

Effect of haemodilution, acidosis, and hypothermia on the activity of recombinant factor VIIa (NovoSeven®)

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Background. A range of plasma volume expanders is used clinically, often in settings where haemostasis may already be impaired. The haemostatic agent, recombinant activated factor VII (rFVIIa, NovoSeven®), may be used to improve haemostasis but potential interactions with different volume expanders are poorly understood.

Methods. Clot formation was measured by thromboelastography (TEG) using blood from healthy volunteers. *In vitro* effects of rFVIIa with haemodilution, acidosis, and hypothermia were examined. Conditions were induced by dilution with NaCl (0.9%), lactated Ringer's solution, albumin 5%, or hydroxyethyl starch (HES) solutions [MW (molecular weight) 130–670 kDa]; by adjusting pH to 6.8 with 1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) buffer; or by reducing temperature to 32°C. We also studied the effect of low vs high MW HES (MW 200 vs 600 kDa) and rFVIIa on *in vivo* bleeding time (BT) in rabbits.

Results. Haemodilution progressively altered TEG parameters. rFVIIa improved TEG parameters in the presence of acidosis, hypothermia or 20% haemodilution ($P < 0.05$). At 40% haemodilution, the rFVIIa effect was diminished particularly with high MW HES. *In vivo*, rFVIIa shortened the BT ($P < 0.05$) with low but not high MW HES.

Conclusions. Efficacy of rFVIIa was affected by the degree of haemodilution and type of volume expander, but not by acidosis or hypothermia.

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Administration of intravascular fluids to expand plasma volume is a key component in the treatment of hypovolaemic shock. Practices for selecting plasma volume expanders vary across clinical situations and countries.¹ Important factors include the volume expanding effect, risk of pulmonary oedema, cost, and potential impact on haemostasis.¹ In surgical critical care, where confounding factors such as hypothermia and acidosis may be present, the potential impact of volume expanders on haemostasis is particularly important.²

Common plasma volume expanders include normal saline (NS), lactated Ringer's solution (LR), hydroxyethyl starches (HES), albumin, and gelatins.^{1, 2} Alterations in coagulation function have been documented for each of these, with both the level of haemodilution and the nature of the colloid molecule playing a role. Crystalloids (NS and LR) have been shown *in vitro* and *in vivo* to induce a

hypercoagulable state.^{3, 4} While the effects on coagulation for natural colloids such as albumin and gelatin appear relatively minor, the impact of synthetic colloids such as dextrans and hetastarches can be much more significant.² For hetastarches, higher molecular weight (MW), molar substitution, and C2:C6 ratios have been related to impaired haemostatic parameters.^{5–8}

While maintenance of circulatory volume during surgical procedures is important, haemostasis is the primary task. Coagulopathy can result in continued blood loss from diffuse capillary bleeding, even after surgical repair of vascular structures. Under these conditions, haemostatic agents such

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as recombinant activated factor VII (rFVIIa, NovoSeven[®], Novo Nordisk A/S, Bagsvaerd, Denmark) may be useful. rFVIIa acts at the site of injury to enhance thrombin generation, leading to a stable fibrin plug.⁹ This drug is established for the treatment of bleeding episodes and for the prevention of bleeding during surgery or invasive procedures in patients with congenital haemophilia A and B with inhibitors to coagulation factors VIII (FVIII) or IX (FIX) or an expected high anamnestic response to FVIII or FIX. Additional indications include acquired haemophilia, congenital FVII deficiency, and Glanzmann's thrombasthenia refractory to platelet transfusion.¹⁰ Based on several case reports and randomized clinical trials, the efficacy of rFVIIa appears to extend to a number of applications outside haemophilia, including trauma¹¹ and others,¹⁰ although these are not yet approved by regulatory agencies.

There appears to be a population of critically ill trauma patients who do not respond to rFVIIa because of factors including haemodilution, acidosis, and hypothermia.^{12–13} Some experimental studies have examined the effect of rFVIIa in the presence of such factors as haemodilution, acidosis, and hypothermia, either individually or in selected combinations.^{14–16} However, no studies have systematically addressed the potential impact of various plasma volume expanders on the efficacy of rFVIIa, or the potential interactions of plasma volume expanders with factors such as hypothermia or acidosis. In this paper, we first present *in vitro* experiments examining the effects of volume expander type, degree of haemodilution, hypothermia, acidosis, and rFVIIa on blood clotting. We then report results of an *in vivo* study that specifically addressed our main *in vitro* finding, the differential effect of low and high MW hetastarches on the apparent efficacy of rFVIIa.

Methods

In vitro coagulation studies

Human WB collection

Blood was obtained by antecubital venepuncture using a 21-gauge needle from 42 healthy volunteers. Informed consent was obtained in accordance with the local human use review committee (Rigshospitalet, Copenhagen, Denmark). Blood was collected into tubes containing sodium citrate (0.13 M) at 1:9 v/v citrate to blood, and the first tube of blood was discarded. After 30 min at room temperature, blood samples were used for *in vitro* experiments.

Haemodilution experiments

A series of seven *in vitro* haemodilution experiments were performed. For each experiment a 3×4 factorial design was used with each of the six subjects represented within each factorial combination. Final concentrations of 25 or 200 nM rFVIIa were used to simulate *in vivo* doses of 90 or 720 µg kg⁻¹, respectively.

Before use, volume expanders were mixed with 0.13 M sodium citrate (10% v/v) to maintain anticoagulation of haemodiluted whole blood (WB). WB was diluted by 20, 40, and 60% (v/v) with one of the following solutions: NaCl (0.9%), LR (Fresenius Kabi, Uppsala, Sweden), human serum albumin (HSA; 5% solution; Sigma-Aldrich, Steinheim, Germany), and the HES Voluven[®] (HES130; average MW 130 kDa/molar substitution ratio 0.4; Fresenius Kabi, Bad Homburg, Germany), Haes-steril[®] (HES200; 10% solution; average MW 200 kDa/molar substitution ratio 0.5; Fresenius Kabi, Bad Homburg, Germany, diluted to 6% with isotonic NaCl before use); Hespan[®] (HES600; 6% hetastarch; average MW 600 kDa/molar substitution ratio 0.7; Braun Medical Inc., Irvine, CA, USA); and Hextend[®] (HES670; 6% hetastarch; average MW 670 kDa/molar substitution ratio 0.75; in Lactated Electrolyte Injection; BioTime Inc., Berkeley, CA, USA). Colloids were in NaCl 0.9% unless otherwise noted.

Effects of acidosis and hypothermia

Blood was manipulated *in vitro* to induce the following three conditions: (1) temperature 37°C, pH 7.4; (2) temperature 32°C, pH 7.4; and (3) temperature 37°C, pH 6.8. Either 0 or 25 nM rFVIIa was added to each condition. Acidosis was simulated by adjusting WB pH using 140 µl of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 1 M buffer to 2 ml WB. Hypothermia was simulated by lowering the temperature of WB to 32°C on the thromboelastograph (TEG).

Combined acidosis, hypothermia, and haemodilution

Blood was haemodiluted 20% using LR, NaCl, albumin, HES200, HES600, or HES670, and pH and temperature were adjusted as mentioned above. Undiluted WB at pH 7.4 and 37°C served as a control. Under each of these conditions, either 0 or 25 nM rFVIIa was added.

TEG WB coagulation analysis

Coagulation was initiated by adding tissue factor 1:50 000 v/v (Innovin[®], Dade Behring, Deerfield, IL, USA) to WB and recalcifying with 15 mM calcium chloride. Blood, tissue factor, and rFVIIa or buffer (HEPES 20 mM, 150 mM NaCl, and BSA 2%) were combined and inverted five times before adding to a TEG cup containing CaCl₂. Clotting time (*R*), velocity of clot formation (alpha angle), and maximum strength of the clot (maximum amplitude, MA) were recorded using a 5000 series TEG analyser (Haemoscope Corporation, Niles, IL, USA).

In vivo bleeding study

Animals

Twenty-four female New Zealand white rabbits (Charles River, Germany) weighing 2.0–2.9 kg were housed in colonies of 8–10 with free access to food and water. The study was approved by the Danish Animal Experiments Inspectorate. After 7 days acclimation, rabbits were

pre-anaesthetized with Diazepam 0.4 mg kg⁻¹ (Stesolid, Alpharma, Oslo, Norway) i.v. Thereafter, pentobarbital sodium (5%) was administered (i.v.) to effect and supplement as needed. Catheters were placed in the right carotid artery and jugular vein, and left femoral artery. The carotid catheter was connected to a pressure transducer (Gould P23 XL transducer and BD 9 recorder, Erik Blichfeldt, Kolding, Denmark). Core temperature (rectal) was maintained at 38°C using a heating blanket.

Baseline bleeding measurements

Each anaesthetized rabbit was positioned on a specially designed table, and a forepaw was placed in a beaker containing 500 ml water (37°C). After 10 min, bleeding was initiated by cutting 2 mm of the nail of the third digit with a spring-loaded sliding blade guillotine nail clipper, to induce combined arterial and venous bleeding. The paw was placed in water at 37°C and bleeding time (BT) was recorded. The total BT was defined as the sum of the duration of all the bleeding episodes (primary and re-bleeding) during the 15 min observation period. If baseline BT exceeded 10 min the rabbit was excluded from the experiment and replaced.

Haemodilution and treatment with rFVIIa

After baseline BT measurements, rabbits were randomly allocated to treatment according to Table 1.

After 10 min stabilization, 20% of the blood volume (estimated as 7% of body weight) was withdrawn via the femoral catheter >5 min. The withdrawn volume was replaced with (37°C) HES200 or HES600 infused through the jugular vein over the next 5 min, followed by a resting period of 5 min. This was repeated twice. Assuming full mixing of blood and haemodiluent at each step, the final haemodilution was 20%+20%×0.8+20%×0.8×0.8=48.8% of total blood volume.

After the final haemodilution step, cuticle bleeding was induced on the paw opposite the baseline paw. After 5 min, animals received either 5 mg kg⁻¹ rFVIIa or vehicle (10 mM Glycyl-glycine buffer; 150 mM NaCl, 10 mM CaCl₂, pH 7.5) via the jugular catheter. BT was recorded. Animals were killed after 60 min.

Statistical analysis

Each *in vitro* experiment was analysed by a three-way mixed analysis of variance model with donor, rFVIIa dose, and experimental condition as factors. The main effects of the three factors and the interaction between experimental condition and rFVIIa dose were considered

Table 1 Random allocation of rabbits to treatment

Group	Number	Haemodilution	Treatment
A	6	60% HES600	Vehicle
B	6	60% HES600	rFVIIa – 5 mg kg ⁻¹
C	6	60% HES200	Vehicle
D	6	60% HES200	rFVIIa – 5 mg kg ⁻¹

fixed; the interactions between donor and the other two factors were considered random. Comparisons were made with normal baseline and with the 0 nM FVIIa dose level within each experimental condition. Data are expressed as a mean percentage of normal baseline and 95% confidence intervals. Means (with 5–95 percentiles) for the normal baselines are also included as general reference points. Data from the *in vivo* study were analysed by Kruskal–Wallis test, followed by Dunns multiple comparison test. Three animals (two from the HES600/rFVIIa group and one from the HES600/vehicle group) died during the experiment, and were excluded from analysis.

Results

In vitro studies

Effect of haemodilution

The *R*-value was significantly reduced by all volume expanders at the three tested dilutions compared with normal undiluted WB except for HAES 130 at 20% haemodilution (Table 2). Addition of rFVIIa 25 or 200 nM further reduced *R* within each fluid and dilution level.

Alpha angle increased when blood was diluted up to 40% with crystalloid or albumin solutions, and remained elevated at 60% dilution with LR. When hetastarch solutions were

Table 2 Effect of *in vitro* haemodilution of WB on clotting time (*R*). Note: plasma volume expanders (fluid) are listed in descending order by MW from lowest to highest. *Per cent change=(*R* at target conditions/*R* at normal baseline)×100; CI, confidence interval. †Statistically different from 0 dose of rFVIIa without haemodilution (*P*<0.05). ‡Statistically different from 0 dose of rFVIIa within haemodilution level (*P*<0.05)

Fluid	rFVIIa dose	Level of haemodilution [per cent change* (lower–upper CI)]		
		20%	40%	60%
NaCl	0	77 (69–86) [†]	68 (61–76) [†]	67 (60–74) [†]
	25	45 (40–50) ^{‡‡}	43 (39–48) ^{‡‡}	44 (40–49) ^{‡‡}
	200	42 (38–47) ^{‡‡}	38 (34–42) ^{‡‡}	46 (41–52) ^{‡‡}
LR	0	80 (68–93) [†]	66 (56–77) [†]	60 (51–70) [†]
	25	42 (35–49) ^{‡‡}	40 (34–48) ^{‡‡}	43 (37–51) ^{‡‡}
	200	38 (32–45) ^{‡‡}	38 (32–45) ^{‡‡}	41 (34–48) ^{‡‡}
Albumin	0	86 (81–92) [†]	80 (75–85) [†]	80 (75–85) [†]
	25	49 (45–53) ^{‡‡}	47(43–51) ^{‡‡}	53 (48–58) ^{‡‡}
	200	43 (40–47) ^{‡‡}	46 (42–50) ^{‡‡}	54 (50–59) ^{‡‡}
HES130	0	123 (111–136) [†]	94 (84–104) [†]	85 (77–95) [†]
	25	54 (49–61) ^{‡‡}	60 (54–67) ^{‡‡}	63 (57–70) ^{‡‡}
	200	54 (48–60) ^{‡‡}	52 (47–58) ^{‡‡}	61 (55–68) ^{‡‡}
HES200	0	89 (81–97) [†]	70 (64–76) [†]	71 (65–77) [†]
	25	45 (41–50) ^{‡‡}	48 (44–54) ^{‡‡}	53 (48–59) ^{‡‡}
	200	44 (40–49) ^{‡‡}	43 (39–48) ^{‡‡}	50 (45–56) ^{‡‡}
HES600	0	89 (84–94) [†]	90 (85–95) [†]	92 (87–97) [†]
	25	51 (48–54) ^{‡‡}	52 (49–55) ^{‡‡}	58 (55–61) ^{‡‡}
	200	45 (42–47) ^{‡‡}	49 (46–52) ^{‡‡}	58 (55–61) ^{‡‡}
HES670	0	94 (82–108)	79 (69–91) [†]	78 (67–89) [†]
	25	56 (45–69) ^{‡‡}	62 (50–76) ^{‡‡}	70 (57–87) ^{‡‡}
	200	53 (43–66) ^{‡‡}	53 (43–66) ^{‡‡}	61 (49–75) ^{‡‡}

Table 3 Effect of *in vitro* haemodilution of WB on the velocity of clot formation (alpha angle). Note: plasma volume expanders (fluid) are listed in descending order by MW from lowest to highest. *Per cent change=(angle at target conditions/angle at normal baseline)×100; CI, confidence interval. †Statistically different from 0 dose of rFVIIa without haemodilution ($P<0.05$). ‡Statistically different from 0 dose of rFVIIa within haemodilution level ($P<0.05$)

Fluid	rFVIIa Dose	Level of haemodilution [per cent change* (lower–upper CI)]		
		20%	40%	60%
NaCl	0	116 (108–125) [†]	118 (110–127) [†]	107 (99–115)
	25	126 (117–136) [†]	122 (114–132) [†]	103 (96–111)
	200	130 (121–140) ^{†‡}	130 (121–140) [†]	105 (97–113)
LR	0	112 (102–124) [†]	126 (113–139) [†]	123 (111–136) [†]
	25	130 (117–145) ^{†‡}	129 (116–144) [†]	106 (95–118) [‡]
	200	142 (127–158) ^{†‡}	140 (125–156) ^{†‡}	120 (108–134) [†]
Albumin	0	111 (104–118) [†]	112 (105–119) [†]	101 (95–107)
	25	134 (124–146) ^{†‡}	130 (119–140) ^{†‡}	100 (92–109)
	200	136 (126–148) ^{†‡}	126 (116–137) ^{†‡}	99 (91–107)
HES130	0	68 (60–76) [†]	74 (66–83) [†]	62 (55–70) [†]
	25	94 (84–106) [‡]	73 (65–82) [†]	60 (53–67) [†]
	200	107 (95–120) [‡]	92 (82–104) [‡]	60 (54–68) [†]
HES200	0	94 (84–105)	92 (82–102)	73 (65–82) [†]
	25	108 (97–121) [‡]	88 (78–99) [†]	62 (56–69) ^{†‡}
	200	115 (103–129) ^{†‡}	102 (92–114) [‡]	65 (58–72) ^{†‡}
HES600	0	94 (85–104)	85 (77–94) [†]	65 (59–71) [†]
	25	106 (96–118) [‡]	90 (81–99) [†]	65 (58–72) [†]
	200	107 (96–119) [‡]	84 (75–93) [†]	63 (57–70) [†]
HES670	0	82 (72–93) [†]	88 (77–100) [†]	61 (53–69) [†]
	25	100 (87–116) [‡]	84 (73–98) [†]	53 (46–61) [†]
	200	106 (92–122) [‡]	88 (76–101)	63 (54–72) [†]

used, the alpha angle was unchanged or decreased at each level of dilution (Table 3). At 20% haemodilution, rFVIIa 25 or 200 nM increased the alpha angle for all fluids, with the alpha angle approaching or exceeding 100% of baseline in each case. At 40% haemodilution, the alpha angle after addition of rFVIIa for albumin, LR, and NaCl followed patterns similar to the respective patterns at the 20% haemodilution. For HES130 and HES200, increases were observed only after addition of 200 nM rFVIIa at 40% haemodilution. Alpha angle was not increased by any dose of rFVIIa when blood was haemodiluted 40% with either HES600 or HES670. At 60% haemodilution the effect of rFVIIa on the alpha angle was abolished.

At 20% haemodilution, MA decreased when albumin or hetastarches were used, but not with LR or NaCl (Table 4). At higher levels of haemodilution, MA decreased for all fluids, with numerically greater decrements for hetastarches. The MA was increased by both rFVIIa concentrations at 20 and 40% haemodilution with albumin and by rFVIIa 200 nM with LR, NaCl, HES130, and HES670. At 60% haemodilution, rFVIIa did not affect MA.

Pooled ($n=42$ subjects) baseline TEG parameters (mean and 5–95 percentiles) were 562 s (340–730) for R , 52.0° (37.6–63.0) for alpha angle, and 61.1 mm (49.6–69.4) for MA.

Table 4 Effect of *in vitro* haemodilution of WB on clot MA. Note: plasma volume expanders (fluid) are listed in descending order by MW from lowest to highest. *Per cent change=(MA at target conditions/MA at normal baseline)×100; CI, confidence interval. †Statistically different from 0 dose of rFVIIa without haemodilution ($P<0.05$). ‡Statistically different from 0 dose of rFVIIa within haemodilution level ($P<0.05$)

Fluid	rFVIIa dose	Level of haemodilution [per cent change* (lower–upper CI)]		
		20%	40%	60%
NaCl	0	100 (94–107)	89 (83–95) [†]	74 (69–80) [†]
	25	100 (94–107)	92 (86–98) [†]	75 (70–81) [†]
	200	104 (97–111)	95 (89–102) [‡]	70 (65–75) [†]
LR	0	97 (93–101)	90 (87–94) [†]	77 (74–80) [†]
	25	100 (96–105)	93 (89–97) [†]	74 (71–77) [†]
	200	103 (98–107) [‡]	95 (91–99) ^{†‡}	77 (74–80) [†]
Albumin	0	93 (88–97) [†]	88 (83–92) [†]	70 (67–74) [†]
	25	105 (100–111) ^{†‡}	94 (90–99) ^{†‡}	69 (66–73) [†]
	200	104 (99–109) [‡]	92 (88–97) ^{†‡}	68 (65–72) [†]
HES130	0	80 (74–86) [†]	61 (57–66) [†]	51 (47–55) [†]
	25	83 (77–90) [†]	63 (58–68) [†]	45 (42–49) ^{†‡}
	200	84 (78–91) [†]	67 (62–73) ^{†‡}	48 (44–52) [†]
HES200	0	88 (80–96) [†]	70 (64–77) [†]	56 (51–61) [†]
	25	86 (79–94) [†]	68 (62–74) [†]	49 (45–54) ^{†‡}
	200	88 (81–97) [†]	74 (67–80) [†]	49 (45–54) ^{†‡}
HES600	0	82 (76–88) [†]	72 (67–77) [†]	54 (50–58) [†]
	25	87 (80–94) [†]	72 (67–78) [†]	55 (51–60) [†]
	200	87 (80–94) [†]	70 (65–76) [†]	54 (50–59) [†]
HES670	0	77 (72–81) [†]	67 (61–73) [†]	53 (50–56) [†]
	25	81 (75–86) [†]	65 (60–69) [†]	48 (44–51) ^{†‡}
	200	83 (78–89) ^{†‡}	69 (64–73) [†]	51 (48–55) [†]

Effects of hypothermia and acidosis

Clotting time (R) was not affected by acidosis, whereas alpha angle and MA were significantly decreased in acidic blood (Table 5). Addition of rFVIIa decreased the R -time, and increased the alpha angle and MA. There was no statistical interaction ($P>0.20$) between acidosis and rFVIIa, indicating that the effect of rFVIIa on these TEG parameters was similar at normal or acidic conditions.

Hypothermia did not statistically alter any TEG parameter (Table 5). Addition of rFVIIa 25 nM significantly decreased R -time and increased alpha angle and MA. There was no statistical interaction ($P>0.20$) between temperature and rFVIIa, indicating that the effects of rFVIIa on these TEG parameters was similar at normal and hypothermic conditions.

Effects of combined acidosis, hypothermia, and haemodilution

The R -value was significantly prolonged for all fluids under combined acidic and hypothermic conditions, while the alpha angle and MA were either numerically or statistically reduced (Table 6). Addition of rFVIIa significantly shortened R compared with baseline with each volume expander. Alpha angle was increased from baseline by the addition of rFVIIa with NaCl, LR, albumin, and HES670,

Table 5 Effect of *in vitro* addition of rFVIIa on TEG parameters in WB from healthy volunteers under acidosis and hypothermia. Data shown as per cent change from baseline. *Significantly different compared with normal WB for R, alpha angle, and MA ($P<0.01$). †Significantly different compared with normal WB for R, alpha angle, and MA ($P<0.05$). ‡Significantly different compared with 0 nM rFVIIa for R, alpha angle, and MA ($P<0.05$)

	Clotting time (R) (mean and CI, n=6)		Alpha angle (mean and CI, n=6)		Maximum amplitude (MA) (mean and CI, n=6)	
	rFVIIa, 0 nM	rFVIIa, 25 nM	rFVIIa, 0 nM	rFVIIa, 25 nM	rFVIIa, 0 nM	rFVIIa, 25 nM
Normal whole blood (WB) (n=6)	100	65 (58–72) ^{**‡}	100	109 (98–123)	100	105 (102–108) ^{**‡}
Acidosis (pH 6.8)	107 (84–136)	69 (54–88) ^{**‡}	82 (72–93) [*]	109 (96–125) [‡]	96 (93–99) [†]	105 (101–109) ^{**‡}
Hypothermia (32°C)	119 (92–153)	66 (51–85) ^{**‡}	94 (82–109)	111 (98–128) [‡]	98 (94–101)	104 (100–108) ^{**‡}

Table 6 Effect of *in vitro* addition of rFVIIa with combined acidosis, hypothermia, and 20% haemodilution on TEG parameters in WB from healthy volunteers. Note: plasma volume expanders (fluid) are listed in descending order by MW from lowest to highest; all final dilutions 20%. *Per cent change=(parameter at target conditions/parameter at normal baseline)×100; CI, confidence interval. †Statistically different from normal baseline ($P<0.05$). ‡Statistically different from normal baseline ($P<0.01$). §Statistically different from 0 dose of rFVIIa within fluid ($P<0.05$). ¶Statistically different from 0 dose of rFVIIa within fluid ($P<0.01$)

Haemodilution agents	Clotting time (R) [per cent change* (mean and CI, n=6)]		Alpha angle [per cent change* (mean and CI, n=6)]		Maximum amplitude [per cent change* (mean and CI, n=6)]	
	rFVIIa, 0 nM	rFVIIa, 25 nM	rFVIIa, 0 nM	rFVIIa, 25 nM	rFVIIa, 0 nM	rFVIIa, 25 nM
NaCl	119 (109–130) [‡]	77 (70–84) ^{§§}	82 (71–94) [‡]	101 (88–116) [§]	80 (75–85) [‡]	87 (82–93) ^{§§}
LR	122 (111–133) [‡]	74 (68–82) ^{§§}	94 (81–108)	107 (93–123)	88 (82–93) [‡]	91 (86–97) [‡]
Albumin	126 (115–138) [‡]	77 (70–84) ^{§§}	64 (56–74) [‡]	90 (79–104) [§]	67 (63–71) [‡]	80 (75–85) ^{§§}
HES130	136 (125–149) [‡]	90 (82–99) ^{†§}	68 (59–78) [‡]	68 (59–78) [‡]	66 (62–70) [‡]	64 (60–68) [‡]
HES600	133 (122–146) [‡]	85 (78–93) ^{§§}	61 (53–70) [‡]	67 (58–77) [‡]	67 (63–71) [‡]	63 (59–67) ^{‡¶}
HES670	129 (117–141) [‡]	80 (74–88) ^{§§}	67 (59–77) [‡]	84 (73–97) ^{†§}	71 (67–76) [‡]	73 (68–78) [‡]

while no significant changes in the alpha angle were observed with HES600 or HES130. MA was improved by the addition of rFVIIa with NaCl and albumin but not with HES600, HES 130, or HES670.

In vivo study

Haemodilution with HES200 or HES600 significantly prolonged primary BT, as 10 out of 11 animals bled continuously for 1 h. The primary BT was significantly reduced after treatment with rFVIIa in animals haemodiluted with HES200 but not HES600 (Fig. 1). Similar patterns were observed for total BT.

Discussion

Data presented here confirm previous findings that although all plasma volume expanders tested shortened initial coagulation time, clot functional characteristics varied greatly. As expected, increased haemodilution progressively compromised clot mechanical characteristics, reflecting at least in part, an impact of reduced fibrinogen and platelet concentrations. However, at a given level of haemodilution, hetastarches, particularly high MW hetastarches, produced the greatest impairment compared with albumin and crystalloid solutions. Additionally, blood diluted with hetastarch solutions appeared less responsive to rFVIIa, especially with high MW hetastarches.

To enable us to examine a range of volume expanders and conditions, we focused on a single method, TEG, to

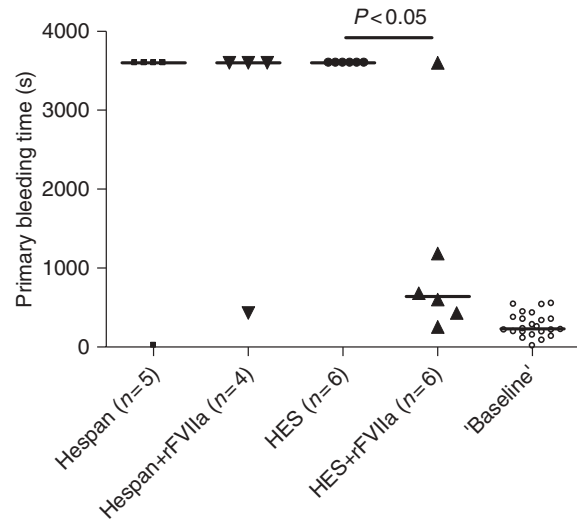


Fig 1 Primary bleeding time (BT) in rabbits haemodiluted with 60% HES600 (MW=600 000) or HES200 (MW=200 000) treated with vehicle or with 5 mg kg⁻¹ rFVIIa 5 min after induction of bleeding. Observation period was 3600 s. Baseline represents BT in the same rabbits from the contralateral paw before haemodilution with an observation period of 900 s. Lines within the graph represent medians.

assess blood clotting *in vitro*. Additionally, we performed an *in vivo* experiment to further investigate a single key finding. We did not specifically address thrombin generation, fibrin assembly, or platelet function. These limitations must temper our interpretation. Because experimental modification of normal blood or animals cannot

fully reflect the pathophysiological mechanisms present in patients, caution should be exercised in extrapolating the present findings to the patient setting. These limitations notwithstanding, we believe that we can draw some conclusions regarding potential mechanisms.

Hetastarches exert a number of effects on the haemostatic systems that are distinct from a simple dilutional effect, including reductions in FVIII and vWF levels,^{6 8 17} impaired fibrin polymerization and cross-linking,^{18–20} and reduced platelet function.⁵ These have been related both to MW and to the molar substitution ratio of the hetastarch, with effects generally more pronounced with higher MW and higher molar substitution ratios.^{5–8} As the lower MW hetastarches used in the present study also had the lowest molar substitutions [HES 130 (130/0.4), HES 200 (200/0.5), HES600 (600/0.7), and HES670 (670/0.75)], it is not possible to differentiate between the potential effects of MW or molar substitution ratio.

In a recent study that examined plasma clotting kinetics after infusion of 20 ml kg⁻¹ high MW hetastarch solution in rabbits (28% estimated blood volume), alpha angle and shear elastic modulus (a measure of clot strength) were reduced by 29 and 73%, respectively.²⁰ These parameters were partially restored by *in vitro* addition of either factor XIII or fibrinogen. Addition of thrombin shortened the *R* time but had no effect on clot development or strength. It was concluded that hetastarch decreased clot propagation and strength primarily because of decreased FXIIIa-fibrin polymer cross-linking. These results suggest that the decrement in clot development and strength cannot be overcome by the actions of thrombin alone, although rate of clot initiation can be enhanced. Recombinant FVIIa functions by enhancing thrombin production at the site of injury.⁹ The fact that *R* was shortened by rFVIIa at all levels of haemodilution in the present study suggests that thrombin production was enhanced by the addition of rFVIIa. Therefore, it does not appear that hetastarch impaired the ability of rFVIIa to enhance thrombin generation. Hetastarch-induced fibrin structural defects may be resistant to correction by thrombin, which may explain why rFVIIa did not consistently improve alpha angle or MA when hetastarch was used in the present study.

Reducing the pH from 7.4 to 7.0 reduces rFVIIa enzymatic activity 60–90%.¹⁶ Therefore, the reduced clinical efficacy of rFVIIa observed in acidosis,^{12 21–23} may be related to lower enzymatic rates. However, it is not clear at what point reduced enzymatic rate might impact clinical efficacy because rFVIIa administration increases circulating FVIIa levels approximately 100-fold. Our findings suggest that rFVIIa may offset pH-related changes in enzymatic rates. This may, in part, explain clinical reports documenting some individual patients who did and others who did not respond to rFVIIa at a pH <7.2.^{12 21 22} Further studies are required to determine the relative roles of blood pH and other acidosis-related factors in determining the efficacy of rFVIIa in acidotic patients.

Our inability to detect a statistical impairment of TEG parameters under hypothermic conditions contrasts with previous findings.^{24 25} Hypothermia reduces coagulation enzymatic rates¹⁶ and platelet enzymatic and secretory rates.²⁶ In a recent report, rFVIIa improved TEG parameters at temperatures of 28–31°C.²⁵ Our data provide further evidence for this effect. The ability of rFVIIa to enhance haemostatic parameters in hypothermia is consistent with its retention of enzymatic activity between 37 and 32°C *in vitro*¹⁶ and with clinical reports of efficacy in hypothermic patients.^{13 21}

When the conditions of hypothermia, acidosis, and 20% haemodilution were combined, *R* was consistently prolonged above normal baseline. This was similar to the pattern observed for undiluted hypothermic blood, but quite different from the effects of haemodilution alone. Alpha angle and MA were reduced below the baseline, a pattern that was also more similar to the responses to hypothermia and acidosis alone than to the responses to 20% haemodilution. These findings suggest that hypothermia and acidosis had the greater impact on TEG parameters than did the type of fluid.

A different pattern was noted when examining the responsiveness to 25 nM rFVIIa. The *R* was shortened by addition of rFVIIa, regardless of the plasma volume expander used, consistent with the results for acidosis, hypothermia, or haemodilution alone. However, the responses for the alpha angle and MA under combined conditions diverged from the rFVIIa responses for acidosis or hypothermia alone, and more closely followed the patterns observed in the isolated haemodilution experiments. When NaCl or albumin was used in the presence of combined acidosis and hypothermia, the alpha angle was normalized by rFVIIa. However, when hetastarches were used in the presence of acidosis and hypothermia, the alpha angle was improved in only one case (HES670) and the post-rFVIIa values remained below the normal baseline for all hetastarches. These data suggest that the response to rFVIIa was more dependent on the type of plasma expander used than on the effects of hypothermia and acidosis.

The potential differential effect of low vs high MW hetastarch on the activity of rFVIIa was further addressed *in vivo*. The consistent extension of BT after haemodilution with hetastarch solutions in normal rabbits in the present study is consistent with results reported for patients undergoing acute normovolemic haemodilution in preparation for surgery.²⁷ Administration of rFVIIa reduced BT in rabbits that were haemodiluted with low MW hetastarch (HES200) but not in rabbits administered with high MW hetastarch (HES600). *In vitro*, we observed that the alpha angle was improved in response to rFVIIa at 40% haemodilution with lower MW hetastarches, HES130, and HES200, but not with higher MW hetastarches, HES600 or HES670. Martinowitz *et al.*¹⁵ reported improved haemostasis in response to rFVIIa after severe liver injury in pigs that were made coagulopathic by induction of hypothermia and

haemodilution with a 200 000 MW hetastarch. However, in a study using a similar animal model, but with a 600 000 MW hetastarch, rFVIIa did not alter blood loss.¹⁴

Infusion of high MW hetastarch yields reductions in vWF and FVIII:c well beyond those expected by simple haemodilution.^{6 8 17} Considering the well-established efficacy of rFVIIa in haemophilia, it does not seem likely that rFVIIa efficacy would be limited by a hetastarch effect on FVIII:c levels. As mentioned earlier, fibrin polymerization is negatively impacted by hetastarches,^{18–20} and this could have affected the efficacy of rFVIIa. High MW has also been related to a greater impairment of platelet function.⁵ It appears that hetastarch molecules coat platelets non-specifically, altering the interaction of GPIIb/IIIa receptors with fibrinogen. The present *in vitro* finding that the alpha angle, a parameter related to platelet function, was more responsive to rFVIIa when low MW than high MW hetastarches were used, is consistent with such a platelet effect. BT procedures are believed to be more dependent on primary haemostasis than secondary haemostasis, suggesting that the effects observed in the present, *in vivo* study might have been related to platelet function.

Conclusion

We found progressive alterations in TEG parameters with increasing haemodilution and observed greater impairments with hetastarches than albumin or crystalloids. At 20% haemodilution, rFVIIa improved TEG parameters for all volume expanders. However, blood diluted with hetastarch solutions, particularly high MW hetastarches, appeared less responsive to rFVIIa when the *in vitro* dilution reached 40%. *In vivo*, rFVIIa improved haemostasis in rabbits after haemodilution with low MW but not high MW hetastarch. It does not appear that hetastarch prevented rFVIIa enhancement of thrombin generation, but instead that hetastarch may have induced changes in fibrin structure and platelet function that were resistant to correction by thrombin, and, therefore, resistant to rFVIIa at high levels of haemodilution. Recombinant FVIIa improved *in vitro* clot formation under conditions of acidosis and hyperthrombia.

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