Fibrinogen depletion after plasma-dilution: impairment of proteolytic resistance and reversal via clotting factor concentrates

Shu He; Hans Johnsson; Michał Zabczyk; Kjell Hultenby; Håkan Wallen; Margareta Blombäck

1Department of Clinical Sciences, Danderyd Hospital, Stockholm, Sweden; 2Department of Emergency Medicine, Karolinska Institutet, Stockholm, Sweden; 3Department of Molecular Medicine and Surgery/Clinical Chemistry, Karolinska Institutet, Stockholm, Sweden; 4Department of Laboratory Medicine, Karolinska University Hospital, Karolinska Institutet, Stockholm Sweden; 5Medical College, Institute of Cardiology, Jagiellonian University, Krakow, Poland

Summary

In trauma patients, resuscitation treatment of intravascular volume may cause haemodilution including blood cell- and plasma-dilution. After plasma-dilution, fibrinogen is the first factor that decreases to critically low concentrations. Fibrin formed in lowered levels is susceptible to fibrinolysis, a natural forerunner for bleeding. To assess whether a fibrinogen concentrate or a factor XIII (FXIII) concentrate can reverse the impairment of fibrin properties after plasma dilution, different laboratory methods were used to determine thrombin generation and fibrin quantity/quality in a normal plasma sample diluted in vitro. Coagulation and clot lysis by plasmin were triggered with tissue factor and rt-PA, respectively. We found that while the endogenous thrombin potential (ETP) was unaffected after plasma-dilution due to postponement of thrombin decay, levels of fibrinogen and hence fibrin were decreased in dilution degree-dependency. The imbalance between influence of the dilution on thrombin activity and fibrin formation brought unexpected outcomes of fibrin properties: the formed clots favoured the degradation by plasmin but the fibrin networks remained tighter/less permeable. This proteolytic tendency was partly overturned by the fibrinogen concentrate added (total fibrinogen ≥ 2 g/l), and much more affected if used in combination with tranexamic acid (a fibrinolysis inhibitor) at small doses. No reversal effect resulted from the FXIII concentrate added. We conclude that plasma-dilution did reduce the proteolytic resistance of formed clots. The fibrinogen concentrate, better together with small doses of tranexamic acid, may reverse the impairment of fibrin property. The FXIII concentrate is not effective in this regard in our in vitro model using platelet-poor plasma.

Keywords

Plasma dilution, coagulopathy, fibrin proteolysis, fibrinogen concentrate, FXIII concentrate, tranexamatic acid

Introduction

During early stages of severe trauma or major surgery with massive bleeding, patients are usually managed with crystalloid infusion. This resuscitation treatment of intravascular volume may lead to haemodilution including dilution of blood cells and plasma; a state of coagulopathy follows (1).

After plasma-dilution, fibrinogen is the first factor that reduces to critically low levels (2–4). The consequent decline in fibrin formation may weaken clot visco-elasticity, resulting in acceleration of fibrin clearance by plasmin – a natural forerunner of haemorrhage (5). Clinical data connected with acute events of injury, gynaecological surgery, neurosurgery, cardiac surgery, etc. suggest that fibrinogen levels below 1.5–2.0 g/l are associated with more severe bleeding (4, 6).

In cases of plasma-dilution, plasma levels of other coagulants decrease in parallel with those of fibrinogen, which down-regulates coagulation process. At present, impairment of haemostasis in such patients is commonly monitored by measuring activated partial thromboplastin time (APTT), thrombin time and the international normalised ratio (INR) (6). However, the standard clotting tests, which reflect only the initiation phase of blood coagulation, are not sensitive enough to facilitate accurate diagnosis while the overall coagulation state is affected. A global thrombin-generation assay, i.e. calibrated automated thrombography (CAT) (7) should be a better approach. Use of this method in an in vitro study confirmed that modest plasma-dilution does not alter the global marker endogenous thrombin potential (ETP) (8).

Thus, it appears that plasma-dilution tends to reduce fibrinogen quantity rather than to depress thrombin potential. This imbalance may impair the properties of fibrin, worsening the bleeding complication in patients after plasma-dilution. In recent years, supplementation with fibrinogen concentrates has been used for managing cases of massive haemorrhage (6, 9). Because
full stabilisation of clots against fibrinolysis requires factor XIII (FXIII), which mediates fibrin cross-linking (10) and inhibition of fibrinolysis by antiplasmin (11), administration of purified FXIII is also recommended (12, 13).

In the present study, we aimed to assess whether or not a fibrinogen concentrate and/or a FXIII concentrate -with or without tranexamic acid (a fibrinolysis inhibitor) - could reverse the impairment of fibrin properties after plasma-dilution. An in vitro model and different laboratory methods that evaluate fibrinogen quality/quantity were employed. The in vitro model was set up with a diluted normal plasma pool, to which recombinant tissue factor together with purified phospholipids and calcium were added to initiate coagulation. The major proportion of tissue plasminogen activator (t-PA) in plasma is involved in complexes with various inhibitors, such as PAI-1, anti-plasmin and C1 inhibitor (14). In our preliminary study without exogenous t-PA, the small proportion of activated t-PA obtained means that natural activation of plasminogen is too slow to offer adequate data on the fibrin degradation by plasmin (data not shown). Therefore, we modified the original protocol of ROTEM: the examined sample was mixed with excess recombinant t-PA prior to addition of the coagulation trigger.

Materials and methods

Plasma samples and reagents

A normal platelet poor-plasma pool (NPP) was obtained from Precision Biologic, Dartmouth, NS, Canada (fibrinogen 3.04 g/l; FXIII 0.91 IU/ml).

A fibrinogen concentrate (FBGc; Haemocomplettan® or Rias-tap®) and a Factor XIII concentrate (FXIIIc; Fibrogammin®; containing trace fibrinogen < 0.1% of total protein) from CSL Behring (Marburg, Germany) were dissolved in water to 1,000 g/l and 100 IU/ml, respectively. One vial of recombinant human tissue factor (Innovin®) from Siemens Healthcare Diagnostics (Marburg, Germany) were dissolved in water to 55.8 g/l and 100 IU/ml, respectively. A purified factor together with purified phospholipids and calcium were added to initiate coagulation. The major proportion of tissue plasminogen activator (t-PA) in plasma is involved in complexes with various inhibitors, such as PAI-1, anti-plasmin and C1 inhibitor (14). In our preliminary study without exogenous t-PA, the small proportion of activated t-PA obtained means that natural activation of plasminogen is too slow to offer adequate data on the fibrin degradation by plasmin (data not shown). Therefore, we modified the original protocol of ROTEM: the examined sample was mixed with excess recombinant t-PA prior to addition of the coagulation trigger.

Sample treatment

• Undiluted NPP, i.e.NPP 4+0 used as a control.
• NPP 4+0 mixed with different proportions of a Tris-NaCl buffer (50 mmol/l tris, 130 mmol/l NaCl, pH 7.4 adjusted with HCl): NPP 3+1 (three parts of NPP 4+0 + one part of buffer), NPP 2+2 (two parts of NPP 4+0 + two parts of buffer) and NPP 1+3 (one part of NPP 4+0 + three parts of buffer).
• NPP 1+3 spiked with one of the concentrates already diluted to different concentrations.
• NPP 1+3 spiked with diluted tranexamic acid, or together with one of the diluted concentrates.

Calibrated automated thrombography (CAT)

Thrombin generation, as reflected by various parameters, i.e. “lag time”, “thrombin peak”, “start tail time” and endogenous thrombin potential (ETP) was determined by using Thrombinscope Kits (BV, Maastricht, Netherlands) according to the instruction for measurement of CAT.

Assays of fibrin network permeability

The fibrin network permeability was determined, in principle according to the approach originally set up by Blombäck et al. (15) and later improved by our group (16, 17). Certain new modifications were involved in this study for increasing its usefulness.

A solution which triggers coagulation (“trigger solution”) was prepared by adding Innovin® (TF, 6000 pmol/l), PPL (500 pmol/l) and CaCl$_2$ (1 mol/l) to a Tris-NaCl buffer, giving the final concentrations 148 pmol/l, 117 pmol/l and 583 mmol/l, respectively. In a plastic test tube, 15 µl of above “trigger solution” was mixed with 425 µl of the plasma sample (see above “Sample treatment”), giving final concentrations 5 pmol/l of TF, 4 µmol/l of PPL and 20 mmol/l of CaCl$_2$. Using a gel-loading tip (diameter 0.5 mm; Labdesign, Stockholm, Sweden) (16), 200 µl of this mixture was immediately pipetted into a plastic cylinder prepared from Combitivity 1.0 ml (Eppendorf, Hamburg, Germany), where a fibrin clot was formed and incubated for 2 hours (h) at room temperature.

The plastic cylinder containing the matured clot was placed vertically on a stand (16). The upper opening of the cylinder was connected to an outer-cup of syringe containing the Tris-NaCl buffer. The dissimilar heights between the “buffer surface” and the “gel surface” led to a difference in hydrostatic pressure (ΔP), which allowed the Tris-NaCl buffer to pass through the fibrin network. During the first 60 minutes (min), soluble substances in the clot were washed away at ΔP of 5 cm$^4$. Then, the liquid percolation was repeated at 5 cm$^4$ and then at 6 cm$^4$; each for 60 min. Volumes of the eluted buffer were measured. A fibrin network permeability value before the normalisation (see “Results”) was shown as mean of permeability coefficients (Ks) tested at the two levels of ΔP. For the permeability coefficient, a calculation formula was:

$$K_s = \frac{Q \cdot L \cdot \eta}{t \cdot A \cdot \Delta P}$$

Q = elution volume, t = percolation time, η viscosity, L = fibrin gel length, A = cylinder inter-area.

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New modifications involved in this study.

Viscosity = 2.414 x 10 –5 – 5 x 10 [247.8 / (T –140)], where “T” had a Kelvin unite (“K”), equaling “273 + room temperature shown in degree Fahrenheit”;


Fibrin gel length = mean of the length values of fibrin gel measured before and after liquid percolation; the measurement was made from outside of the cylinder wall using a digital caliper.
Quantitative determination of clotted fibrinogen-fibrin (18)

Fully washed fibrin clots (after flow measurement) were dissolved in 1 ml alkaline urea solution (8 M urea + 0.2 N NaOH). The optical density of clotted fibrinogen (fibrin quantity) was determined at 282 nm, using a turbidimetric method (Biophotometer, Eppendorf, Hamburg, Germany).

Analysis of SEM images

Fully washed fibrin clots (after flow measurement) were used to prepare SEM images, as earlier reported by Dey (19).

Analysis of fibrin firmness by rotational thromboelastometry (ROTEM) (20, 21)

The ROTEM instrument was provided by TEM Innovations GmbH, Munich, Germany. A fibrinolysis trigger containing recombinant t-PA 2,300 ng/ml (15 μl) and then the coagulation trigger (15 μl) containing TF 148 pmol/l, PPL 117 μmol/l and CaCl₂ 583 mmol/l were added to 410 μl plasma sample. After mixing fully, 350 μl of the sample mixture was used for ROTEM assay. The final concentrations were TF 5 pmol/l, PPL 4 μmol/l, CaCl₂ 20 mmol/l and t-PA 78 ng/ml; this concentration of t-PA is at least 10-fold higher than the physiological level but lower than the mean therapeutic levels during thrombolysis (22).

Standard thrombelastograms are shown in Figure 1. Not all the ROTEM variables offered by the thromboelastometry were included in the present study. We collected the raw data of clot amplitude i.e. clot firmness (mm), as a function of running time (sec).
Based on these data, the GraphPad Prism® software for Windows (San Diego, CA, USA) was used to determine three variables: clotting time (CT), maximum clot firmness (MCF) and clot lysis time (CLT), considered being necessary for our investigation.

We have modified the approach earlier reported (18) for CLT assay: i) Along the proteolysis course of the control thromboelastometry (Figure 1, upper panel), a clot firmness value equivalent to 10% of MCF value was detected. ii) For each of unknown samples (Figure 1, lower panel), a CLT value was shown as time needed to reach a reduced value of clot firmness which represented 10%MCF in the control sample. Thus, the new protocol of CLT calculation is restricted by the MCF value in the unknown sample itself; the proteolysis rate determined like this should be more responsive for the changes in fibrinogen concentration.

The ROTEM assays were performed in NPP diluted with Tris-NaCl buffer, and also in NPP diluted with Ringer-acetate; however, the latter sample set did not include the concentrates and tranexamic acid.

Normalisation of assay results
In each experiment, NPP_{4+0} was included as control (100%). Results from the examined samples were normalised (100 × examined sample / control sample), and shown as “% of control”.

Statistical analysis
Pearson’s correlation coefficient (r) was calculated to assess the correlation between two groups (unpaired values). Values of p of <0.05 and <0.01 were considered to be significant and highly significant, respectively.

Results
Thrombin generation determined by CAT assay
Plasma-dilution did not affect thrombin generation rates, seen as similar values of “lag time” in all samples with or without the dilution. However, “peak thrombin” levels decreased along with the increasing extent of sample dilution; in NPP_{1+3} alone, “peak thrombin” showed a mean value about 50% lower than in controls (NPP_{4+0}). ETP was not modified compared with controls, while the “start tail time” was delayed in a dilution-dependent way.

Fibrin quantity determined by the turbidimetric method
In the control sample, i.e. NPP_{4+0}, the mean quantity (± standard deviation) of clotted fibrinogen i.e. fibrin indicated by the optical
Figure 3: Quantitative determination of clotted fibrinogen (fibrin) and fibrin network permeability in diluted NPP to which a fibrinogen concentrate (FBGc) or a factor XIII concentrate (FXIIIc) was added. A) Fibrinogen (FBG) and factor XIII (FXIII) levels were 3.04 g/l and 0.91 IU/ml, respectively, in NPP\(_{4+0}\), and were 0.76 g/l and 0.23 IU/ml, respectively, in NPP\(_{1+3}\). B and C) To NPP\(_{1+3}\), addition of FBGc at 0.44, 1.24, 2.24, 3.24 and 5.24 g/l elevated total fibrinogen levels in plasma to 1.20, 2.00, 3.00, 4.00 and 6.00 g/l, respectively, while addition of FXIIIc at 0.97, 1.77, 3.77 and 5.77 IU/ml elevated total FXIII levels to 1.00, 2.00, 4.00 and 6.00 IU/ml, respectively. NPP\(_{1+3}\) alone (red, panel A) was the positive control for the assays shown in panels B and C.

Figure 4: Scanning electron microscopy (SEM) images of fibrin networks derived from NPP diluted to different degree. Scale bar = 200 nm.
density (OD) was 0.70 ± 0.04 (▶ Figure 2A). Dilution of 3+1, 2+2 and 1+3 made the OD values decline to 0.68 ± 0.17, 0.38 ± 0.04 and 0.17 ± 0.02, respectively. On addition by FBGc at 0.44, 1.24, 3.24 and 5.24 g/l to NPP1+3 (▶ Figure 2B), fibrin quantities rose from 0.17 ± 0.02 (control) to 0.19 ± 0.0, 0.48 ± 0.04, 0.75 ± 0.04 and 1.41 ± 0.03, respectively. No important change of fibrin quantity was brought about by addition by FXIIIc at 0.97, 1.77, 3.77 and 5.77 IU/ml (▶ Figure 2C).

Fibrin network permeability determined by flow measurement

The permeability of fibrin network, measured as the Ks value, was decreased by plasma dilution. However, this reduction was moderate; even in the sample with the highest extent of plasma-dilution (NPP1+3), Ks dropped only from 100% to 74% (▶ Figure 3A).

In NPP1+3, addition by FBGc at 0.44, 1.24, 3.24 and 5.24 g/l caused further reduction from 74 to 36, 10, 2 and 0.9% of control values, respectively (▶ Figure 3B). When NPP1+3 was spiked with increasing doses of FXIIIc (▶ Figure 3C), fibrin network permeability was in principle unchanged.

SEM images of fibrin fibres and network porosity

Greater degrees of plasma dilution rendered the fibres thinner and the network tighter than in controls (▶ Figure 4C vs A and D vs A). These changes were more profound as regards NPP1+3 vs. NPP2+2 (▶ Figure 4D vs C).

The SEM image shown in ▶ Figure 4D (NPP1+3 alone, total fibrinogen 0.23 g/l), is also presented in ▶ Figure 5A, as a control in evaluation of the influences by fibrinogen or FXIII. Compared with this control, FBGc added to NPP1+3 at 1.24 g/l gave a total fi-

![Figure 5: Scanning electron microscopy (SEM) images of fibrin networks derived from diluted NPP to which different doses of the fibrinogen concentrate (FBGc) or FXIII concentrate (FXIIIc) were added. Scale bar = 200 nm. To NPP1+3 originally containing fibrinogen (FBG) 0.76 g/l and factor XIII (FXIII) 0.23 IU/mL, addition of FBGc at 1.24 and 3.24 g/l increased total FBG to 2.00 g/l and 4.00 g/l, respectively, and addition of FXIIIc at 1.77 and 5.77 IU/mL increased total FXIII to 2.00 and 6.00 IU/mL, respectively. Yellow arrows = major fibrin network (in panel C, the major fibrin fibers tend to aggregate laterally); red arrows = “additional” fibrin networks.
brinogen concentration of 2.00 g/l and further reduced fibre thickness and network porosity (▶ Figure 5B vs A). Between the major fibres (yellow arrows, ▶ Figure 5B), "additional" networks were formed where minimization of fibre diameters and network pores had proceeded (red arrows, ▶ Figure 5B).

A different SEM image was obtained when a higher dose of FBGc was added (total fibrinogen 4.00 g/l). Though the "additional" network still existed (red arrows), the major fibres tended to become associated laterally, forming thick fibrin bundles (yellow arrows, ▶ Figure 5B).

After addition of FXIIIc at 1.77 IU/ml and 5.77 IU/ml (total FXIII 2.00 IU/ml and 4.00 IU/ml respectively), the appearance of the fibrin fibres and network porosity were similar to those in the controls (▶ Figure 5D and E vs A).

Clot firmness and clot resistance to fibrinolysis determined by ROTEM

1) In diluted NPP alone
- In NPP diluted with Tris-NaCl buffer: Thromboelastograms derived from undiluted (control) and diluted NPP are shown in the left panel of ▶ Figure 6. For dilution degrees 3+1 and 2+2, there were no important changes in CT vs. control (top right panel, samples B and C vs sample A). However, for dilution degree 1+3 (sample D), CT was prolonged to about 270% of the control value. MCF and CLT decreased significantly with increasing dilution (bottom right panel).
- In NPP diluted with Ringer-acetate, CT, MCF and CLT values were similar to those in NPP diluted with Tris-NaCl buffer (data not shown).

2) In diluted NPP spiked with one of the concentrates
Thromboelastograms derived from undiluted (control) and diluted NPP are shown in the left panels of ▶ Figure 7 and ▶ Figure 8.
- In NPP1+3 spiked with the FBGc alone (▶ Figure 7).
  - In NPP1+3 alone (panel A), CT was about 270% of the control value. When the FBGc was added giving total fibrinogen concentrations of 1.20 g/l (panel B) and 2.00 g/l (panel C), CT decreased to about 250% (panel B) and 190% (panel C) of the control value. Practically no further shortening of CT was detected at higher concentrations of total fibrinogen (panels D–F).
  - The mean MCF value in NPP1+3 alone (panel A) was about 26% of the control value. After addition of the FBGc giving total fibrinogen concentrations of 1.20 g/l (panel B) to 6.00 g/l (panel F), MCF values were enhanced, reaching about 150% of the control level.
  - Mean CLT in NPP1+3 alone (A) was about 17% of the control value. When total fibrinogen concentrations in NPP1+3 were...
elevated to 1.20 g/l (panel B) and 2.00 g/l (panel C) after addition of the FBGc, CLT values rose to 58% and 67% of the control value, respectively. No important change was found at higher levels of total fibrinogen i.e. 3.00 and 4.00 g/l (panels D and E, respectively). In the presence of total fibrinogen at 6 g/l (panel F), CLT values increased to 97% of the control value.

- In NPP_{1+3} spiked with the FXIIIc alone (Figure 8).
  - In NPP_{1+3} alone (panel A), mean CT and MCF values were about 270% and 27% of control values, respectively. Addition of FXIIIc at 0.97 IU/ml (total FXIII concentration 1.20 IU/ml) shortened CT to about 220% of control and increased MCF to about 39% of control (panel B); both these ROTEM variables in principle showed no further change when increasing doses of the FXIIIc were added (panels C–E). In NPP_{1+3} alone (panel A), CLT was about 35% of control. At all doses of the FXIIIc, this variable remained at a similar level.
  - In NPP_{1+3} spiked with the FBGc and tranexamic acid (Figure 9).
  - In NPP_{1+3} spiked with different doses of tranexamic acid, most MCF values were too low to be of use in evaluating CLT, except in the samples containing the inhibitor at 10 μmol/l. In NPP_{1+3} spiked with the FBGc (total fibrinogen 2 g/l), increasing doses of tranexamic acid did not alter MCF importantly but did prolong CLT in a dose-dependent manner when used at concentrations higher than 1.3 μmol/l. At 2.5 or 10 μmol/l of tranexamic acid, the CLT value was normalised or the fibrinolysis was fully blocked, respectively.

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**Figure 7:** ROTEM parameters derived from NPP_{1+3} to which the fibrinogen concentrate (FBGc) was added. Left panel: Thromboelastograms of the different samples. Right panel: CT, MCF and CLT values observed in samples A–F.
Influence of plasma-dilution on thrombin activity in CAT assay

As expected, we have found that the “thrombin peak” values decreased in a dilution-dependent way. This may be considered as a result of prothrombin dilution. However, the global marker ETP was not affected by the dilution up to the extent of 1+3 (Figure 2). Since the area under the CAT curve displayed obvious broadening when “peak thrombin” had been reached, ETP remaining at the original level is most likely due to postponement of thrombin decay. In other words, though the plasma-dilution can prolong the time-to-start of detectable thrombin and lower the maximum level of generated thrombin, the coagulation enzyme maintains its effect on fibrin polymerisation for a longer time. In an earlier study by other authors (8), this fact was attributed to a greater response of thrombin generation to dilution of tissue factor pathway inhibitor than to dilution of coagulants. The authors also suggested that the anti-thrombophilic effects of the protein C pathway were weakened as a consequence of protein S dilution.

As indicated by the turbidimetric data (Figure 3A, left), clotted fibrinogen was quantitatively reduced in a dilution-dependent way. Conflicting with the ordinary causal relationship between a lower fibrinogen concentration and formation of a looser fibrin network, the SEM images and flow measurements proved that the clotted fibrinogen in smaller amounts obtained from NPP$_{1+3}$ and NPP$_{2+2}$ had obviously tighter fibrin networks with a clear decrease in permeability (Figure 3, left) or porosity (Figure 4, right). This unexpected finding seems to be a result of the “extended-time” effect by thrombin (Figure 2, start tail time), which is “over-influential” in contrast to the poor source of fibrinogen.

In the ROTEM assay, CT reflects the time needed to find the first signal of fibrin visco-elasticity (Figure 6, right – top).
NPP<sub>1+3</sub>, the prolongation of CT detected by ROTEM but not by CAT indicate that plasma-dilution causes a decrease in fibrin formation rather than thrombin activity. In addition, a tight fibrin network is generally known to down-regulate the transport of fibrinolytic components and thus reduce plasmic digestion (16, 23). However, the declines in fibrin network porosity/permeability by plasma-dilution, as mentioned above, could not overcome the opposing effect of fibrinogen depletion; clots arising from the small amounts of fibrin were more liable to proteolysis, shown as the significant shortening of CLT in NPP<sub>1+3</sub> (Figure 6, right – bottom).

**Effects by the FBGc**

The FBGc added to NPP<sub>1+3</sub> increased the quantity of formed fibrin (Figure 3B, left), and made the fibrin network permeability which was already reduced to a moderate extent by plasma-dilution more evident (Figure 3B, right).

The confocal 3D microscopy was previously employed by our group (16), which indeed assisted in evaluation of fibrin network porosity. On a basis of the micrographic data, we earlier assumed that the major fibrin network is created at a definite stage of gelation, reflected in clotting time (CT). Later-formed fibrin may be incorporated into the existing network and increase fibrin fibre thickness (24). Since the SEM technique was introduced to our group, we have achieved better images with much higher resolution (17). In the present study, when NPP<sub>1+3</sub> was spiked with the FBGc to give a total fibrinogen concentration of 2.00 g/l, the SEM images clearly showed that the de novo network with further minimisation in fibrin fibre diameters and pore sizes was formed within the liquid space of the major fibrin network (Figure 5B). Because thrombin inactivation was delayed by the plasma-dilution (Figure 2), this phenomenon could be explained by the extended effect of thrombin on insufficient amounts of fibrin(ogen). The increase of fibrin fibre diameters subsequent to CT (24), as we earlier inferred, is negligible.

After addition of the FBGc, the enhancement of total fibrinogen from 2.00 to 4.00 g/l led to parallel increase in MCF (Figure 7C-F, right). However, the fibrinolysis which followed was independent on the concentrate doses; the declined tendency of CLT in the mentioned samples was comparably sharp despite the differences of fibrinogen levels. We consider that when the fibrinogen source became rich due to addition of FBGc, the formed fibrin fibres in greater numbers did aggregate laterally into thick bundles (Figure 5B, C). Since plasminogen is bound along the length of fibrin fibres, a cross-cleavage of individual fibres can be initiated by plasmin. The generated fibrin fragments re-create larger pores in the fibrin network, favouring transport of fibrinolytic components (25). By way of this mechanism, we may better understand that in NPP<sub>1+3</sub> containing total fibrinogen concentrations higher than 2.00 g/l (3.00–4.00 g/l), clots were conditional on fibrinolysis promoted by the formation of thick fibrin bundles, reflected by no further prolongation in CLT.
Effects by the FXIIIc

Fibrin cross-linking itself cannot be detected with SEM or flow measurement (18); however, the induced changes in fibrin viscoelasticity are probably evaluated using the technique of ROTEM (26, 27). We found no important difference in fibrin network porosity or permeability after addition of FXIIIc to NPP_{1+3} (Figure 3C, right; Figure 5D and E), but ROTEM analysis brought a moderate increase of MCF when the total FXIII concentration rose from 0.22 to 2.00 IU/ml (Figure 8, right - bottom). Even with the improvement of fibrin visco-elasticity by the FXIIIc, all the examined samples with total FXIII higher than 1 IU/ml had CLT values similar to the original level (NPP_{1+3} alone; Figure 8, right - bottom). This observation seems to negate data we reported recently, where increased concentrations of FXIII prolonged CLT more potently than FBGc in the NPP sample containing a thrombin or factor Xa inhibitor (18). However, different behavior of FXIII was found in different circumstances, where fibrinogen was depleted by plasma dilution but remained analogous to control when thrombin or factor Xa was inhibited. Fibrinogen is the basic material of fibrin cross-linked by FXIII. In samples lacking sufficient fibrinogen and hence fibrin, FXIII – even at relatively high levels can never strengthen clots by creating more covalent bonds between fibrin monomers. Thus, we assumed that only when fibrinogen insufficiency is already overcome and greater numbers of fibrin fibre are formed, could FXIIIc increase the proteolytic resistance to plasmin. This hypothesis was not verified by our in vitro investigation with NPP_{1+3} to which both FBGc and FXIIIc were added (data not shown). However, we used platelet-poor plasma, and according to recent findings it was found that the effect of exogenous FXIII is dependent on the presence of functional platelets(32, 33). We are planning new experiments to investigate the importance of functional platelets in our in vitro model.

Effects by the FBGc in the presence of a fibrinolysis inhibitor

Different does of FBGc (1.24 – 3.24 g/l) added to NPP_{1+3} can normalise fibrinogen concentrations in the plasma up to 2–4g/l. However, this treatment could not fully normalise the proteolytic resistance of fibrin clots, where the increased CLT values were only 67–70% of control (Figure 7, right – bottom).

Tranexamic acid is a synthetic derivative of the amino acid lysine, with an antifibrinolytic effect through a reversible blockade of lysine-binding sites on plasminogen molecules (28). The plasma level of tranexamic acid to inhibit fibrinolysis in vitro is 10 µmol/l (29), while a recent study reported that the therapeutic levels in plasma were much higher (30).

Accordingly, we assumed that the FBGc used in combination with a fibrinolysis inhibitor (such as tranexamic acid) would be more advantageous for improving the clot proteolytic resistance after plasma dilution. To assess this proposition, tranexamic acid at 0.4–10 µmol/l was added to NPP_{1+3} spiked with or without FBGc. In the absence of added FBGc, 0.4–2.5 µmol/l tranexamic acid was not effective at increasing clot stability (Figure 9), since the unchanged depletion of fibrinogen brought about a low MCF and hence the undetectable CLT. Notably, when fibrinogen depletion in NPP_{1+3} was already overcome by the addition of FBGc, this antifibrinolytic agent in the same concentrations did slow the proteolytic rate; a concentration of 2.5 µmol/l of the antifibrinolytic agent was sufficiently functional to normalise the CLT value. Although tranexamic acid alone at its therapeutic level of 10 µmol/l can entirely prevent plasmic digestion, for a fibrinolytic inhibitor if used in clinical settings, a lower dose is safer than a higher dose as regards the possible occurrence of thrombosis. Moreover, co-utilisation with the FBGc may not only aid the reversal of fibrinogen depletion after plasma-dilution, but also affect other haemostasis aspects, such as interactions of fibrinogen (ogen) with platelets, endothelial cells, leukocytes and some growth-factors (31).

Conclusion

Imbalance between influence of plasma-dilution on the global thrombin potential and fibrinogen concentration led to unexpected outcomes of fibrin properties i.e. the formed clot tended to be degraded by plasmin though its network structure remained obviously tighter/less permeable. This impairment of fibrin property

What is known about this topic?

- In traumatic patients, resuscitation treatment of intravascular volume may cause plasma dilution.
- Normal thrombin potential and fibrinogen depletion, together with a bleeding tendency, are frequently shown in these patients.
- The lack of thrombin insufficiency is explained by dilution of coagulation inhibitors rather than by dilution of coagulants.
- Fibrinogen depletion may result in increase of fibrin network porosity/permeability, reducing clot stability against proteolysis.
- Fibrinogen concentrates and a factor XIII (FXIII) concentrates have been used for bleeding control in different clinical trials.

What does this paper add?

- This is the first study in vitro using different laboratory methods to detect multiple changes in coagulation and proteolysis after plasma dilution.
- An imbalance between influence of plasma-dilution on thrombin potential and fibrinogen concentration led to unexpected outcomes of fibrin properties: the formed clot tended to be degraded by plasmin though its fibrin network remained tighter/less permeable.
- The high resolution of SEM images and the modifications of ROTEM assay brought about better understanding of fibrin fibre behaviours that regulate the proteolytic resistance.
- The fibrinogen concentrate, better used in combination with tranexamic acid, may reverse the fibrinogen depletion and also slowdown the proteolytic rate. The FXIII concentrate seems unhelpful in this regard at least in our in vitro model.
can be reversed by the fibrinogen concentrate, especially when used in combination with small doses of tranexamic acid. The FXIII concentrate is not effective in this regard, at least in our in vitro model using platelet-poor plasma. Although the artificial test system is helpful to show above interesting findings in vitro, further studies should address this issue in vivo. Moreover, the modified approaches of fibrin network permeability and of ROTEM analyses need to be standardised for optimising future work in this aspect.

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Conflicts of interest
None declared.

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