Fibrinogen and FXIII dose response effects on albumin-induced coagulopathy.

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Dose–response of fibrinogen and factor XIII concentrate for correcting albumin-induced coagulopathy

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Running head: Fibrinogen and FXIII in treating coagulopathy

Abstract

Objectives: Natural colloid albumin induces a lesser degree of dilutional coagulopathy than synthetic colloids. Fibrinogen concentrate has emerged as a promising strategy to treat coagulopathy, and factor XIII (FXIII) works synergistically with fibrinogen to correct coagulopathy following haemodilution with crystalloids. Objectives were to examine the ability of fibrinogen and FXIII concentrates to reverse albumin-induced dilutional coagulopathy.

Methods: High and low concentrations of both fibrinogen and FXIII were used to reverse coagulopathy induced by 1:1 dilution *in vitro* with 5% albumin of blood samples from healthy volunteers, monitored by rotational thromboelastometry (ROTEM).

Results: Haemodilution with albumin significantly attenuated EXTEM maximum clot firmness (MCF), alpha angle (AA), clotting time (CT) and clot formation time (CFT), and FIBTEM MCF ($p<0.001$). Following haemodilution, both doses of fibrinogen significantly corrected all ROTEM parameters ($p\leq0.02$), except the lower dose did not correct AA. Compared to the lower dose, the higher dose of fibrinogen significantly improved FIBTEM MCF and EXTEM MCF, AA and CFT ($p<0.001$). The lower dose of FXIII did not significantly correct any of the ROTEM parameters, and the high dose only improved EXTEM CT ($p=0.004$). All combinations of high/low concentrations of fibrinogen/FXIII significantly improved all ROTEM parameters examined ($p\leq0.001$). Fibrinogen concentration generally had a greater effect on each parameter than did FXIII concentration; the best correction of ROTEM parameters was achieved with high-dose fibrinogen concentrate and either low- or high-dose FXIII.

Conclusions: Fibrinogen concentrate successfully corrected initiation, propagation and clot firmness deficits induced by haemodilution with albumin, and FXIII synergistically improved fibrin-based clot strength.

Key words:

Albumin; dilutional coagulopathy; factor XIII concentrate; fibrinogen concentrate; ROTEM; thromboelastometry

Introduction

Critically ill patients commonly require volume resuscitation with intravenous colloids or crystalloids; however, volume resuscitation can lead to dilutional coagulopathy. The extent of dilutional coagulopathy depends on a number of factors including blood type [1], the degree of dilution and type of fluid used for resuscitation [2-6]. Haemodilution with colloids affects coagulation earlier and more extensively than haemodilution with crystalloids such as Ringer's solution and saline [3-6]. However, colloids such as albumin, gelatin, hydroxyethyl starch (HES) and dextran confer advantages over crystalloids, including enhanced duration of haemodynamic effects and less capillary leakage [7,8]. A number of studies have shown that synthetic colloids including gelatin, hydroxyethyl starches and dextran, affect haemostatic parameters in whole blood viscoelastic tests (thromboelastography [TEG®] and rotational thromboelastometry [ROTEM®]), to a greater extent than albumin [9-14]. Therefore, an initial volume substitution regime with 4-5% albumin, together with a restricted crystalloid regime, may be favourable.

To correct coagulopathy, fresh frozen plasma (FFP) is commonly used; however, FFP is slow to administer as it must be thawed prior to use and is associated with side-effects such as transfusion-associated circulatory overload and transfusion-related lung injury. In addition, FFP may exacerbate dilutional coagulopathy as it only contains coagulation factors at physiological levels, and so a large dose may be required in order to increase their levels.

Treatment with fibrinogen concentrate has emerged as a promising strategy to improve haemostasis during massive bleeding. Fibrinogen is the precursor of fibrin, which is cross-linked to form the basis of the clot, and is the first coagulation factor to fall to critical levels during massive bleeding [15,16]. Therapy with fibrinogen concentrate allows a precise dose of fibrinogen to be delivered quickly and in a small volume, and has been shown to

effectively reverse dilutional coagulopathy *in vitro* following haemodilution with albumin [9], HES [3], dextran [3] or Ringer's acetate [17,18].

Factor XIII (FXIII) is a transglutaminase that introduces fibrin-fibrin and fibrin-inhibitor cross-links, resulting in more mechanically stable clots. In addition, FXIII attenuates hyperfibrinolysis [19,20]. It has recently been shown that addition of FXIII alone does not enhance clot strength *in vitro* following haemodilution with Ringer's acetate [17]. However, FXIII synergistically enhances the reversal of dilutional coagulopathy with fibrinogen concentrate, following haemodilution with Ringer's acetate [17,18] or gelatin [21].

Haemodilution with albumin induces a lesser degree of coagulopathy than haemodilution with synthetic colloids; however, it remains unknown whether FXIII and fibrinogen work in synergy to restore clot strength following haemodilution with albumin. Moreover, some studies examining the correction of colloid-induced haemodilution with FXIII and fibrinogen have used doses far beyond the normal therapeutic range [17], and therefore do not represent the situation *in vivo* or address the question of whether the treatment strategy is economically viable. The aim of this study was to evaluate the ability of high and low doses of fibrinogen and/or high and low doses of FXIII concentrate to reverse albumin-induced dilutional coagulopathy *in vitro*, monitored by ROTEM plus a point-of-care device to measure whole blood prothrombin time (PT) and activated partial thromboplastin time (aPTT). We also evaluated the therapeutic cost per patient for each combination of therapies with factor concentrates.

Methods

Sampling

Blood samples (45 mL) were collected from ten healthy volunteers who had given written consent, and who had not taken medication for 7 days preceding blood sampling. The study was approved by the Ethical Board, Lund, Sweden (Registration Number: DNR 484). All procedures were performed in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Venipuncture was performed with a 22-gauge needle (BD Vacutainer PrecisionGlide™ Multi-Sample Needle, Plymouth, UK). Blood samples were collected in plastic vacuum tubes (2.7 mL PET BD Vacutainer® Coagulation Tubes, Stadler, Germany) containing 0.109 M buffered sodium citrate. The first tube for each sample was discarded. All samples were incubated in plugged tubes in a heating block at 37°C for 30 min directly before haemodilution, treatment and viscoelastic testing, as described by Sørensen et al [22].

Haemodilution and treatment

Following incubation at 37°C, citrated whole blood was diluted 1:1 with 5 % human albumin pre-warmed to 37°C (CSL Behring GmbH, Marburg, Germany; lot number 36144331A).

Human fibrinogen concentrate (RiaSTAP®, CSL Behring, Marburg, Germany) was dissolved in sterile water according to the manufacturer's instructions, to a final concentration of 20 mg/mL. Human FXIII concentrate (Fibrogammin®, CSL Behring, Marburg, Germany) was dissolved in sterile water, to a final concentration of 62.5 IU/mL. Following reconstitution, both test solutions were kept at 37°C. Varying doses of fibrinogen concentrate and/or FXIII were added to 1-mL samples of undiluted, pre-warmed whole blood or whole blood diluted 1:1 with 5% albumin, as described in Table 1. Samples were carefully mixed before being pipetted immediately into pre-warmed ROTEM plastic cups, as described below.

Analysis of coagulation parameters

Thromboelastometry was carried out using a ROTEM® analyser (TEM International GmbH, Munich, Germany) according to the manufacturer's instructions, and ROTEM-based assays were run for 60 min. Citrated test samples were recalcified with STARTEM® and the final concentration of ionised calcium was measured using a calcium electrode in a blood gas analyser. The concentration of calcium was confirmed to be >0.95 mmol/L in all samples, including for those containing albumin which is known to chelate calcium, thus the effect of ionised calcium concentration on ROTEM parameters was deemed to be negligible [23]. The extrinsic coagulation assay (EXTEM), was initiated with tissue factor and the following coagulation parameters were measured: maximum clot firmness (MCF), clotting time (CT), clot formation time (CFT) and alpha-angle (AA). MCF is the maximum firmness of the clot, and fibrin, platelets and FXIII all influence clot strength. CT measures time from the start of measurement until the initiation of clotting (when MCF of 2 mm has been reached). CFT is the time from initiation of clotting until a clot firmness of 20 mm is detected, and AA is the angle of tangent of clot firmness over time at 2 mm amplitude; both CFT and AA represent the speed at which the clot forms, and are primarily influenced by platelets and fibrinogen.

The FIBTEM assay was performed as for the EXTEM assay but with the addition of the platelet inhibitor cytochalasin D, in order to measure the fibrin-based component of clot strength. A surrogate measure of platelet activity was determined by calculating the difference between EXTEM MCF and FIBTEM MCF.

A point-of-care Hemochron® Junior device (ITC, Edison, US) was used to measure PT and aPTT in citrated blood samples. PT and aPTT were measured in both undiluted blood and

blood diluted 1:1 with albumin, and samples were also treated with 0.1 mL fibrinogen, 0.01 mL FXIII, or both.

Statistical analysis

A paired t-test was performed to assess the effect of dilution with albumin on ROTEM parameters. A two-way analysis of variance (ANOVA) was used to study the influence of fibrinogen concentrate and FXIII on coagulation, with a Dunnett's correction to allow for multiple comparisons. The level of statistical significance was set at $p<0.05$. Statistical analyses were performed using Stata software (version 12.0).

Results

Coagulopathy induced by haemodilution with albumin

In the first part of this study, the effects of haemodilution with 5% albumin were investigated. Haemodilution significantly attenuated all ROTEM parameters examined ($p<0.001$). An increase in EXTEM CT and CFT, along with a decrease in EXTEM AA, indicated that the clot was slower to form upon haemodilution (Figure 1). Decreases in EXTEM MCF and FIBTEM MCF indicated a reduction in the strength of the clot (Figure 2).

Effect of fibrinogen or *FXIII concentrates on albumin-induced coagulopathy*

In the second part of the study, the effects of adding different doses of fibrinogen and/or FXIII concentrate on ROTEM parameters following haemodilution were examined. Following dilution with albumin, the low dose of fibrinogen significantly corrected all measured parameters ($p\leq0.02$) except AA, and the high dose of fibrinogen concentrate significantly improved all measured ROTEM parameters including AA ($p<0.001$) [Table 2; Figures 1 and 2]. Compared to the lower dose of fibrinogen concentrate, the higher dose resulted in a

slightly improved FIBTEM MCF and EXTEM MCF and AA, and a lower CFT (all $p<0.001$) [Figures 1 and 2]. This demonstrated that addition of fibrinogen concentrate to samples diluted with albumin improved clot propagation and clot strength in a dose-dependent manner. A low dose of FXIII did not significantly correct any of the ROTEM parameters measured in samples diluted with albumin, and a high dose of FXIII significantly improved only EXTEM CT ($p=0.004$) [Figure 1a]. No statistical differences between the high and low doses of FXIII alone were observed for any ROTEM parameter.

Effect of fibrinogen concentrate plus *FXIII on albumin-induced coagulopathy*

All combinations of fibrinogen concentrate and FXIII concentrate significantly improved all ROTEM parameters examined ($p\leq0.001$) [Figures 1 and 2]. All ROTEM parameters were significantly improved ($p<0.001$) in samples spiked with high-dose FXIII and high-dose fibrinogen, as compared to those spiked with high-dose FXIII alone [Figures 1 and 2]. However, the concentration of fibrinogen generally had a greater effect on each parameter than did the concentration of FXIII; the best correction of ROTEM parameters was achieved with high-dose fibrinogen concentrate and either low- or high-dose FXIII. As compared to samples containing fibrinogen concentrate alone (either high or low dose), the addition of high-dose FXIII resulted in a significant increase in FIBTEM MCF. An increase in EXTEM MCF was also seen for samples spiked with high-dose fibrinogen. Compared with samples treated with low-dose fibrinogen alone, samples with low-dose fibrinogen plus high-dose FXIII had a significantly higher FIBTEM MCF and also EXTEM AA.

Effect of fibrinogen and FXIII on the contribution of platelets to clot strength following haemodilution

The contribution of platelets to clot strength was calculated by subtracting FIBTEM MCF from EXTEM MCF (data not shown). Dilution with albumin significantly reduced the platelet

contribution to clot strength ($p<0.001$). This attenuation was not significantly improved with any combination of high- or low-dose fibrinogen, and/or high- or low-dose FXIII (Table 2).

Effect of fibrinogen and FXIII on PT and aPTT following haemodilution

The integrity of both the extrinsic and intrinsic coagulation pathways was assessed before and after dilution with 5% albumin, by measuring PT and aPTT, respectively (data not shown). Both PT and aPTT were significantly prolonged following dilution with 5% albumin ($p<0.001$). Addition of high-dose fibrinogen concentrate and/or high-dose FXIII had no effect upon either parameter.

Discussion

Here, we show that fibrinogen and FXIII concentrates synergistically reverse albumin-induced coagulopathy. Treatment with both a high and low dose of fibrinogen concentrate was effective in correcting all measured ROTEM parameters, with the higher dose improving all parameters, except CT, to a significantly greater degree. The *in vitro* addition of either dose of FXIII had little effect on ROTEM parameters when added alone; however, when added to samples containing fibrinogen concentrate it resulted in a significant increase in fibrin-based clot strength.

The degree of impairment of ROTEM parameters induced by haemodilution with albumin is in agreement with an earlier study from our group [9]. It has previously been shown that addition of FXIII alone has no or little effect on ROTEM parameters following haemodilution with either Ringer's solution [17,18] or gelatin [21], and our results suggest that this is also

the case for the natural colloid albumin. In addition, our results are in agreement with *in vitro* studies that have examined the effect of fibrinogen and FXIII concentrates in correcting coagulopathy induced by dilution with a crystalloid or synthetic colloid [17,18,21]. All three studies also reported a synergistic effect when FXIII was added to samples containing fibrinogen concentrate. Taken together with the results from our study, these data suggest that FXIII and fibrinogen concentrates are effective for correcting dilutional coagulopathy induced by a range of resuscitation fluids.

It is important to characterise dilutional coagulopathy for different resuscitation fluids, and the ways to best treat them, as the mechanisms of coagulopathy vary. Both colloids and crystalloids can cause coagulopathy by dilution of coagulation factors such as fibrinogen and platelets, resulting in a reduction in clot strength, while haemodilution with synthetic colloids causes coagulopathy through inhibitory effects on clotting factors [24-26], platelet function [27,28] and fibrin polymerisation [10,29].

The contribution of FXIII, fibrinogen and also factor II (FII) to restoring clot strength following haemodilution has been explored in detail by Nielsen et al. [10], who suggested that colloids may compromise factor II (FII)-mediated FXIII-fibrin polymer interactions, leading to a decrease in clot initiation, propagation and strength as shown by thrombelastographic assays. Supplementation with either fibrinogen, FXIII or FII partially reversed these effects. Fibrinogen is the precursor of fibrin, which forms the basis of the clot, and FXIII stabilises the clot by improving the cross-linking of fibrin, platelets and matrix proteins during thrombus formation. This is reflected by results from our study, which show that addition of FXIII to haemodiluted samples containing fibrinogen results in an increase in fibrin-based clot strength. FXIII further enhances clot quality by increasing resistance of the clot to fibrinolysis

through FXIIIa-mediated covalent binding of α-plasmin inhibitor to fibrin molecules [30], and increased resistance to fibrinolysis was demonstrated *in vitro* following treatment with fibrinogen and FXIII in samples haemodiluted with Ringer's acetate [18].

In our study, we found that treatment with albumin significantly increased PT and aPTT. PT and aPTT measure the integrity of the extrinsic and intrinsic clotting pathways, in which thrombin plays a key role. Thrombin generation can also be measured using new ultra-specific, ultra-sensitive assays such as the recalcified coagulation assay (RECA). Using this assay, Stief demonstrated that haemodilution with 20% albumin (5% in our study) may increase thrombin generation, varying with albumin preparations from different sources [31].

Treatment with fibrinogen and FXIII had no effect on PT or aPTT following haemodilution with albumin in the present study. Similar results were seen following haemodilution with Ringer's solution [18]. Shenkman *et al.* [18] suggested that, following haemodilution with 60% Ringer's solution, thrombin generation declined by around 30%, which is consistent with the observation that patients with dilutional coagulopathy following major surgery also exhibit reduced thrombin generation [32]. Since FXIII and fibrinogen operate downstream in the coagulation cascade from thrombin, they are not expected to affect thrombin generation, and thus PT or INR. Interestingly, FFP does contain factors that promote thrombin generation, and would be expected to increase PT and INR following haemodilution. However, FFP was less effective than fibrinogen concentrate alone, or fibrinogen plus FXIII concentrates in correcting ROTEM parameters following haemodilution with Ringer's solution [17].

A study by Haas et al [17] showed that following 60% *in vitro* dilution with lactated Ringer's solution, a synergistic effect was evident upon addition of 70 IU/kg FXIII to samples

containing fibrinogen. In our study, we selected two doses of FXIII, with the lower corresponding to 20 IU/kg BW, which is within the dose range recommended by the manufacturer. However, in the study by Haas et al., a dose of FXIII corresponding to ~70 IU/kg BW was used, which is well beyond the recommended level *in vivo*. Despite using a lower dose, we still observed a synergistic effect when FXIII concentrate was used in addition to fibrinogen concentrate. Adding a higher dose of FXIII to samples containing fibrinogen concentrate did not further improve ROTEM parameters, suggesting that a lower dose is sufficient. Coagulation concentrates are costly, and although *in vitro* studies such as ours and others may indicate synergistic effects, the efficacy of combining fibrinogen and FXIII therapies needs to be tested *in vivo*.

It has been shown that levels of FXIII decrease significantly after major surgery, following haemodilution and blood loss [33,34]; however, critical thresholds for FXIII therapy remain unknown [35]. Further studies are needed to establish these thresholds, and to determine the effect of FXIII in reversing dilutional coagulopathy and correcting ROTEM parameters *in vivo*.

Haemodilution studies performed *in vitro,* such as the present study, have a number of limitations. *In vitro* models do not account for factors such as activation of procoagulation or fibrinolytic pathways in response to tissue trauma or shear stress, which contribute to impairment of blood clotting and coagulopathy *in vivo*. Also, this study was performed using blood from healthy volunteers, whereas trauma patients or those undergoing surgery may exhibit a wide range of coagulopathies, as well as hypothermia and shock, both of which affect coagulation.

Conclusions

Fibrinogen concentrate successfully corrected the initiation, propagation and maximum clot firmness deficits induced by haemodilution with albumin. FXIII concentrate had little impact on albumin-induced coagulopathy when added alone; however, when added at a physiological dose in combination with fibrinogen concentrate, FXIII synergistically improved fibrin-based clot strength. Further studies are needed to establish whether therapy with FXIII and fibrinogen concentrate can be used to treat dilutional coagulopathy *in vivo*, and to establish critical thresholds for FXIII therapy.

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Conflict of interest: Ulf Schött has received honoraria from CSL Behring. Fibrinogen concentrate (RiaSTAP®) was provided by CSL Behring for *in vitro* use in this study. Jennifer Hanna has no conflicts of interest to declare.

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Tables

Table 1. Treatment of haemodiluted blood samples with fibrinogen and factor XIII concentrates.

Sample	Fibrinogen concentrate		FXIII concentrate		Clinical cost
	Concentration	Corresponding dose in a 70-kg adult	Concentration	Corresponding dose in a 70-kg adult	(Euro)
1.	-	-	-	-	-
2	1 mg/mL	5 g (70 mg/kgbw)	-	-	15,000
3	2 mg/mL	10 g (150 mg/kgbw)	-	-	30,000
4	-	-	0.3 IU/mL	1500 IU (20 IU/kgbw)	3,600
5	-	-	0.6 IU/mL	3000 IU (40 IU/kgbw)	7,200
6	1 mg/mL	5 g (70 mg/kgbw)	0.3 IU/mL	1500 IU (20 IU/kgbw)	18,600
7	1 mg/mL	5 g (70 mg/kgbw)	0.6 IU/mL	3000 IU (40 IU/kgbw)	22,200
8	2 mg/mL	10 g (150 mg/kgbw)	0.3 IU/mL	1500 IU (20 IU/kgbw)	33,600
9	2 mg/mL	10 g (150 mg/kgbw)	0.6 IU/mL	3000 IU (40 IU/kgbw)	37,000

Table 2. Effect on ROTEM parameters of *in vitro* addition of fibrinogen concentrate and/or FXIII to samples diluted with albumin. Parameters were compared using a two-way ANOVA, with a Dunnett's correction to allow for multiple comparisons. Bold = statistically significant ($p<0.05$).

	Comparison of untreated sample with:							
	Low fibrinogen	High fibrinogen	Low FXIII	High FXIII	Low fibrinogen + low FXIII	High fibrinogen + low FXIII	Low fibrinogen + high FXIII	High fibrinogen + high FXIII
EXTEM CT	**<0.001**	**<0.001**	0.46	**0.004**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
EXTEM CFT	**0.02**	**<0.001**	0.41	1.00	**<0.001**	**0.001**	**<0.001**	**<0.001**
EXTEM AA	0.10	**<0.001**	0.88	1.00	**<0.001**	**<0.001**	**<0.001**	**<0.001**
EXTEM MCF	**<0.001**	**<0.001**	1.00	1.00	**<0.001**	**<0.001**	**<0.001**	**<0.001**
FIBTEM MCF	**<0.001**	**<0.001**	1.00	1.00	**<0.001**	**<0.001**	**<0.001**	**<0.001**
EXTEM MCF − FIBTEM MCF	1.00	1.00	1.00	1.00	1.00	**0.02**	1.00	0.20

Figures

Figure 1. a) Clotting time, b) clot formation time, and c) alpha angle in the EXTEM assay, with or without 1:1 dilution with 5% albumin and *in vitro* addition of fibrinogen concentrate and/or FXIII concentrate. Low fib = 1 mg/mL fibrinogen concentrate; high fib = 2 mg/mL fibrinogen concentrate; low FXIII = 0.3 IU/mL FXIII concentrate; high FXIII = 0.6 IU/mL FXIII concentrate.

*Significant difference as compared to samples diluted with albumin, without fibrinogen and/or FXIII concentrate

Figure 2. a) EXTEM maximum clot formation, and b) FIBTEM maximum clot formation, with or without 1:1 dilution with 5% albumin and *in vitro* addition of fibrinogen concentrate and/or FXIII concentrate. Low fib = 1 mg/mL fibrinogen concentrate; high fib = 2 mg/mL fibrinogen concentrate; low FXIII = 0.3 IU/mL FXIII concentrate; high FXIII = 0.6 IU/mL FXIII concentrate.

*Significant difference as compared to samples diluted with albumin, without fibrinogen and/or FXIII concentrate