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Citrate artificially masks the haemostatic effect of recombinant factor VIIa in dilutional coagulopathy

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Abstract Most often, thrombelastographic analyses are carried out using citrated blood and re-calcification. However, calcium chelation may affect dynamics of tissue-factor-initiated thrombin generation. The present study investigates the effect of sample anticoagulant on the response of a colloid induced dilutional coagulopathy model to recombinant activated factor VII (rFVIIa) as measured by thrombelastography. Thrombelastographic evaluation of whole blood coagulation activated with minute amounts of tissue factor in a model of in vitro haemodilution with hydroxyethyl starch (HES) 130/0.4 in a prospective laboratory study. Whole blood coagulation was evaluated before and after 30% dilution with HES 130/0.4, and following in vitro addition of rFVIIa to whole blood collected into tubes containing citrate, corn trypsin inhibitor (CTI), and no stabilizers. Haemodilution with HES 130/0.4 induces a coagulopathy characterised by a reduced maximum rate of clot formation and a pronounced reduction in the final clot firmness. With all test mediums investigated, rFVIIa significantly shortened clot initiation phase. In cases of native whole blood and CTI-stabilised whole blood, rFVIIa shortens the clotting time but also demonstrated an acceleration of the maximum velocity of clot formation. When citrate is used as anticoagulants in thrombelastographic clotting assays, these may artificially mask the haemostatic effect of rFVIIa in colloid haemodilution. The

effect in vitro of rFVIIa in citrated blood samples may underestimate the haemostatic potential of rFVIIa.

Keywords Haemodilution · Trisodium citrate · Corn trypsin inhibitor · Plasma substitutes · Recombinant activated factor VII · Thrombelastography

Introduction

During a number of years, haemostatic monitoring and intervention has become a major issue in initial assessment and management of trauma and serious bleeds. Terms such as acidosis, hypothermia, dilution, consumption, hyperfibrinolysis, multiple transfusions and dysfunctional fibrinogen resulting from resuscitation with colloid plasma expanders [1, 2] add to the complexity and reflect elements of the pathogenesis in the development of coagulopathy in trauma. The potential benefit of thrombelastography as a point-of-care tool in evaluation of dynamics of whole blood formation has been reported in a number of studies [3, 4]. Recombinant activated factor VII (rFVIIa) has been reported as a potent and potentially useful haemostatic agent in the control of excessive bleeding in some traumatised patients [5, 6]. Our laboratory has developed a thrombelastographic model of continuous whole blood coagulation, employing activation with minute amounts of tissue factor and novel data processing of the raw coagulation data signal to provide dynamic parameters of clot formation [7]. Recently, our haemostasis centre forwarded that rFVIIa may fail in correction with dilutional coagulopathy induced by hydroxyethyl starch (HES) 130/04 in vitro [8]. This observation has been supported by results from Engstrom et al. [9]. In addition, other investigations performed in a rabbit model have indicated that colloid haemodilution may attenuate the haemostatic potential of rFVIIa [10]. In contrast, clinical experiences and several case reports point towards a beneficial effect of rFVIIa in control of massive bleeding with haemodilution by excessive use of volume substitution [6, 11, 12].

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Most often, thrombelastographic analyses are carried out using citrated blood and re-calcification. Citrate acts as reversible anticoagulants by chelating calcium ions, thereby blocking calcium ion complex formation between gamma-carboxylated groups of coagulation factors and phospholipids.

Recent pioneering work has revealed that citrate-dependent calcium chelation significantly affects the dynamics of tissue-factor-initiated thrombin generation as well as whole blood thrombelastography [13]. The exact mechanisms are not known; however, citrate may interfere with the enzymatic properties of coagulation factors [13]. Moreover, it has been documented that citrate and calcium chelation interferes with the metabolic processes in platelets [14].

Recombinant factor VIIa has exerted its function via a tissue-factor-dependent [15] as well as a tissue-factor-independent–platelet-dependent mechanism of action [16]. The association of rFVIIa with the phospholipid surface of platelets is mediated by a calcium-dependent conformational change in the GLA domain [17]. It may be speculated whether laboratory evaluation of the haemostatic effect of rFVIIa might be flawed by a citrate-specific mechanism interacting with coagulation and interfering with platelet function. An alternative approach to stabilise blood samples and prevent clotting in a way which do not influence calcium homeostasis is blocking of contact activation using corn trypsin inhibitor (CTI) that specifically inhibits factor XII [18].

The aim of the present study was to investigate the haemostatic effect of rFVIIa in a laboratory whole blood model of colloid haemodilution using citrate-stabilised blood in comparison with native whole blood and whole blood stabilised with CTI. We hypothesised that the haemostatic effect of rFVIIa was significantly better in native and CTI-stabilised blood as compared to citrate-stabilised blood.

Materials and methods

Study subjects

The study was approved by the local Ethics Committee (approval number 2006-0015). Following informed consent, 11 healthy male volunteers with a mean age of 30 years (range 26–38 years) were enrolled in the study. Exclusion criteria were use of acetyl–salicylic acid or non-steroid anti-inflammatory drugs during a period of 7 days prior to blood sampling. To minimise the known inter-gender variation [7], the present study enrolled only male volunteers.

Test reagents

Recombinant activated factor VII (NovoSeven®) was from NovoNordisk (Bagsværd, Denmark) and HES 130/0.4

(Voluven® 6%) from Fresenius Kabi (Bad Homburg, Germany)

Blood sampling and processing

Blood samples were drawn from an antecubital vein using minimum stasis and a 21-gauge butterfly needle. The first tube aspirated was discarded. Samples were collected into: (1) Venoject® tubes (Terumo Europe, Leuven, Belgium, trisodium citrate 0.129 mol/L: 3.2% *W/V*), at a volume ratio of 1:10, (2) open plastic polystyrene tubes corn trypsin inhibitor (Haematologic Technologies Inc., Essex Junction, USA) at a final concentration of 100 µg/mL and (3) no anticoagulants (denoted native blood). Since all tubes utilised in the study were made of plastic; we assumed that spontaneous contact activation was limited and comparable

Thrombelastographic whole blood coagulation analysis

Dynamic whole blood coagulation profiles were recorded in parallel using thrombelastography (ROTEM® Thrombelastometry, Pentapharm Co., Munich, Germany) as previously described [7]. In brief, pre-warmed (37°C) ROTEM® plastic cups were loaded with 0 or 90 µL HES 130/0.4 to achieve immediate dilution of 0% or 30% regarding HES 130/0.4 followed by addition of 300 or 210 µL of whole blood, respectively. Subsequently, the reaction mixture was spiked with 20-µL buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 20 mM, NaCl 150 mM, pH 7.4) or 20 µL rFVIIa (final concentration=4 µg/mL, roughly corresponding an *in vivo* dose of 200 µg/kg). The coagulation process was activated with tissue factor (Innovin® Dade Behring, Marburg, Germany) at a final dilution of 1:50,000. Further, citrated blood was re-calcified with the addition of 0.2 mol/L CaCl₂. Hence, in all cases, the final volume in the ROTEM® cup was 340 µL. Native and CTI-stabilised blood were analysed immediately whereas citrate-stabilised blood rested approximately 30–45 min according to previous recommendations [7]. All analyses were processed in duplicate for a minimum of 45 min. Standard thrombelastographic parameters such as the clotting time (CT, [s]) and maximum clot firmness (MCF, [mm]) were recorded. The digitalised raw signal was further processed using a software programme (DyCoDerivAn, AvordusoL, Risskov, Denmark) to obtain the dynamic velocity parameter maximum velocity (MaxVel, [mm×100/s]) and time until maximum velocity (t, MaxVel [s]).

Ca²⁺ measurements

Approximately 1 mL of blood was collected into a heparin-containing blood-sampling device (Pico 50, Radiometer Medical A/S, Copenhagen, Denmark) for determination of the concentration of ionised calcium using a Radiometer ABL

Table 1 ROTEM® parameters in native whole blood and whole blood stabilised with citrate and CTI

	Native	Citrate	CTI
Clot initiation phase			
CT (s)	486±90	493±130	528±111
Clot propagation phase			
MaxVel (mm×100/s)	6.2±1.4	8.3±1.8	5.8±1.3
T, MaxVel (s)	660±116	607±171	811±169 ^a
Clot stabilisation			
MCF (mm)	54±4.5	54±3.8	54±3.6

N=11. Data presented as mean±SD.

CT Clotting time, MCF maximum clot firmness, MaxVel maximum velocity of clot formation

^aStatistically significant from citrate.

625 analyser (Radiometer Medical A/S, Copenhagen, Denmark). Values were adjusted to a physiological temperature of 37°C.

Statistics

Statistical analyses were performed using the statistical software Analyse-it (Analyse-it Software, Ltd. Leeds, UK). The minimum sample size was estimated as *n*=9 based on an expected effect of rFVIIa of 20% on the MaxVel in non-citrated whole blood compared to citrated whole blood, SD=0.15, alpha=0.05, power=0.8. A normal distribution was found based on the interpretation of Q-Q plots and histograms. Hence, intra-group comparison was performed using a parametric Student's *t* test. A *p* value less than 1% (*p*<0.01) was considered statistically significant.

Results

Whole blood clot initiation

Clotting time At baseline (A samples), a non-significant increased CT was observed in CTI-stabilised whole blood compared to native and citrated whole blood (Table 1). Haemodilution with HES 130/0.4 (B samples) significantly shortened CT in native whole blood but not in CTI and citrated whole blood. Addition of rFVIIa (C samples) improved the clot initiation in all test combinations as evidenced by a significant reduction in CT, although the effect found in citrated whole blood (Fig. 1) was less pronounced.

Whole blood clot propagation

Maximum velocity At baseline, the whole blood clot propagation, as evaluated by the MaxVel, was elevated in citrated

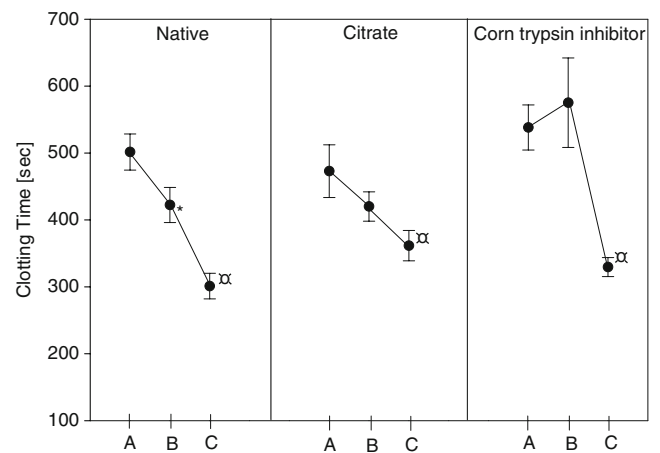


Fig. 1 Clotting time before (A), after 30% haemodilution with HES 130/0.4 (B) and following substitution with rFVIIa (C) in native or whole blood stabilised with citrate and corn trypsin inhibitor. Data presented as mean and standard error. *N*=11. **p*<0.01 compared to A. ^α*p*<0.01 compared to B

whole blood, although not statistically different from native and CTI-stabilised whole blood (Table 1). Following haemodilution with HES 130/0.4 (B samples), the MaxVel was significantly reduced in all mixtures. In vitro addition of rFVIIa (C samples) failed to improve the MaxVel of whole blood clot formation in citrated whole blood. In contrast, rFVIIa normalised the MaxVel to pre-dilutional levels in native whole blood and CTI-stabilised whole blood (Fig. 2).

Time until MaxVel Significant prolonged *t*, MaxVel was observed at baseline (A samples) in CTI-stabilised whole blood compared to native and citrated whole blood (Table 1).

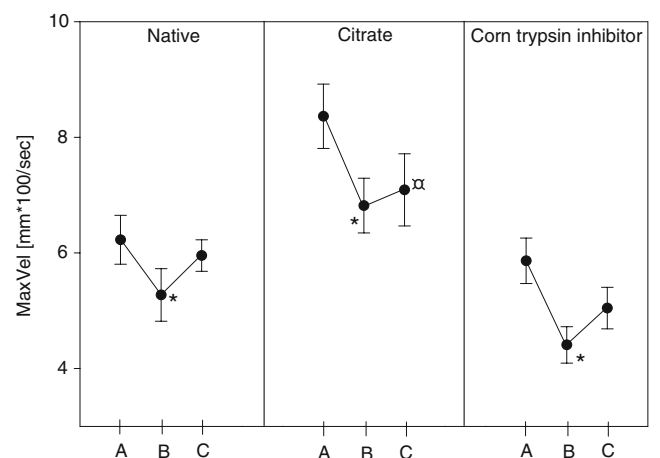


Fig. 2 Maximum velocity (MaxVel) before (A), after 30% haemodilution with HES 130/0.4 (B) and following substitution with rFVIIa (C) in native or whole blood stabilised with citrate and corn trypsin inhibitor. Data presented as mean and standard error. *N*=11. **p*<0.01 compared to A. ^α*p*<0.01 compared to B

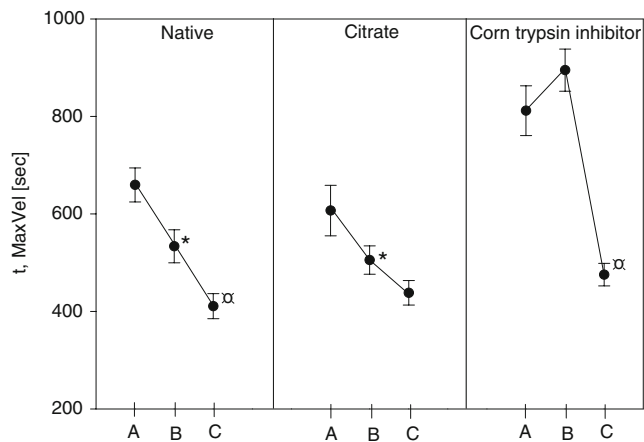


Fig. 3 Time until maximum velocity (t , MaxVel) before (A), after 30% haemodilution with HES 130/0.4 (B) and following substitution with rFVIIa (C) in native or whole blood stabilised with citrate and corn trypsin inhibitor. Data presented as mean and standard error. $N=11$. * $p<0.01$ compared to A. ^α $p<0.01$ compared to B

Haemodilution with HES 130/0.4 (B samples) significantly reduces t , MaxVel in native and citrated whole blood but not in CTI-stabilised blood. In vitro addition of rFVIIa (C samples) failed to improve t , MaxVel in citrated blood but returned significantly reduced values in native and CTI-stabilised blood (Fig. 3).

Whole blood clot stabilisation

Maximum clot firmness MCF was comparable within all groups (Table 1). Haemodilution with HES 130/0.4 (B samples) significantly reduced whole blood clot stabilisation as evidenced by a decrease in the MCF with no improvement detectable in the presence of rFVIIa (C samples; Fig. 4).

Ca²⁺ levels

Table 2 lists Ca²⁺ levels in native, citrated and CTI-stabilised blood before and after haemodilution with HES 130/0.4 and after re-calcification. Physiological concentrations (~1.3 mmol/L) were observed in native and CTI-stabilised whole blood. No calcium was detectable in

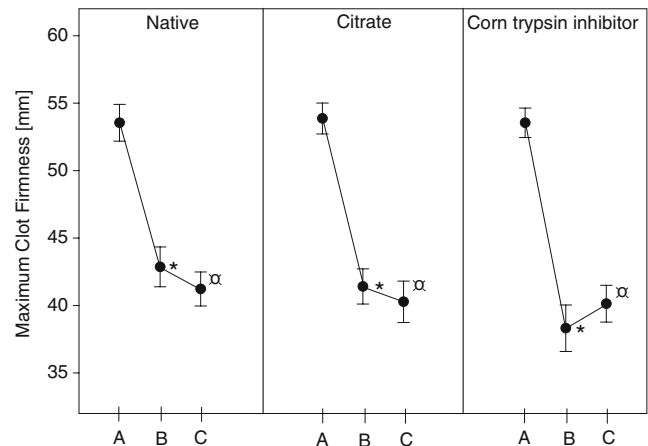


Fig. 4 Maximum clot firmness before (A), after 30% haemodilution with HES 130/0.4 (B) and following substitution with rFVIIa (C) in native or whole blood stabilised with citrate and corn trypsin inhibitor. Data presented as mean and standard error. $N=11$. * $p<0.01$ compared to A. ^α $p<0.01$ compared to B

citrate-chelated blood. Re-calcification resulted in supra-physiological levels of calcium at 3.75 mmol/L (citrate).

Discussion

The present study evaluated the haemostatic effect of rFVIIa in a laboratory model of colloid haemodilution using citrate-stabilised whole blood in comparison with native whole blood and whole blood stabilised with CTI. The study verified previous observations showing that haemodilution with HES 130/0.4 induces a coagulopathy characterised by a reduced maximum rate of clot formation as well as a pronounced reduction in the final maximum clot firmness [1, 19]. However, in contrast to our previous findings suggesting that rFVIIa fails to correct a dilutional coagulopathy induced by colloid plasma expanders [8], the present study shows that the use of whole blood stabilised with citrate may artificially mask the haemostatic effect of rFVIIa in colloid haemodilution. Hence, using native whole blood or CTI-stabilised whole blood rFVIIa not only shortened the CT but also revealed the capacity to accelerate the MaxVel of clot formation. The compromised MCF was unchanged with rFVIIa indicating that

Table 2 Ca²⁺ levels in native, citrated and CTI-stabilised blood before and after haemodilution with HES 130/0.4 and after re-calcification. $N=1$

	Native	Citrate	CTI
Before in vitro haemodilution with HES 130/0.4 (mmol/L)	1.24	Φ	1.22
After in vitro haemodilution with HES 130/0.4 (mmol/L)	0.87	Φ	0.88
After re-calcification (mmol/L)	No re-cal	3.75	No re-cal

Φ Immeasurable, No re-cal no re-calcification

defects in the function of fibrinogen and fibrin polymerisation is the predominant cause of haemostatic dysfunction in dilutional coagulopathy [1]. The observation of a slightly shortened CT in the B sample compared with the A sample may partly be explained by spontaneous contact activation of coagulation in the ROTEM® cup. However, since the time course from activation and analysis of the A, B and C samples was less than 45 s, it seems unlikely that spontaneous contact activation may be the only explanation for the shorter CT in the B sample. HES 130/0.4 investigated in the present study were not reported to activate the kallikrein system, unlike other HES species as reported by Nielsen [20]

Calcium chelation does not prevent contact activation of FXII which might explain the shortened clotting time and reduced time until maximum velocity observed in native and citrate-stabilised compared to CTI-stabilised whole blood [21, 22]. Further, spontaneous contact activation in the reaction cup, as a result of ex vivo mixing of the reagents, could also mask the haemostatic efficacy of rFVIIa in our system. The underlying mechanism explaining the differences in the haemostatic response patterns to rFVIIa was not explored in the present study. However, it may be speculated whether citrate could induce changes of the platelet function or hinder the association of rFVIIa with the surface of the activated platelet by interfering with the calcium-dependent conformational changes of the GLA domain of rFVIIa. To differentiate platelet from plasmatic effects, an assay employing platelet inhibitor like the cytochalasin D would be interesting.

In contrast to a clinical situation, our laboratory model of haemodilution with addition of rFVIIa was carried out in vitro. The system selected has the potential to illustrate some pharmacokinetic and pharmacodynamic properties. In the laboratory model, confounders can be controlled such as the degree of dilution, level of pH, temperature, consumption, etc. Furthermore, in investigations of the haemostatic potential of rFVIIa, it seems crucial to use human whole blood containing viable platelets. In animal models employing other species like rabbits, rats or swine, rFVIIa has a distinctly different pharmacodynamic profile [23]. Dosage of rFVIIa corresponding to an in vivo administration of 200 µg/kg was chosen in the present laboratory study to ensure an adequate and detectable effect.

In conclusion, we have shown that the use of citrate or whole blood anticoagulants in thrombelastographic clotting assays may artificially mask the haemostatic effect of rFVIIa in dilutional coagulopathy induced by synthetic colloid plasma expanders. The in vitro evaluation of rFVIIa in a matrix of citrated whole blood samples may lead to artificial underestimation of the haemostatic capacity of rFVIIa. In the clinical setting, when the haemostatic potential of rFVIIa is tested in vitro to judge whether

administration of rFVIIa might be efficacious, its role in improvement of haemostasis may be misinterpreted, delayed or excluded.

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