

Impact of experimental haemodilution on platelet function, thrombin generation and clot firmness: effects of different coagulation factor concentrates

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Background. Haemodilution during resuscitation after massive haemorrhage may worsen the coagulopathy and perpetuate bleeding.

Materials and methods. Blood samples from healthy donors were diluted (30 and 60%) using crystalloids (saline, Ringer's lactate, Plasmalyte™) or colloids (6% hydroxyethylstarch [HES130/0.4], 5% human albumin, and gelatin). The effects of haemodilution on platelet adhesion (Impact R), thrombin generation (TG), and thromboelastometry (TEM) parameters were analysed as were the effects of fibrinogen, prothrombin complex concentrates (PCC), activated recombinant factor VII (FVIIa), and cryoprecipitates on haemodilution.

Results. Platelet interactions were already significantly reduced at 30% haemodilution. Platelet reactivity was not improved by addition of any of the concentrates tested. A decrease in TG and marked alterations of TEM parameters were noted at 60% haemodilution. HES130/0.4 was the expander with the most deleterious action. TG was significantly enhanced by PCC whereas rFVIIa only caused a mild acceleration of TG initiation. Fibrinogen restored the alterations of TEM parameters caused by haemodilution including those caused by HES 130/0.4. Cryoprecipitates significantly improved the alterations caused by haemodilution on TG and TEM parameters; the effects on TG disappeared after ultracentrifugation of the cryoprecipitates.

Discussion. The haemostatic alterations caused by haemodilution are multifactorial and affect both blood cells and coagulation. In our *in vitro* approach, HES 130/0.4 had the most deleterious effect on haemostasis parameters. Coagulation factor concentrates did not improve platelet interactions in the Impact R, but did have favourable effects on coagulation parameters measured by TG and TEM. Fibrinogen notably improved TEM parameters without increasing thrombin generation, suggesting that this concentrate may help to preserve blood clotting abilities during haemodilution without enhancing the prothrombotic risk.

Keywords: haemodilution, coagulation factor concentrates, platelet function, thrombin generation, thromboelastometry.

Introduction

Haemorrhage continues to be one of the main causes of death following severe trauma. Massive bleeding is a leading cause of death worldwide standing already as the most important cause of death in individuals aged 5 to 45 years after severe trauma¹. Trauma patients are particularly susceptible to the early development of coagulopathy and the most severely injured patients are coagulopathic on admission to hospital. Uncontrolled bleeding initially causes loss of coagulation factors, red blood cells and platelets. Hypothermia, acidosis, and dilution from standard resuscitation practices can worsen this coagulopathy and perpetuate bleeding². Moreover, trauma-induced exposure of tissue factor to flowing

blood induces the activation of coagulation, which may trigger consumptive coagulopathy³. Administration of fluids for resuscitation after a massive blood loss causes clotting factor levels to decrease significantly, exacerbating the initial coagulopathy^{1,2,4}. The type of fluid and, more specifically, some colloids are being blamed for an additional deleterious effect on haemostasis, with altered fibrin polymerisation and decreased platelet adhesive and aggregating properties⁵. The vicious cycle of coagulopathy, fluid resuscitation and massive transfusion results in thrombocytopenia, delayed clot generation due to various clotting factor deficiencies, alterations of clot firmness, fibrin polymerization and impaired clot stability due to hyperfibrinolysis⁶.

Guidelines for the management of massive blood loss recommend that patients receive packed red blood cells, platelet concentrates and fresh frozen plasma (FFP). Although the proportions are not precisely defined^{6,7,8}, it has been suggested that current practice with FFP could be insufficient to reverse severe coagulopathies⁹. Chowdary *et al.*¹⁰ demonstrated that the standard doses of FFP recommended by current guidelines should be doubled or tripled to correct severe coagulation factor deficiencies in critically ill patients. Since the volume of transfused products contributes to the coagulopathy, the rationale for a change in current practices towards an earlier and more resolute use of concentrates of coagulation factors (fibrinogen, activated recombinant factor VII [rFVIIa], and prothrombin complex concentrates [PCC]) could reverse severe coagulopathy more efficiently and consequently reduce transfusion needs.

Evaluating the alteration in haemostasis and decision making during haemodilutional coagulopathy is a controversial subject. Standard coagulation tests such as prothrombin time (PT) and activated partial thromboplastin time (aPTT) do not seem to correlate with bleeding symptoms¹¹. In recent years, several reports have encouraged the use of algorithms based on thromboelastometry (TEM) methodology to support the use of coagulation factor concentrates¹²⁻¹⁵. Unfortunately, there is a lack of comparative studies of TEM technologies with routine coagulation tests which could validate such algorithms. We must accept that the emergency of the clinical situation in severe trauma patients - with isolated exceptions¹⁶ - imposes limitations to the design of prospective, randomised, comparative clinical trials that could generate evidence-based decisions for transfusion strategies to treat coagulopathy developing after massive bleeding.

Alterations in haemostasis caused by dilutional coagulopathy imply the interaction of complex cellular and plasma mechanisms. With this rationale in mind, we analysed modifications on platelet adhesive and cohesive functions, viscoelastic properties of clots and thrombin generation parameters induced by experimental haemodilution. Different crystalloid and colloid solutions currently used for resuscitation were administered to produce levels of haemodilution levels of 30 and 60%, theoretically corresponding to situations of severe bleeding equivalent to blood losses of 1.5 L or 3 L, respectively, for an average-sized adult. Once we had characterised the alterations caused by haemodilution on the different parameters studied, we evaluated the potential action of different factor concentrates: fibrinogen, PCC, rFVIIa, or cryoprecipitate in the correction or reversion of the experimentally induced coagulopathy.

Materials and methods

Study material

Following approval from the local Ethics Committee and written informed consent from each participant, the study group consisted of eight healthy volunteers. Individuals who had received acetylsalicylic acid, non-steroidal anti-inflammatory or antiplatelet drugs within 7 days before blood sampling were excluded. Blood samples were collected into tubes (BD Vacutainer, Franklin Lakes, United States of America) containing citrate (final concentration of 13 mM).

Experimental design

Samples of whole blood were diluted *in vitro* with different crystalloids, including isotonic saline (NaCl 0.9%, Baxter S.L., Valencia, Spain), Ringer's lactate (LacRinger™, Braun Medical S.A., Barcelona, Spain), and Plasmalyte™ (Baxter S.L.); or colloids such as 6% hydroxyethylstarch HES 130/0.4 (Voluven™, Fresenius Kabi S.A., Barcelona, Spain), 5% human albumin (Grifols S.A., Barcelona, Spain), and gelatin (Gelifundina™, B. Braun Medical S.A.). Diluted samples were used to evaluate: (i) platelet interactions using the cone platelet analyser method (Impact R™, Matis Medical, Beersel, Belgium); (ii) clot firmness by thromboelastometry (ROTEM, Tem International GmbH, Munich, Germany); and (iii) thrombin generation using the fluorogenic assay Technothrombin TGA (Technoclone GmbH, Vienna, Austria). One crystalloid and one colloid were selected to continue with the second stage of the study.

In the second stage, the following haemostatic components were spiked to the haemodiluted sample to evaluate their potential corrective effect on above mentioned technologies: fibrinogen concentrate (Haemocomplettan™, CSL Behring GmbH, Marburg, Germany), PCC (Beriplex™, CSL Behring GmbH), rFVIIa-NovoSeven™ (NovoNordisk, Bagsvaerd, Denmark), and cryoprecipitates (Bang de Sang i Teixits, Barcelona, Spain). All haemostatic components were investigated at concentrations comparable to those commonly used in patients presenting with massive bleeding: fibrinogen at 2 g/L, PCC at 35 IU/kg, rFVIIa at 6 µg/mL, and cryoprecipitates at 2 units /10 kg of weight).

Routine laboratory determinations

Platelet count, haematocrit, and other haematological parameters were determined in an Advia 2120 Haematology System (Siemens, Deerfield, Illinois, United States of America). Fibrinogen levels, PT, and aPTT were determined in the BCS™ XP system (Siemens, Deerfield, Illinois, United States of America). The von Willebrand factor antigen (VWF:Ag) was

evaluated by and enzyme-linked immunosorbent assay (ELISA; DG-EIA vWF, Grifols).

Platelet interaction: the cone platelet analyser procedure

The cone platelet analyser method was used as previously described¹⁷. Briefly, 130 μ L of whole blood or haemodiluted blood samples were placed in a well polystyrene plate and subjected to a shear rate of 1,800 s^{-1} for 2 minutes using a rotating TeflonTM (Beersel, Belgium) cone. The wells were subsequently washed thoroughly with tap water, stained with May-Grünwald stain and analysed with an inverted light microscope connected to an image analysis system. Two parameters of platelet adhesion were evaluated: percentage of surface coverage (% SC), which is the percentage of total area covered by platelets (single platelets and clusters/aggregates), and the average size (AS, measured as μm^2) of the polystyrene-bound platelet clusters/aggregates.

Thromboelastometry studies

In order to evaluate the influence of haemodilution, and the effect of the different coagulation factor concentrates on the clot formation, we used the ROTEM analyser¹⁸. The technique was performed according to the manufacturer's instructions. For simplicity, we focused on the analysis of the exTEM test in TEM studies. Briefly, exTEM uses tissue factor as an activator and is sensitive for measuring changes to the extrinsic pathway of coagulation, fibrinogen and fibrin polymerisation as well as platelet function. This technique provides information through different parameters. We assessed four variables. The clotting time (CT), defined as the time elapsed from the start of the measurement until the amplitude of the forming clot reaches 2 mm. The clot formation time (CFT) is the time from the start of clot formation until the tracing reaches 20 mm of amplitude. The clot amplitude after 10 min (A10) and the maximum clot firmness (MCF) were evaluated as measures of clot firmness. In the experiments performed in the presence of the different plasma concentrates, only the A10 was measured to assess clot firmness. Recently, it has been reported that A10 is a good linear predictor of MCF¹⁹. CT and CFT are indicators of the dynamics of clot formation. The clot amplitude gives information about clot strength and stability, which is largely dependent on fibrinogen and platelets. All analyses were performed for a minimum of 45 min.

Thrombin generation assay

We evaluated thrombin generation in citrated platelet-rich plasma (PRP) samples, haemodiluted PRP, and haemodiluted PRP with the coagulation factor concentrates at the appropriate dosage. For specific

experimental purposes, cryoprecipitates were subjected to ultracentrifugation at $6 \times 10^5 \times g$ for 10 min at 10 °C in an ultracentrifuge (Sorval MTX 150 Micro-UltraCentrifuge, Thermo Fisher Scientific, Kanagawa Prefecture, Japan).

Thrombin generation on citrated PRP was assessed with the fluorogenic assay Technothrombin TGATM following the manufacturer's instructions and indications in previous publications^{20,21}. This assay is based on the fluorescence generated by the cleavage of a fluorogenic substrate by thrombin over time. The activation of the coagulation cascade was triggered by a reagent (RCL) consisting of low concentration micelles of negatively charged phospholipids containing 71.6 pM of recombinant human tissue factor and $CaCl_2$. The fluorescence generated was measured at 1 min intervals throughout 90 min. The assay provides thrombin concentration as well as other parameters, such as the lag time (min) to trigger thrombin generation (lag phase), peak of maximal thrombin concentration (nM) and the velocity index of thrombin generation.

Statistics

Data are expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed with raw data using ANOVA. The SPSS statistical package 17.0.0 (SPSS Inc, Chicago, IL, United States of America) was used for all analyses. P values less than 0.05 were considered statistically significant.

Results

Description of the effect of haemodilution

Table I summarises the effects of 30% and 60% haemodilution with Ringer's lactate and HES 130/0.4 on platelet counts, haematocrit, fibrinogen, PT, aPTT, and VWF levels. Similar results were observed with other diluents used in our studies (*data not shown*). Alterations in these parameters followed the severity of the haemodilution, reaching maximal differences at 60% haemodilution. No significant differences were observed depending on whether the haemodilution was produced with crystalloids or colloids.

Influence on platelet interactions

Haemodilution had a dramatic influence on platelet interactions, as assessed by Impact-R technology. Platelet surface coverage in citrated whole blood samples reached values of $9.1 \pm 1.2\%$ (mean \pm S.E.M, $n=8$). A 30% haemodilution with any of the crystalloids or colloids caused reductions in platelet interactions with coverage values declining to 6%. As an example, 30% haemodilution with Ringer's lactate reduced the surface coverage to $5.5 \pm 0.8\%$ ($n=8$; $P < 0.05$). Undetectable levels of platelet coverage were observed at more intense

Table I - Effects of haemodilution with Ringer's lactate and HES 130/04 on various haematological parameters.

	Platelet count ($10^9/\mu\text{L}$)	Hematocrit (%)	Fibrinogen (g/L)	PT (ratio)	aPTT (s)	VWF (U/dL)
Baseline	170.8 \pm 16.8	42.4 \pm 1.8	2.8 \pm 0.3	1.05 \pm 0.03	28.4 \pm 1.5	154.7 \pm 6.1
Ringer's lactate						
30% Haemodilution	117.0 \pm 2.7*	30.0 \pm 1.3***	1.7 \pm 0.2*	1.48 \pm 0.08**	35.9 \pm 2.5*	95.0 \pm 8.0*
60% Haemodilution	69.0 \pm 6.4*	17.0 \pm 0.5***	1.0 \pm 0.1**	2.55 \pm 0.13**	104.0 \pm 45.4	26.1 \pm 3.4**
HES 130/0.4						
30% Haemodilution	139.0 \pm 14.2**	30.2 \pm 1.3***	2.1 \pm 0.2*	1.40 \pm 0.08*	86.1 \pm 51.3	102.7 \pm 21.9
60% Haemodilution	84.3 \pm 6.9**	17.0 \pm 0.2***	1.4 \pm 0.2*	1.92 \pm 0.2**	109.0 \pm 44.0	34.2 \pm 5.3**

Legend Data are expressed as mean \pm S.E.M (n=8). *: P<0.05; **: P<0.01; ***: P<0.001 vs baseline.

levels of haemodilution (60%) with any of the diluents used in our experiments (*data not shown*).

None of the concentrates tested improved reductions in platelet surface coverage observed after haemodilution. Figure 1 exemplifies representative microscopic fields obtained in experiments before, after 30% hemodilution with Ringer's lactate and following *in vitro* addition of fibrinogen to the haemodiluted sample. Figure 2 summarises independent values for all the concentrates tested in blood samples haemodiluted with Ringer's lactate (fibrinogen, PCC, rFVIIa, and cryoprecipitates). Similar tendencies were observed for haemodilution with other crystalloids or colloids or after *in vitro* addition of the different concentrates.

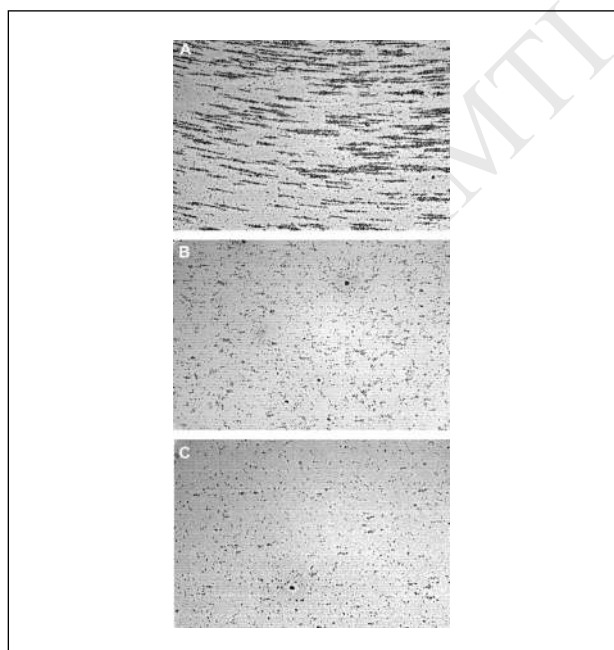


Figure 1 - Micrographs showing platelet interactions as observed with the cone and plate technology (Impact R).

Representative microscopic fields in: (A) Experiments performed with whole blood; (B) a 30% haemodiluted sample using Ringer's lactate; (C) a 30% haemodiluted sample using Ringer's Lactate after *in vitro* addition of fibrinogen (2 mg/mL).

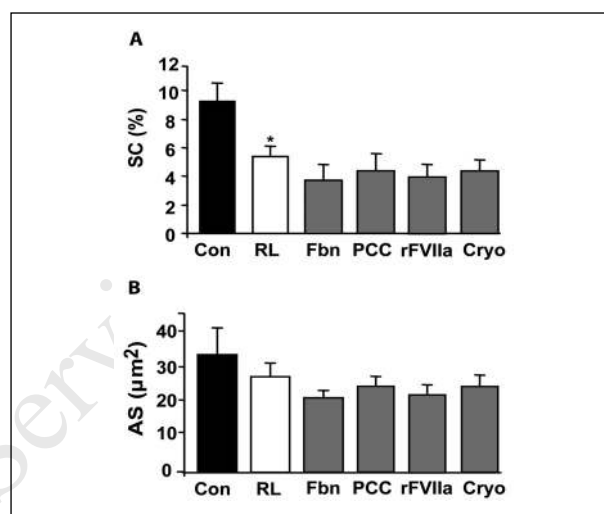


Figure 2 - Influence of different concentrates on platelet interactions using the cone and platelet technology. Bar diagrams indicate the percentage of surface covered by platelets with whole blood (Con), after 30% haemodilution using Ringer's lactate (RL), and after *in vitro* addition of fibrinogen (Fbn) at 2 mg/mL, prothrombin complex concentrates (PCC) at 35 IU/kg, activated recombinant factor VII (rFVIIa) at 6 μg/mL, and cryoprecipitates (cryo) at a dose of 2 cryo/10 kg of weight. Panel A shows the total percentage of surface covered (SC) by platelets and panel B shows the average size (AS) of the aggregates formed, expressed in μm². Results are expressed as mean \pm SEM, n=8 (*P<0.05 vs whole blood).

Modifications in viscoelastic properties of clots

The viscoelastic properties of clots were progressively altered with the intensity of the haemodilution, always being more evident at 60%. Figure 3 summarises the most significant alterations in TEM parameters observed at a 60% haemodilution for each of the solutions tested. HES 130/0.4 proved to be the expander with the most deleterious effect (P<0.001). Crystalloids produced a delay in the initiation of clot formation, showing significant increases of CT and CFT. Decreased levels of A10 and MCF indicate a reduction of the clot firmness (see Table II). Haemodilution with colloids resulted in more significant impairment as reflected by the greater delay in CT and

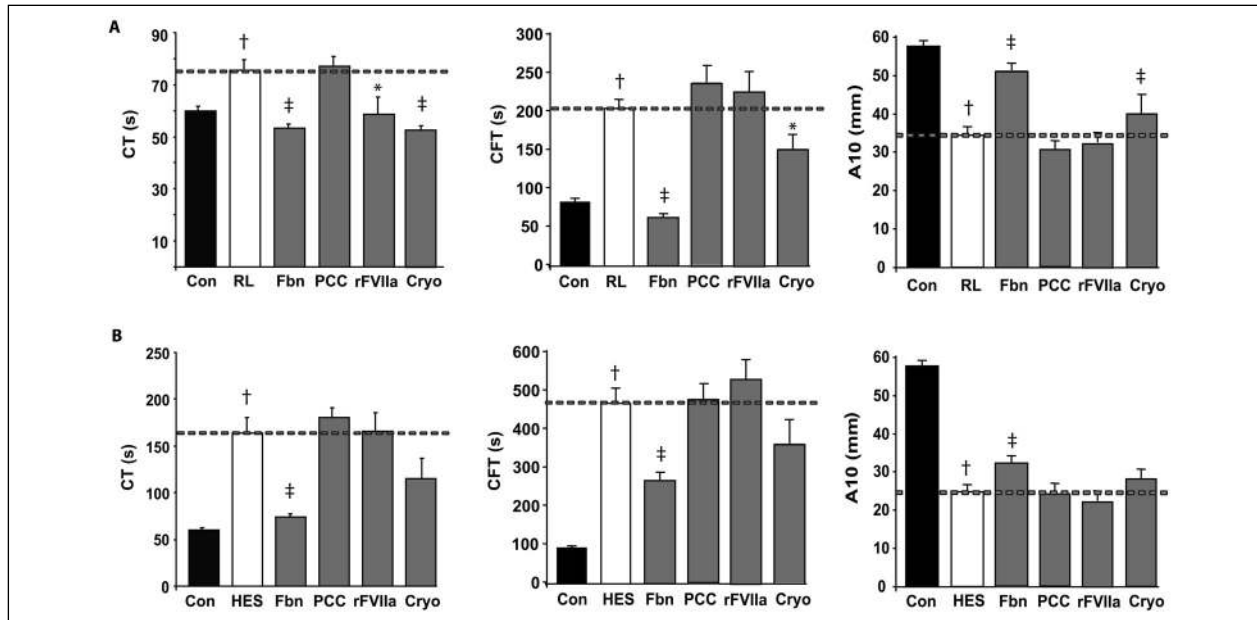


Figure 3 - Effects of different plasma concentrates on the viscoelastic properties of clots evaluated by ROTEM.

Each panel corresponds to a different fluid used for haemodilution of the sample: Ringer's lactate (A), representative of crystalloids, and HES 130/0.4 (B), representative of colloids. Each panels shows the clotting time (CT), clot formation time (CFT) both expressed in seconds; and clot amplitude after 10 min (A10) expressed in mm. The dashed line provides the reference for undiluted control samples (Con), samples 60% haemodiluted with Ringer's lactate (RL) or HES 130/0.4 (HES), before and after addition of: 2 mg/mL of fibrinogen (Fbn), 35 IU/kg of prothrombin complex concentrates (PCC), 6 µg/mL of activated recombinant factor VII (rFVIIa), or cryoprecipitates (Cryo) at an equivalent dose of 2 cryo/10 kg of weight. Results are expressed as mean ± S.E.M (n=8); (†) P <0.01 vs control samples, (*) P <0.05 vs haemodilution (‡), and P <0.01 vs haemodilution.

Table II - Effect of 60% haemodilution with different crystalloids and colloids on viscoelastic properties of forming clots.

	Baseline	Saline	Ringer lactate	Plasmalyte	HES 130/0.4	5% Albumin	Gelatin
CT (s)	60.9±2.0	88.6±3.0**	75.5±4.3*	70.3±3.2*	163.5±17.1**	111.3±4.6**	104.3±22.7*
CFT (s)	79.6±3.6	205.4±16.5**	202.9±15.4**	210.7±17.1**	457.9±42.5**	243.7±16.4**	344.0±63.2**
A10 (mm)	57.0±1.1	33.7±1.3**	34.3±1.1**	33.8±1.3**	23.6±1.3**	31.3±1.0**	28.7±3.5**
MCF (mm)	65.0±1.6	42.7±2.3**	44.7±2.3**	41.0±2.1**	36.5±3.5**	40.7±1.8**	40.8±2.3**

Legend Data are expressed as mean ± S.E.M. (n= 8). *: P <0.05; **: P <0.001 vs baseline.

more intense reduction of the clot firmness than those observed with crystalloids (see Table II).

As shown in Figure 3, the *in vitro* addition of fibrinogen significantly improved the alterations caused by haemodilution with any of the diluents tested, even those with HES 130/0.4. Cryoprecipitates significantly improved alterations of TEM parameters induced by haemodilution with crystalloids but seemed less efficient than fibrinogen at correcting the alterations caused by haemodilution with HES 130/0.4. The effect of rFVIIa only was evident in the first stage of clot formation, shortening the CT when crystalloids were used. The *in vitro* addition of PCC did not have any effect on TEM parameters (Figure 3).

Influence on thrombin generation

Table III summarises the effects of the greatest

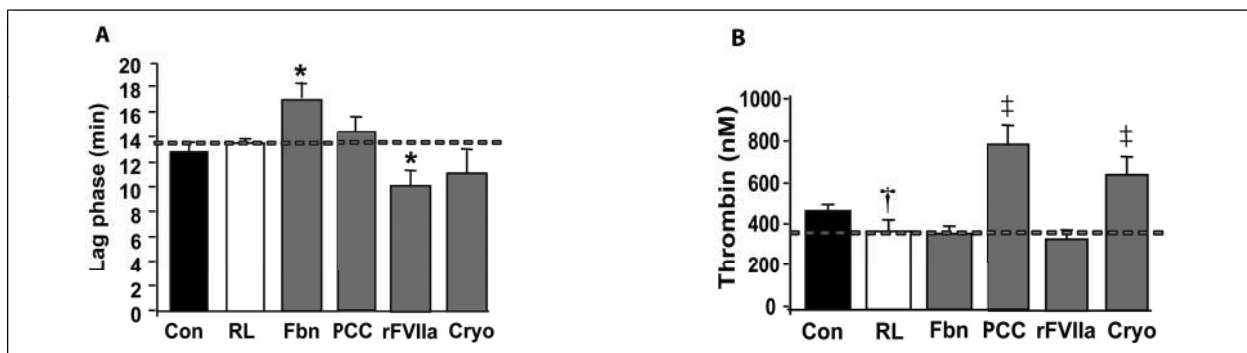
haemodilution on thrombin generation. The lag phase preceding the initiation of thrombin generation was significantly delayed (P <0.05) at 60% haemodilution with saline, plasmalyte™, HES 130/0.4, and 5% albumin. Lag phases were also slightly delayed after haemodilution with Ringer's lactate and gelatin although the differences did not reach the levels of statistical significance. Significant reductions in thrombin peaks and slower velocity indices were observed after 60% haemodilution with the different crystalloids and colloids tested (P <0.05 vs baseline conditions). Haemodilution with albumin seemed to have a more profound influence on thrombin generation as confirmed by the lowest maximal concentration of thrombin reached at peak (P <0.01 vs baseline).

With regards to the different concentrates studied, the thrombin generation peak was significantly elevated by PCC and cryoprecipitates (Figure 4). *In vitro* addition

Table III - Modifications in thrombin generation parameters after 60% haemodilution with different crystalloids and colloids.

	Baseline	Saline	Ringer lactate	Plasmalyte	HES 130/0.4	5% Albumin	Gelatin
Lag phase (min)	10.9±0.2	13.6±0.4*	12.0±0.6	13.1±0.5*	14.1±0.9*	12.1±0.3*	11.6±0.2
Thrombin peak (nM)	471.0±48.7	346.8±36.0*	375.9±38.5*	325.8±37.5*	355.6±40.5*	230.1±24.9**	368.5±37.9*
Velocity index	85.5±10.7	47.9±8.2**	51.8±7.8*	40.4±6.3*	47.3±7.4*	37.6±6.4*	62.9±9.1*

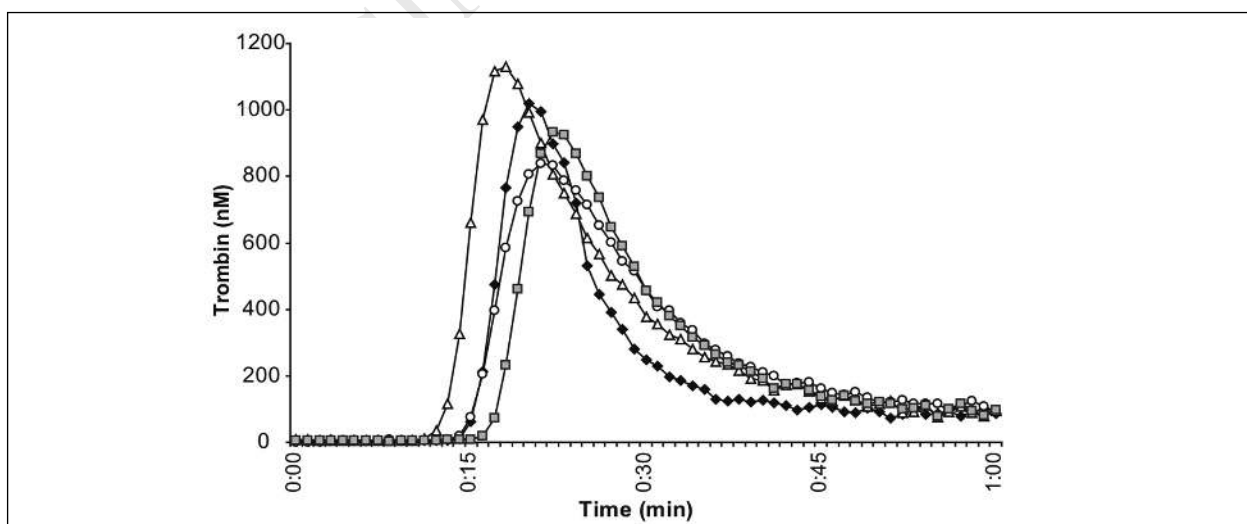
Legend Data are expressed as mean ± S.E.M. (n=8). *: P < 0.05; **: P < 0.001 vs baseline.

**Figure 4** - Effect of the different factor concentrates on thrombin generation.

Bar diagrams summarise modifications in (A) lag time (min) to trigger thrombin generation (lag phase) and (B): the peak of maximal thrombin concentration (nM) in samples of platelet-rich plasma (PRP) from blood haemodiluted to 60% with Ringer's lactate. The different bars represent values in control undiluted samples (Con), haemodiluted samples before (RL), and after addition of 2 mg/mL of fibrinogen (Fbn), 35 IU/kg of prothrombin complex concentrates (PCC), 6 µg/mL of activated recombinant factor VII (rFVIIa), or cryoprecipitates (Cryo) at a dose equivalent to 2 cryo/10 kg of weight. Results are expressed as mean ± S.E.M (n=8); (*) P < 0.05 vs haemodiluted samples, (†) P < 0.01 vs control samples, and (‡) P < 0.01 vs haemodilution.

of rFVIIa to haemodiluted samples caused a moderate acceleration of thrombin generation. Addition of fibrinogen to haemodiluted blood samples caused a slight delay of the initiation of thrombin generation without any significant effect on total thrombin generation. Interestingly,

cryoprecipitates significantly improved the alterations caused by haemodilution on thrombin generation (Figure 4). However, the previously mentioned effect disappeared when cryoprecipitate was ultracentrifuged, suggesting that contaminating particulates could account for this effect (Figure 5).

**Figure 5** - Effect of ultracentrifugation of cryoprecipitate on thrombin generation using samples of platelet-rich plasma (PRP) from blood haemodiluted to 60% with Ringer's lactate.

Graphs display representative thrombin generation kinetics (n=4) in control undiluted samples (♦), haemodiluted PRP (O), haemodiluted PRP plus cryoprecipitate at a dose of 2 cryo/10 kg of weight (Δ), and haemodiluted PRP plus supernatant of ultracentrifuged cryoprecipitates at equivalent doses (■). Enhanced thrombin generation induced by cryoprecipitates disappeared after ultracentrifugation.

Discussion

Our results demonstrate that haemodilution has a dramatic impact on platelet-mediated haemostasis at high shear with an additional influence on thrombin generation and subsequent alterations of the viscoelastic properties of clots generated under low shear conditions. All the concentrates showed favourable effects, correcting different phases of the coagulation process, but failed to compensate the platelet defect. Fibrinogen and cryoprecipitates clearly improved the viscoelastic properties of forming clots, cryoprecipitates and PCC increased thrombin generation, whereas rFVIIa accelerated the initiation of thrombin generation initially delayed by the haemodilution.

There is a general agreement that alterations in haemostasis induced by haemodilution are multifaceted and affect both cellular and coagulation mechanisms. It is also accepted that routine standard coagulation tests (PT, aPTT) have limited value for predicting bleeding²² or for monitoring replacement therapy^{4,7} in patients subjected to surgery. Although haemostatic alterations caused by haemodilution should be evaluated through specific laboratory tests, the emergency of the situation in critical bleeding patients and the need for rapid management of the coagulopathy have promoted the introduction of point of care technologies with an ability to evaluate haemostasis globally^{14,23,24}. TEM technology has rapidly become established as a useful method for quick monitoring of coagulopathies and applied to transfusional decision-making in the care of perioperative bleeding.

In the present study we used an experimental model of dilutional coagulopathy that was characterized through three different laboratory approaches to investigate platelet interactions, thrombin generation and clot formation after haemodilution. The experimental dilutional coagulopathy we produced is compatible with the clinical situation in severely bleeding patients who may require intensive transfusion. Special attention was paid to results produced at 60% haemodilution considering that this is the situation in which clotting factor concentrates would have the potential for fast reversal.

A dramatic reduction in platelet interactions with adhesive surfaces was observed in our studies at the elevated shear rates of the cone and plate analyser. None of the concentrates tested improved adhesive or aggregating properties of platelets in a system that is also sensitive to the negative haemorheological influence of the haematocrit reduction, though we cannot exclude possible limitations of the cone and plate technology in our experimental setting. It is accepted that the haemorheological effect of red blood cells is critical for the maintenance of correct haemostasis under flow

conditions^{25,26}. The transfusion of packed red blood cells with the objective of raising the haematocrit above 30% has a favourable impact on the haemostatic performance of circulating platelets^{27,28}.

An additional finding of our present studies, which could explain the dramatic reduction in platelet interactions was the significant reduction in VWF levels after severe haemodilution. Levels of VWF reached values compatible with a diagnose of type 1 von Willebrand's disease. It is interesting in this respect that transfusion algorithms based on TEM technology promote the use of fibrinogen or PCC to correct alterations in TEM parameters, but disregard the use of concentrates containing VWF to compensate the obvious deficiency in this adhesive protein.

Haemodilution with colloids or crystalloids caused significant alterations in the viscoelastic properties of forming clots as evaluated by TEM technology. Viscoelastic parameters were more severely affected when HES 130/0.4 was used as the haemodilution fluid. Fibrinogen and cryoprecipitate were found to be very effective at improving the alterations of the viscoelastic properties of thrombi induced by haemodilution as evaluated by TEM. These findings are basically in agreement with those of previous experimental and clinical studies^{13,29}. Interestingly, PCC or rFVIIa did not show any relevant correction of the alterations of viscoelastic clot parameters produced in our experimental model of haemodilution. The favourable results in the TEM parameters in our studies were not significantly potentiated by further addition of PCC or rFVIIa (*data not shown*).

With the exception of fibrinogen, all the products tested in our experimental setting (PCC, rFVIIa and cryoprecipitate) had a favourable action, enhancing and accelerating alterations in thrombin generation caused by haemodilution. PCC appeared to be the most efficacious at reversing the alterations caused by haemodilution. The modifications induced by rFVIIa on thrombin generation were apparently milder than those observed with PCC, but correlated with the shorter CT produced by rFVIIa in the TEM. Previous studies demonstrated that rFVIIa had a marked impact on thrombin generation in plasma from patients with haemophilia³⁰. It is very likely that the by-passing effects of rFVIIa on thrombin generation are less evident when there is an overall haemodilution of all coagulation factors as would be the case during severe haemodilutional coagulopathy.

Cryoprecipitates were the only concentrate that improved the viscoelastic properties of the clots and, surprisingly, thrombin generation. Previous studies by George *et al.*³¹ revealed that platelet membrane microparticles, highly concentrated in cryoprecipitates, could contribute to their therapeutic effect. The fact that

the enhanced thrombin generation we observed in our studies disappeared after ultracentrifugation suggests that contaminant materials present in the cryoprecipitates could have been responsible for the effects on thrombin generation. Cryoprecipitate is a heterogeneous plasma component³² that may exert a complex haemostatic action other than restoring fibrinogen concentration. On the other hand, cryoprecipitates are the only concentrate, used in our studies, providing an additional supply of VWF, an adhesive protein that we found significantly to be reduced after severe haemodilution.

Our results indicate that TEM technology, currently applied for a global evaluation of coagulation in emergency, surgical or intensive care units, is very sensitive at detecting the effects of fibrinogen and cryoprecipitates, but less sensitive for detecting those of PCC or rFVIIa. According to our findings, point-of-care TEM devices are not designed to evaluate the mechanism of primary haemostasis with regards to the adhesive functions of platelets. Moreover, in our experimental setting the exTEM seemed to have limited sensitivity for identifying the effects of some concentrates (PCC and FVIIa) which, on the other hand, did have a definite action on thrombin generation. The information provided by the point-of-care TEM devices is useful for providing an early diagnose of the coagulopathy and for facilitating transfusion decisions in emergency situations³³, but haemostatic alterations in the critically ill patient are far more complex than revealed by this technology.

Our findings have implications that can be extrapolated to clinical situations. First, the amounts of fluids used for resuscitation in severe trauma situations should be kept to a minimum to avoid dilutional coagulopathy. Second, hydroxyethyl starches may have an additional deleterious effect on coagulation parameters, as measured by TEM. Third, fibrinogen concentrates may be useful for improving or restoring blood clotting without increasing thrombin generation if the severity of bleeding has resulted in haemodilution. Finally, PCC, rFVIIa or cryoprecipitates could be used to boost thrombin generation in patients with uncontrollable bleeding resistant to the previous transfusional approach. Recent evidence indicates that the combined use of concentrates capable of enhancing thrombin generation may result in thrombotic complications^{34,35}.

Our experimental studies indirectly suggest that fibrinogen concentrates might be useful to preserve blood clotting capacities during transportation of severely injured patients who require resuscitation with fluids. Fibrinogen concentrates could help to prevent the alterations of viscoelastic properties of clots altered by haemodilution without increasing thrombin generation.

Limitations of our studies

Our experimental model of haemodilution cannot reproduce the additional consumptive coagulopathy and associated hyperfibrinolysis related to the exposure of damaged tissue components occurring in severe trauma patients.

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Conflicts of interest disclosure

Gines Escolar has received honoraria/consultant fees from CSL Behring and from NovoNordisk. None of the other Authors have any potential conflicts of interest.

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