0.2 mg kg\textsuperscript{-1}, i.v.) was administered to facilitate laparotomy. Finally, splenectomy and cystotomy were performed. To compensate for blood loss associated with spleen removal, RL (37°C) was administered at three-fold the spleen weight. Subsequently, the animals were allowed a period of 30 min for haemodynamic parameters to stabilize.

The animals then underwent a stepwise haemodilution of \(\sim 80\%\) of their estimated blood volume to achieve fibrinogen concentrations of 50–60 mg dl\textsuperscript{-1}.\textsuperscript{13} To compensate for the difference in baseline fibrinogen concentrations and to achieve this low concentration of fibrinogen, we had to perform a two-step haemodilution model with intermittent re-transfusion of washed red blood cells (RBCs). In the first step, blood was withdrawn at a rate of 55 ml kg\textsuperscript{-1} and blood loss was compensated with the infusion of 6% HES 130/0.4 (total infusion rate 50 ml kg\textsuperscript{-1}; Voluven\textsuperscript{9}, Fresenius, Bad Homburg, Germany) and RL. The collected blood was processed using a continuous cell saver system (Cell Saver 5\textsuperscript{8}, Haemonetics, Munich, Germany) and erythrocytes were re-transfused. To avoid coagulation in the cell saver system, sodium citrate (3.8% in a ratio of 1:1.7) was used. In the second step, blood was withdrawn at a rate of 55–70 ml kg\textsuperscript{-1} and as well substituted with HES and RL. Finally, levels of fibrinogen were measured. If levels of fibrinogen were above 80 mg dl\textsuperscript{-1}, further blood was withdrawn and substituted with RL. Subsequently, washed RBCs were again re-transfused to avoid early death from severe anaemia. Total blood withdrawal [median (range)] between the groups [control group: 3800 (3650–3850) ml; fibrinogen group: 3800 (3650–3850) ml; rFVIIa group: 3600 (3500–3800) ml; fibrinogen+rFVIIa group: 3700 (3650–3700) ml] and the total volume of RL infused [control group: 2250 (2150–2300) ml; fibrinogen group: 2250 (2150–2350) ml; rFVIIa group: 2050 (1900–2250) ml; fibrinogen+rFVIIa group: 2200 (2050–2350) ml] were comparable between the groups.

**Fibrinogen substitution**

Using sealed envelopes containing the group number, the animals were randomly assigned to the following intervention groups (all \(n=7\)): Group 1, control; Group 2, fibrinogen; Group 3, rFVIIa; Group 4, fibrinogen+rFVIIa. Animals received either fibrinogen (Haemocomplettan\textsuperscript{10}, CSL Behring, Marburg, Germany) at a dose of 70 mg kg\textsuperscript{-1} (for the fibrinogen and fibrinogen+rFVIIa groups) or an equal amount of normal saline (control and rFVIIa groups). This time point was termed ‘Fibrinogen substitution’.

**Liver injury and rFVIIa substitution**

According to the Liver Injury Scale of the American Association for the Surgery of Trauma (AAST),\textsuperscript{16} a reproducible Grade III blunt liver injury was induced using a custom-made instrument.\textsuperscript{15} The same investigator, who was also blinded to fibrinogen infusion and subsequent rFVIIa substitution, performed the liver injury procedure on all animals. Adequate exposure of the liver was achieved by retraction, and the base of the instrument plate was positioned beneath the right middle lobe. The injury was induced by one-time clamping of the instrument through the parenchyma with a force of 185–260 N. The force of injury was analysed in real-time (time of registration was set to 500 ms) after amplifying
rFVIIa and fibrinogen after liver injury

(VG140, ATR Industrie-Elektronik, Krefeld, Germany) and digitizing the force signal (NI USB-6009, National Instruments, Austin, TX, USA) with custom-made software (LabView 8.8, National Instruments).

After liver injury, the abdomen was loosely closed with surgical clamps. Further manipulations were avoided to prevent any clot removal. rFVIIa (dose: 180 μg kg⁻¹; NovoSeven™, Novo Nordisk) was infused post-injury for 30 s in animals from the rFVIIa and fibrinogen+rFVIIa groups at a concentration of 600 μg ml⁻¹. The dose of rFVIIa was chosen based on findings of previous studies. Pigs from the control and fibrinogen groups received the same volume of vehicle. Independent of mean arterial pressure (MAP), 5 min after injury, the animals received RL at a rate of 4 ml kg⁻¹ min⁻¹ over 8 min for resuscitation. After resuscitation, the rate of RL was set to 25 ml kg⁻¹ h⁻¹ until the end of the experiment. The observation period lasted up to 120 min after the time of injury. Pulseless electrical activity, an MAP below 10 mm Hg was defined as death, whatever occurred first. Animals surviving for more than 2 h were euthanized with a high dose of fentanyl, propofol, and potassium chloride. Immediately after death, the abdomen was reopened, the vena cava was clamped, and the intraperitoneal blood was collected to determine the blood loss after liver injury.

Blood sampling and analytical methods

Arterial blood sample collection and blood analyses were performed 10 min after splenectomy (‘baseline’), at the end of haemodilution (‘haemodilution’), after fibrinogen substitution (‘fibrinogen substitution’), and 120 min after liver injury (‘trauma’) or immediately after death. Haemoglobin (Hb) concentration, pH value, partial oxygen (Po₂) tension, and partial carbon dioxide (Pco₂) tension were measured with a blood gas analyzer (ABL500, Radiometer, Copenhagen, Denmark). Prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen concentrations (the Clauss method) were determined by standard laboratory methods using the appropriate tests from Dade Behring (Marburg, Germany) with a coagulometer (KC4, Baxter, Newbury, UK). To confirm the results of concentrations of fibrinogen by a coagulometer, a subset of samples were analysed using a pig enzyme-linked immunosorbent assay (ELISA, E-5F1B, Immunology Consultants Laboratory, Newberg, OR, USA) (data not shown). Thrombin–antithrombin (TAT) complexes (Enzygnost, Dade Behring) and rFVIIa antigen concentration were measured by ELISA. Thromboelastometry was carried out on the ROTEM™ coagulation analyzer (ROTEM®, Pentapharm, Munich, Germany). Using the EXTEM™ assay (Pentapharm), the clotting time (CT, s), clot formation time (CFT, s), and the maximum clot firmness (MCF, mm) were assessed.

Pathological examination

Post-mortem, the entire liver was removed and inspected for the degree of liver injury. Injured areas of the liver were fixed in 10% neutral buffered formalin and cut into 3-mm-thick slices. Only areas of maximum depth of injury and vessel rupture were chosen for further histological examination. In addition to these samples, representative tissues of the lung, liver, and heart were processed to explore areas with frequent thrombotic events. After paraffin embedding, histological sections 3 μm in thickness were prepared for examination by microscopy. The samples were stained by haematoxylin/eosin and a standard Elastica-van Gieson protocol. Further, sections of lung and liver tissues were immunostained for fibrinogen and von Willebrand factor (vWF) using a polyclonal rabbit anti-human fibrinogen antibody (DAKO A0080, polyclonal rabbit, DAKO, Glostrup, Denmark) and polyclonal rabbit vWF VIII antibody (DAKO, A0082, polyclonal rabbit), both at a dilution of 1:100, for staining. Proteins were seen using the Vectastain universal ABC kit (Vector Laboratories, Burlingame, CA, USA), and haematoxylin was used as counter stain. Histology was assessed using light microscopy (Eclipse 50i, Nikon, Duesseldorf, Germany). Microscopic examination was performed by a pathologist blinded to the treatments to assess the degree of fibrin deposition in vessels and microthrombi formation and to quantify the liver trauma.

Statistical analysis

SPSS version 16 (SPSS, Chicago, IL, USA) was used for statistical analyses. Statistical tests for all four sampling points (baseline, haemodilution, fibrinogen substitution, and trauma) were initialized with application of the Kruskal–Wallis test. Significant differences were subsequently analysed by the unpaired Wilcoxon test followed by the Bonferroni adjustment. To investigate the effects of haemodilution, a non-parametric Friedman analysis was applied. Parameters are presented as median and inter-quartile ranges (IQRs) if not otherwise indicated. Data on survival were analysed using the log-rank test. Statistical tests were performed two-tailed and the level of significance was defined as P<0.05.

Results

Baseline measurements and coagulation parameters

Baseline parameters including animal weight, haemodynamic, and coagulation measurements and also RBC count were comparable between all groups (Tables 1 and 2). The dilutional coagulopathy caused a significant alteration of all coagulation parameters and a significant decrease in platelet count. PT increased from 9.5 (9.2–10) to 20 (18–22) s (Fig. 1a), whereas fibrinogen concentrations (Fig. 1b) decreased from 298 (275–322) to 59 (55–61) mg dl⁻¹ (P<0.001). Thromboelastometry parameters showed a significant prolongation of CT and CFT after dilutional coagulopathy (Fig. 2a and e). In contrast, the MCF decreased (Fig. 2c).

After fibrinogen substitution, treated animals had significantly greater fibrinogen concentrations compared with groups without fibrinogen substitution (P<0.05) (Fig. 1a). In addition, fibrinogen-substituted animals had a mean PT that was shortened to 12 (11–12) s (P<0.05) (Fig. 1a), and a decrease in CT and CFT. Corresponding to these decreased measures, MCF significantly increased (P<0.05).
### Table 1: Laboratory parameters. Median (IQR) including Hb, platelet count, aPTT, TAT complex at baseline, after haemodilution, after fibrinogen substitution, and at the end of the observation period (trauma). *P<0.001 vs control; ‡P<0.05 vs fibrinogen

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Haemodilution</th>
<th>Fibrinogen substitution</th>
<th>Trauma</th>
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</thead>
<tbody>
<tr>
<td><strong>Haemoglobin (g litre⁻¹)</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>8.7 (8.1–9.1)</td>
<td>7.5 (7.1–8.1)</td>
<td>7.6 (7.1–8.2)</td>
<td>3.5 (3.3–4.3)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>8.4 (8.0–8.6)</td>
<td>7.8 (7.6–8.2)</td>
<td>7.6 (7.4–8.3)</td>
<td>3.7 (3.5–4.9)</td>
</tr>
<tr>
<td>rFVIIa</td>
<td>8.6 (8.2–9.1)</td>
<td>7.7 (7.1–8.4)</td>
<td>8.0 (7.6–8.2)</td>
<td>6.3 (5.0–6.5)‡‡</td>
</tr>
<tr>
<td>Fibrinogen+rFVIIa</td>
<td>8.6 (8.1–9.0)</td>
<td>8.2 (7.3–8.6)</td>
<td>7.8 (7.4–8.2)</td>
<td>6.2 (5.9–7.4)‡‡</td>
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<tr>
<td><strong>Platelet count (10³ µl⁻¹)</strong></td>
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<tr>
<td>Control</td>
<td>243 (208–270)</td>
<td>76 (74–84)</td>
<td>81 (75–91)</td>
<td>41 (36–50)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>246 (205–317)</td>
<td>81 (77–101)</td>
<td>79 (77–92)</td>
<td>51 (34–68)</td>
</tr>
<tr>
<td>rFVIIa</td>
<td>271 (187–325)</td>
<td>93 (75–110)</td>
<td>91 (77–105)</td>
<td>83 (74–112)‡‡</td>
</tr>
<tr>
<td>Fibrinogen+rFVIIa</td>
<td>261 (249–296)</td>
<td>88 (81–102)</td>
<td>90 (83–101)</td>
<td>81 (80–111)‡‡</td>
</tr>
<tr>
<td><strong>aPTT (s)</strong></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>13 (12–14)</td>
<td>23 (19–26)</td>
<td>23 (18–26)</td>
<td>30 (21–40)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>12 (11–13)</td>
<td>23 (19–30)</td>
<td>23 (20–23)</td>
<td>29 (24–33)</td>
</tr>
<tr>
<td>rFVIIa</td>
<td>14 (10–14)</td>
<td>27 (23–30)</td>
<td>23 (19–27)</td>
<td>27 (20–29)</td>
</tr>
<tr>
<td>Fibrinogen+rFVIIa</td>
<td>13 (12–14)</td>
<td>26 (26–30)</td>
<td>24 (22–26)</td>
<td>25 (21–28)</td>
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<tr>
<td><strong>TAT (µg litre⁻¹)</strong></td>
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<tr>
<td>Control</td>
<td>10.2 (9.2–13.4)</td>
<td>7.4 (6.0–13.0)</td>
<td>7.6 (4.8–19.0)</td>
<td>14.0 (13.3–19.8)</td>
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<tr>
<td>Fibrinogen</td>
<td>6.6 (5.9–13.1)</td>
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<td>5.0 (3.7–8.7)</td>
<td>13.0 (7.5–13.6)</td>
</tr>
<tr>
<td>rFVIIa</td>
<td>6.9 (5.4–11.7)</td>
<td>4.7 (2.2–6.9)</td>
<td>4.0 (2.0–7.0)</td>
<td>17.4 (10.9–29.7)</td>
</tr>
<tr>
<td>Fibrinogen+rFVIIa</td>
<td>8.1 (4.7–11.1)</td>
<td>2.9 (2.0–3.9)</td>
<td>2.4 (2.0–4.7)</td>
<td>12.8 (6.5–17.2)</td>
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</tbody>
</table>

### Table 2: Haemodynamic parameters. Median (IQR) including CO, central venous pressure (CVP), heart rate (HR), MAP, and MPAP at baseline (after splenectomy), after haemodilution, after fibrinogen substitution, and at the end of the observation period (trauma). *P<0.001 vs control; ‡P<0.05 vs fibrinogen

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Haemodilution</th>
<th>Fibrinogen substitution</th>
<th>Trauma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HR (beats min⁻¹)</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Control</td>
<td>95 (73–104)</td>
<td>91 (78–115)</td>
<td>100 (85–103)</td>
<td>133 (118–187)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>95 (76–101)</td>
<td>95 (82–110)</td>
<td>84 (75–101)</td>
<td>143 (139–171)</td>
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<tr>
<td>rFVIIa</td>
<td>92 (77–103)</td>
<td>106 (85–107)</td>
<td>96 (89–105)</td>
<td>180 (153–181)</td>
</tr>
<tr>
<td>Fibrinogen+rFVIIa</td>
<td>76 (70–101)</td>
<td>93 (74–94)</td>
<td>83 (75–96)</td>
<td>130 (111–150)</td>
</tr>
<tr>
<td><strong>MAP (mm Hg)</strong></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>80 (73–88)</td>
<td>75 (73–90)</td>
<td>77 (72–79)</td>
<td>13 (9–14)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>79 (71–82)</td>
<td>73 (64–79)</td>
<td>73 (71–76)</td>
<td>31 (25–37)*</td>
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<tr>
<td>rFVIIa</td>
<td>88 (85–92)</td>
<td>75 (74–87)</td>
<td>74 (63–86)</td>
<td>42 (32–47)*</td>
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<tr>
<td>Fibrinogen+rFVIIa</td>
<td>88 (79–89)</td>
<td>83 (74–88)</td>
<td>83 (73–84)</td>
<td>52 (49–57)*‡‡</td>
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<tr>
<td><strong>CVP (mm Hg)</strong></td>
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<tr>
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<td>7 (6–11)</td>
<td>7 (6–11)</td>
<td>2 (1–2)</td>
</tr>
<tr>
<td>Fibrinogen</td>
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<td>7 (6–9)</td>
<td>7 (7–8)</td>
<td>4 (3–5)</td>
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<tr>
<td>rFVIIa</td>
<td>8 (7–8)</td>
<td>9 (7–10)</td>
<td>9 (7–10)</td>
<td>5 (4–6)*‡</td>
</tr>
<tr>
<td>Fibrinogen+rFVIIa</td>
<td>7 (6–8)</td>
<td>7 (6–8)</td>
<td>8 (6–9)</td>
<td>6 (6–7)*‡‡</td>
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<tr>
<td><strong>MPAP (mm Hg)</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>17 (16–21)</td>
<td>20 (17–21)</td>
<td>17 (16–21)</td>
<td>7 (7–9)</td>
</tr>
<tr>
<td>Fibrinogen</td>
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<td>18 (17–20)</td>
<td>10 (8–12)</td>
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<tr>
<td>rFVIIa</td>
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<td>21 (19–23)</td>
<td>20 (18–22)</td>
<td>16 (13–18)*</td>
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<tr>
<td>Fibrinogen+rFVIIa</td>
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<td>18 (16–19)</td>
<td>20 (17–21)</td>
<td>16 (14–16)*‡‡</td>
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<tr>
<td><strong>CO (litre min⁻¹)</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.1 (3.7–4.5)</td>
<td>4.0 (3.5–4.6)</td>
<td>3.5 (3.4–4.0)</td>
<td>1.2 (1.1–1.6)</td>
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<td>Fibrinogen</td>
<td>3.8 (3.7–4.0)</td>
<td>3.6 (3.1–4.0)</td>
<td>3.7 (3–0–3.9)</td>
<td>2.0 (2.0–2.3)*‡</td>
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<td>rFVIIa</td>
<td>4.0 (3.7–4.2)</td>
<td>4.2 (3.6–4.5)</td>
<td>4.0 (3.7–4.2)</td>
<td>2.7 (2.4–2.9)*</td>
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<td>Fibrinogen+rFVIIa</td>
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<td>3.5 (3.4–4.1)</td>
<td>3.6 (3.5–4.6)</td>
<td>3.2 (3.1–3.3)*‡‡</td>
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</tbody>
</table>
After trauma, fibrinogen concentrations decreased in all groups. Besides, the fibrinogen-induced decrease in PT was partially reversed in the fibrinogen-substituted groups. In contrast, therapy with rFVIIa under hypofibrinogenaemia resulted in a significant reduction in PT, CT, and CFT. Therapy with rFVIIa after replacing fibrinogen (fibrinogen + rFVIIa) caused a significant decrease in PT and an increase in fibrinogen concentration (P, 0.05) compared with the rFVIIa-treated or fibrinogen-substituted group. At the end of the experimental protocol, PT, CT, and CFT were highest in the control group, and MCF, fibrinogen concentration, and platelet concentration were lowest in this group.

aPTT was significantly prolonged after haemodilution, without showing any significant differences between the groups. Similarly, TAT complexes increased in all groups after trauma without significant differences between the groups (Table 1).

Plasma concentrations of rFVIIa reflected the increase in circulating levels of rFVIIa after its substitution with a decrease over time (Fig. 3).

**Haemodynamic variables**

All haemodynamic variables were comparable between the groups and over the time between the baseline period and after the fibrinogen substitution period (Table 2). After liver injury and before resuscitation, all animals again developed haemorrhagic shock with a decrease in arterial pressure of ~40% (data not shown). At the end of the observation period, MAP, cardiac output (CO), and mean pulmonary artery pressure (MPAP) in the fibrinogen and rFVIIa groups were lower than in the fibrinogen + rFVIIa group (P<0.05). MAP and CO were significantly less in the control group than in the three intervention groups.
Blood loss and survival
Total blood loss (ml) measured at the end of the observation period was lowest in the fibrinogen+rFVIIa group, followed by the rFVIIa, fibrinogen, and control groups (Fig. 4). Accordingly, all animals treated with rFVIIa or fibrinogen+rFVIIa survived, whereas three of seven animals (42%) in the fibrinogen group died before the end of the observation time ($P=0.023$) (Fig. 5).

Macroscopic and microscopic analysis
Post-mortem gross sectioning of liver injuries and microscopic evaluation showed parenchyma injury with an average depth of 1.5–2 cm (~60–90% of total lobe depth). Immunostaining with vWF revealed comparable laceration of venous vessels (2–4 mm). Histological slides from representative sections of kidney, liver, and heart tissues showed no thromboembolic events. Immunostaining of lung tissue with fibrinogen antibody revealed no specific thrombus formation in all animals of the intervention groups.

Discussion
This study investigated the haemostatic effects that result from the treatment with rFVIIa under conditions of hypofibrinogenaemia and after the prior substitution with fibrinogen in a model of blunt liver injury in coagulopathic pigs. rFVIIa was effective to restore coagulation parameters and reduce blood loss under critical concentrations of fibrinogen. However, prior substitution with fibrinogen improved the efficacy of rFVIIa treatment, which was both reflected upon a further reduction in blood loss and additive effects on coagulation parameters.

To induce dilutional coagulopathy and to achieve plasma fibrinogen concentrations of 50–60 mg dl$^{-1}$, a haemodilution of ~80% had to be performed. This degree of dilutional coagulopathy resulted both in a significant prolongation of PT and clot formation and in a reduction in clot strength. The substitution of 70 mg kg$^{-1}$ exogenous fibrinogen led to an overall improvement of impaired coagulation parameters, which was probably caused by improved fibrin polymerization adding to the overall clot stability. This result is also in accordance with data from various published studies, which showed that the fibrinogen substitution successfully reversed colloid- and crystalloid-induced coagulopathy. However, after the infliction of trauma, loss and consumption of coagulation factors resulted in decreased concentrations of fibrinogen, and the observed effects of fibrinogen-induced improvement of thromboelastometry parameters were partially reversed (fibrinogen group without rFVIIa treatment).

In contrast, despite pre-injury hypofibrinogenaemia, the treatment of rFVIIa was associated with a higher degree of reduction in blood loss when compared with the pre-injury correction of coagulation parameters induced by fibrinogen. Treatment with rFVIIa improved both PT and thromboelastometry measurements. The reduction in blood loss and the improvement of coagulation parameters may be explained...
by the enhanced rate of thrombin generation on thrombin-activated platelet localized to the site of injury after administration of therapeutic doses of rFVIIa.\textsuperscript{21} Even though the residual thrombin concentration is normally sufficient to cleave fibrinogen in highly diluted plasma, studies have shown that thrombin generation and clot formation are reduced in a plasma dilution of more than 40%.\textsuperscript{22} These findings underscore the importance of rapid thrombin generation to achieve sufficient haemostasis in the presence of active bleeding, as FXa and thrombin have a short half-life.\textsuperscript{23} Furthermore, the observed reduction in blood loss might be explained by the enhanced clot stability caused by thrombin and rFVIIa-induced activation of thrombin-activatable fibrinolysis inhibitor and FXIII.\textsuperscript{24}

However, the treatment with rFVIIa after fibrinogen substitution had the greatest impact on blood loss and coagulation parameters. The additive haemostatic effects can be explained by their different mechanisms. The enhanced levels of thrombin (induced by rFVIIa) combined with the exogenous provision of its substrate fibrinogen allowed a higher rate of fibrinogen cleavage, contributing to improved clot strength. This observation is in agreement with recently published in vitro studies.\textsuperscript{25–27} The significantly higher platelet count in both rFVIIa-substituted groups at the end of trauma may also have favoured the reduction in blood loss. However, the platelet count after haemodilution was comparable between the groups and the observed difference after trauma was most likely caused by varying amounts of blood loss. As the study drugs were either substituted before the infliction of trauma or directly after trauma, the impact of the platelet count on blood loss in relation to the study drug can probably be ignored.

Several animal studies on haemorrhage in pig models have been performed to measure the impact of rFVIIa under varying pathophysiological conditions.\textsuperscript{16, 28–34} The findings of these studies showed contradictory results of rFVIIa therapy, which might be due to variability in severity of injury, subsequent interventions (e.g. abdominal packing), varying haemodilution solutions, and resuscitation life-saving protocols. A recent published retrospective analysis from a database of combat casualty patients with severe trauma (injury severity score >25) and massive transfusion (RBC > 10 units 24 h\textsuperscript{-1}) showed that the early use of rFVIIa was associated with both decreased mortality at both 24 h and 30 days.\textsuperscript{35} Also, in two placebo-controlled randomized trials, a significant reduction in the primary endpoints, RBC units transfused, and need for massive transfusion (defined as >20 units of RBCs) was observed in patients with blunt trauma who survived for more than 48 h and the need for massive transfusion was reduced by nearly 20%.\textsuperscript{36}

However, clinically fresh frozen plasma (FFP) is normally used as first-line therapy to reverse dilutional coagulopathy after major trauma associated with massive bleeding or if a prolongation of PT, respectively, activated thromboplastin time of more than 1.5 times is accompanied by signs of microvascular bleeding.\textsuperscript{37} Drawbacks of FFP therapy include immunological reactions such as transfusion-related lung injury, and anaphylaxis and haemolysis in cases of ABO incompatibility. In contrast, the use of single factors allows a fast reversal of coagulopathy and requires only small volume, and products are either virus inactivated (e.g. fibrinogen) or recombinant. However, to date, no clinical relevant studies have investigated the effects of FFP vs single-factor therapy, and this needs to be evaluated in future studies.

There is some concern that the use of rFVIIa in patients without an underlying coagulation disorder may increase the risk for thromboembolic adverse events, resulting in serious morbidity and mortality.\textsuperscript{38} The risk of thromboembolic events may be aggravated by the concomitant application of other haemostatic agents.\textsuperscript{39} In the present study, no thrombi were observed in any of the rFVIIa-, fibrinogen-, or rFVIIa-fibrinogen-treated animals. This finding is in accordance with other animal studies, where no enhanced risk for fibrinogen or rFVIIa treatment-associated thromboembolic events was founded.\textsuperscript{16, 28–34}

There are some limitations of our study that need to be acknowledged. To standardize the degree of coagulopathy, haemodilution and fibrinogen application had to be induced before the induction of injury. Usually, under circumstances of haemodilution—as a result of blood loss (e.g. by major trauma) followed by the infusion of large volumes—haemostatic agents are given as coagulopathy occurs. In addition, the induction of injury was performed in anaesthetized healthy pigs. Thus, the physiological response to things such as pain and inflammation may have additional effects on haemostasis, which are not reflected in our model. Regarding new concepts of damage control resuscitation protocols that aim to early plasma substitute with RBC transfusion in a ratio of 1:1, it is unlikely that low plasma concentrations of fibrinogen of 50 mg dl\textsuperscript{-1} will occur.\textsuperscript{40} However, this study was designed to mimic a realistic clinical scenario rather than to investigate the effectiveness of rFVIIa under hypofibrinogenemia.

In conclusion, isolated therapy with rFVIIa during hypofibrinogenemia was an effective treatment to reduce liver bleeding in coagulopathic pigs. Although the substitution with fibrinogen reversed de facto dilutional-induced coagulopathy, the efficacy of the sole administration of this haemostatic agent might be limited by low concentrations of other coagulation factors in cases of highly impaired haemostasis. Thus, the treatment with rFVIIa after supplementing fibrinogen showed the most convincing positive effects on coagulation and reduction of blood loss. These findings are in alignment with current proposed recommendations of a European expert panel and the Israeli Multidisciplinary rFVIIa Task Force, which advice to optimize several precondition factors including sufficient levels of fibrinogen and platelets to enhance rFVIIa efficacy.\textsuperscript{41, 42} As the use of rFVIIa in traumatized patients remains an off-label indication, the administration of this substance should be considered as a last rescue therapy after carefully weighing up the benefit vs potential harm.
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Conflict of interest

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