A calcium-containing electrolyte-balanced hydroxyethyl starch (HES) solution is associated with higher factor VIII activity than is a non-balanced HES solution, but does not affect von Willebrand factor function or thromboelastometric measurements - results of a model of *in vitro* haemodilution

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Background. Hydroxyethyl starch (HES) is known to impair blood coagulation. The impact of calcium-containing, balanced carrier solutions of HES on coagulation is controversial. We investigated the effects of increasing degrees of haemodilution with modern 6%, electrolyte-balanced HES *vs* non-balanced HES on coagulation *in vitro*, and compared the balanced HES to a balanced crystalloid solution for an internal control.

Materials and methods. Blood samples from ten healthy volunteers were diluted *in vitro* by 20%, 40% and 60% with either calcium-containing balanced 130/0.42 HES, non-balanced 130/0.4 HES or balanced crystalloid. In all samples, blood counts, prothrombin time ratio, activated partial thromboplastin time, ionized calcium, factor VIII activity, von Willebrand factor antigen, von Willebrand factor collagen binding activity, and von Willebrand factor activity were determined, and activated rotational thromboelastometry (EXTEM and FIBTEM assays) was performed.

Results. Haemodilution impaired coagulation in a dilution-dependent manner as determined by both conventional laboratory assays and thromboelastometry. Ionized calcium increased with balanced HES ($p \leq 0.004$), but decreased with non-balanced HES ($p \leq 0.004$). Prothrombin time ratio ($p \leq 0.002$) and factor VIII levels ($p = 0.001$) were better preserved with balanced HES than with non-balanced HES in dilutions ≥40%. Thromboelastometry showed no differences between values in blood diluted with the balanced or non-balanced HES.

Discussion. *In vitro*, a balanced calcium-containing carrier solution of 6% HES 130/0.42 preserved coagulation better than did non-balanced HES 130/0.4 as quantified by conventional coagulation assays, but not in activated thromboelastometry. One explanation could be the increased ionized calcium levels after dilution with calcium-containing carrier solutions.

Keywords: blood coagulation disorder, balanced solutions, hydroxyethyl starch, von Willebrand factor, thromboelastometry.

Introduction

Crystalloid and colloid solutions are used for volume replacement in patients requiring haemodynamic support[1,2]. These fluids were shown to induce a dilutional coagulopathy[3,4]. In addition, colloids cause specific impairment to coagulation[4-6]. The current understanding of the mechanisms involved in hydroxyethyl starch (HES)-induced coagulopathy include impaired fibrin polymerisation[4,7], and a von Willebrand type 1-like syndrome[8].

Modern HES is characterised by a mean molecular weight (MW) of 130 kDa and a degree of substitution (DS) of 0.4-0.42[9]. Latest developments have focused on carrier solutions[9,10]. Electrolyte-balanced solutions were found to have positive effects on electrolytes and pH homeostasis[10,11], which may also affect coagulation[12].

Recent studies investigating modern, electrolyte-balanced and non-balanced HES have involved thromboelastometric analyses[13,14], which did not confirm a benefit of balanced carrier solutions in HES-induced coagulopathy. At the time of writing this paper there is a lack of controlled data on conventional laboratory tests of coagulation and the von Willebrand-like syndrome.

The aim of this study was to investigate the impact of a calcium-containing balanced *vs* non-balanced carrier solution of 6% HES 130/0.4-0.42 compared to a balanced crystalloid solution on plasma coagulation, on the von Willebrand factor and on thromboelastometry in an *in vitro* model of haemodilution.

Materials and methods

This study was approved by the Ethics Committee of Charité-University Medicine Berlin, Campus Charité Mitte (EA2/006/07, amendment 2). It was performed in compliance with the Declaration of Helsinki. Written informed consent to participation in the study was obtained prior to any study-related activity.

Blood sampling and processing

Ten healthy volunteers (five women/five men, four with blood group O, six with blood group A) participated in this study. Their median age was 27.5 years (interquartile range [IQR] 23.0-37.3 years), median body mass index 22.2 kg/m^2 (IQR 20.8-26.0 kg/m^2). Criteria for exclusion from the study were a history of bleeding, history of renal, hepatic or haematological diseases, intake of any medication known to be capable of altering coagulation and/or platelet function in the 14 days prior to blood withdrawal, age below 18 years, and lack of written informed consent.

Blood sampling. Free-flowing venous blood samples were taken from a cubital vein by sterile, gentle aspiration using a 21-gauge butterfly cannula. The first 2 mL of blood for blood gas analysis and blood group verification were collected using syringes containing dry, electrolyte-balanced heparin (PICO50, Radiometer Medical ApS, Brønshøj, Denmark). Blood for coagulation assays including thromboelastometry was collected using 20 mL-syringes prefilled with 2 mL of 3.13% sodium citrate (Natriumcitrat-Lösung 3.13% Eifelfango®, Eifelfango Chem. Pharm. Werke, Bad Neuenahr-Ahrweiler, Germany), resulting in a final concentration of sodium citrate 1:9 v/v. Two millilitres of blood were collected for blood count measurements using plastic tubes containing potassium ethylenediaminetetraacetic acid (BD Vacutainer® EDTA Tubes, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). All tubes and syringes were gently inverted repeatedly to ensure that the blood was thoroughly mixed with the anticoagulants.

In vitro haemodilution. Three solutions were selected for *in vitro* haemodilution: an electrolyte-balanced HES (balHES) 6%, MW 130 kDa, DS 0.42 (Tetraspan 6%®, B. Braun Melsungen AG, Melsungen, Germany), a non-balanced HES (nbalHES) 6%, MW 130 kDa, DS 0.4 (Voluven®, Fresenius Kabi Deutschland AG, Bad Homburg, Germany), and an electrolyte-balanced crystalloid (balCry) (Jonosteril®, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany). The physicochemical characteristics of these solutions are given in Table I. All solutions were prediluted 1:9 v/v with 3.13 % sodium citrate (Natriumcitrat-Lösung 3.13 % Eifelfango®, Eifelfango Chem. Pharm. Werke, Bad Neuenahr-Ahrweiler, Germany) to obtain stable concentrations of sodium citrate in both undiluted and diluted citrated blood samples. Aliquots of citrated blood were either diluted by balHES, nbalHES or balCry by 20%, 40% and 60% to a total volume of 5 mL. Undiluted aliquots served as control samples.

Platelet-poor, citrated plasma. When measurements of blood counts, blood gas analyses and thromboelastometry had been performed, remaining samples where centrifuged twice for 10 minutes at 4,000 rpm each. The supernatant was filled into separate tubes after each step and was flash-frozen. Plasma samples were stored at −80 °C until further analysis.

Table I - Physicochemical characteristics of the diluent solutions for *in vitro* haemodilution.

	(1) Electrolyte-balanced 6% HES 130/0.42	(2) Non-balanced 6% HES 130/0.4	(3) Electrolyte-balanced crystalloid
Basis	Poly(O-2-hydroxyethyl) starch	Poly(O-2-hydroxyethyl) starch	n.a.
Concentration [g/L]	60.0	60.0	n.a.
Mean molecular weight [kDa]	130	130	n.a.
Molar substitution	0.42	0.38-0.45	n.a.
Sodium [mmol/L]	140	154	137
Potassium [mmol/L]	4.0	n.a.	4.0
Calcium [mmol/L]	2.5	n.a.	1.65
Chloride [mmol/L]	118	154	110
Acetate [mmol/L]	24	n.a.	36.8
Magnesium [mmol/L]	1.0	n.a.	1.25
Malate [mmol/L]	5.0	n.a.	n.a.
Theoretical osmolarity [mOsmol/L]	296	308	291
pH	5.6-6.4	4.0-5.5	5.0-7.0

For in vitro haemodilution an electrolyte-balanced HES 6%, MW 130 kDa, DS 0.42, a non-balanced HES 6%, MW 130 kDa, DS 0.4, and an electrolyte-balanced crystalloid were used as diluents solutions. The physicochemical characteristics of the three solutions are presented as outlined in the official product information. Abbreviations: HES: hydroxyethyl starch; n.a.: not applicable.

Laboratory analysis

Blood count. An automatic blood count using a Sysmex XE-2100 (Sysmex Deutschland GmbH, Norderstedt, Germany) was performed for baseline determination of haematocrit and platelet count in EDTA-blood, and for haematocrit (Hct) in all samples to check on the degree of haemodilution.

Ionized calcium. Ionized calcium (Ca^{2+}) levels at baseline and in diluted samples prior to re-calcification were determined by blood gas analyses using an ABL 725, Radiometer Medical ApS, Brønshøj, Denmark.

Blood group. We verified blood groups of the ABO system using a bedside test (Medtrokarte®, Medtro GmbH, Leimen-Gau, Germany).

Conventional coagulation parameters. Conventional coagulation parameters were measured at the Institut für Medizinische Diagnostik Oderland, Frankfurt (Oder), Germany. Frozen plasma samples were thawed in approved water baths (GFL-1003, Gesellschaft für Labortechnik mbH, Burgwedel, Germany). Activated partial thromboplastin time (aPTT), prothrombin time ratio (PTR) according to Quick, plasma factor VIII activity (FVIII), von Willebrand factor antigen level (vWF:Ag), and von Willebrand factor activity (vWF:Act) were measured, immediately after thawing the samples, on a fully automated coagulation analyser (ACL TOP, Instrumentation Laboratory GmbH, Kirchheim bei München, Germany). PTR, aPTT, and FVIII were quantified turbidimetrically using reagents from Instrumentation Laboratory GmbH (Kirchheim bei München, Germany). Results of FVIII were primarily given as percentage activity of commercially available reference plasma (Instrumentation Laboratory GmbH, Kirchheim bei München, Germany). vWF:Ag and vWF:Act were measured using latex immunoassays (HemosIL™ von Willebrand Factor Antigen Kit and HemosIL™ von Willebrand Factor Activity Kit, Instrumentation Laboratory GmbH, Kirchheim bei München, Germany). The automated vWF:Act assay quantifies vWF activity using specific anti-vWF monoclonal antibodies that are directed against the platelet-binding site of vWF[15]. Additionally, von Willebrand factor collagen-binding activity (vWF:CBA) was assessed using the Technozyme® vWF:CBA Enzyme Linked Immunosorbent Assay (ELISA)-Kit (Technoclone GmbH, Vienna, Austria).

Thromboelastometry

Activated rotational thromboelastometry, as previously described in detail[16], was carried out on a ROTEM® delta (TEM International GmbH, Munich, Germany) according to the manufacturer's instructions, using the recommended test kits provided by the manufacturer. All samples were analysed within 4 hours after venipuncture by two activated tests in parallel: EXTEM, using tissue factor as an activator, and FIBTEM, which is activated the same way but uses cytochalasin D for platelet inhibition[16]. Run time was predefined as 30 minutes at 37 °C. The following thromboelastometric parameters were analysed in an EXTEM test: clotting time (CT) and clot formation time (CFT) in seconds, maximum clot firmness (MCF) in millimetres. Of the FIBTEM test, only MCF was considered.

Statistical analysis

Given the gender dependence of haematocrit and blood group dependence of factor VIII and the vWF parameters[17], reference values were introduced for each participant, the results of the undiluted plasma sample being defined as the reference (100%), and results of diluted plasma samples being relative to that reference value. Ionized calcium values in diluted samples are also given as a percentage of the corresponding undiluted value.

Since this analysis was designed as an exploratory investigation, no statistical sample size calculation was conducted. Due to the non-symmetrical distribution of data only non-parametric tests were performed. Results are given as median and interquartile ranges. The effect of haemodilution on ionized calcium, i.e. intragroup comparisons, was analysed using Wilcoxon's test for paired observations. We applied the Mann-Whitney U-test for intergroup analysis to evaluate the effects of the diluent solutions.

The primary α level was set at 5%. Corrections for multiple testing were made using Bonferroni's method. Consequently, p values of ≤ 0.005 were considered statistically significant for ionized calcium, whereas for all other parameters p values ≤ 0.008 were considered statistically significant. All numerical calculations and statistical analyses were carried out using PASW Statistics for Windows (version 18, SPSS Inc., Chicago, IL, USA, licensed for Charité-University Medicine Berlin, Berlin, Germany).

Results

Baseline blood counts (EDTA blood), values of standard coagulation parameters (citrated blood), and ionized calcium (heparinised blood) were within normal ranges (Tables II and III). Only vWF antigen and vWF activity in the sample from one volunteer exceeded normal ranges (Table II). Thromboelastometry-derived parameters (CT and CFT in seconds, MCF in mm) were within normal ranges at baseline (Figure 1). The values of haematocrit, PTR, aPTT, and Ca^{2+} measured in citrated blood samples at baseline and after haemodilution are given in Table III, whereas the results for FVIII and vWF parameters are presented in Table IV.

Haemodilution resulted in the aimed for degrees of dilution (Table III). As expected from the calcium content of the diluents, compared to undiluted samples, progressive haemodilution significantly increased Ca^{2+}-levels in calcium-containing balHES ($p \leq 0.004$) at all dilutional degrees and in balCry in 40% and 60% dilutions ($p \leq 0.004$), whereas Ca^{2+} significantly decreased in nbalHES ($p \leq 0.004$) at all degrees of dilution (Table III).

Table II - Baseline values of blood counts, ionized calcium, and von Willebrand factor parameters.

	Reference range	Baseline values
Haematocrit [%]		
Male (n=5)	40-52	43 (43-44)
Female (n=5)	35-47	40 (39-40)
Platelet count [$\times 10^9$/L]	150-400	231 (181-277)
Ionized calcium [mmol/L]	1.15-1.29	1.21 (1.20-1.24)
Factor VIII [%]	50-150	62 (51-68)
vWF-Ag [%]		
Blood group 0 (n=4)	42-141	89 (81-142)
Blood group A (n=6)	66-176	119 (103-137)
vWF:Act [%]		
Blood group 0 (n=4)	40-126	80 (74-129)
Blood group A (n=6)	40-163	100 (91-112)
vWF:CBA [U×10-3/L]	0.6-1.3	1.0 (0.7-1.2)

Absolute values of haematocrit and platelet count in EDTA-blood, ionized calcium in heparinised blood, and absolute values of factor VIII and the vWF parameters vWF antigen (vWF:Ag), vWF activity (vWF:Act), and vWF collagen-binding activity (vWF:CBA) prior to haemodilution. Results are given as median (IQR).

Effect of the carrier solution

balHES vs *nbalHES*. Ca^{2+} levels were higher ($p<0.001$) in balHES-diluted samples than in nbalHES-diluted samples at all degrees of dilution. With balHES, PTR was higher in dilutions of 40% and above ($p \leq 0.002$) (Table III). FVIII levels were higher in balHES solutions at 40% and 60% dilution ($p=0.001$) (Table IV). Except for higher values of vWF:CBA in nbalHES at 40% dilution ($p=0.003$), there were no differences in aPTT, vWF:Ag, vWF:Act or vWF:CBA between the two HES groups (Tables III and IV). In terms of thromboelastometry, there were no differences between the calcium-containing balanced and the non-balanced HES regarding the analysed parameters (Figure 1).

balHES vs *balCry*. A higher PTR ($p=0.002$) was observed after 40% dilution with balHES compared to balCry (Table III). Ca^{2+} and FVIII levels were higher in balHES in dilutions of 40% and above ($p_{Ca2+}<0.001$,

Table III - Effect of in vitro haemodilution on haematocrit, conventional coagulation parameters, and ionized calcium.

	(1) Electrolyte-balanced 6% HES 130/0.42	(2) Non-balanced 6% HES 130/0.4	(3) Electrolyte-balanced crystalloid
$Haematocrit_{rel}$ [%]			
Undiluted samples	100	100	100
Diluted by 20 %	80.8 (79.8-81.5)	80.8 (79.8-81.5)	80.8 (79.8-81.5)
Diluted by 40 %	61.1 (59.7-61.5)	59.5 (58.9-61.0)	60.0 (59.1-61.1)
Diluted by 60 %	40.0 (38.9-40.9)	38.9 (38.6-40.3)	38.9 (38.6-40.3)
Prothrombin time ratio [%]			
Undiluted samples	90.0 (88.5-95.5)	90.0 (88.5-95.5)	90.0 (88.5-95.5)
Diluted by 20 %	79.5 (77.8-83.5)	75.0 (73.8-79.5)	78.0 (75.0-79.3)
Diluted by 40 %	63.5 (61.0-66.5)[b,c]	54 (50-58.8)[a]	56.5 (49.0-59.0)[a]
Diluted by 60 %	46.0 (42.8-53.3)[b]	35 (32.8-38.5)[a]	43.0 (41.0-46.8)
aPTT [s]			
Undiluted samples	31.5 (31.0-34.0)	31.5 (31.0-34.0)	31.5 (31.0-34.0)
Diluted by 20 %	31.5 (30.0-32.5)	32.0 (31.0-35.0)	32.5 (32.0-34.5)
Diluted by 40 %	34.0 (32.0-35.0)	36.5 (33.5-39.0)	38.0 (36.0-40.5)
Diluted by 60 %	46.0 (43.0-54.0)	55.0 (51.0-77.0)	57.0 (52.0-67.5)
Ca^{2+} [%]			
Undiluted samples	100	100	100
Diluted by 20 %	116.7 (116.1-135.0)[b]	83.3 (77.9-83.9)[a]	100 (100.0-117.5)
Diluted by 40 %	150.0 (140.5-152.5)[b,c]	58.6 (50-66.7)[a]	116.7 (114.3-117.5)[a]
Diluted by 60 %	177.4 (164.3-187.5)[b,c]	41.4 (33.3-51.8)[a]	116.7 (114.3-135.0)[a]

Haematocrit, activated partial thromboplastin time (aPTT), and ionized calcium (Ca2+) before and after in vitro haemodilution with nbalHES, balHES, and balCry. Reference ranges are: prothrombin time ratio 70 - 130%, aPTT 27.6 - 34.3 seconds. Results are given as median (IQR). The superscripts indicate statistically significant differences ($p \leq 0.008$ for aPTT, $p<0.0056$ for Ca2+) if applicable: a = significant in comparison to balHES, b = significant in comparison to nbalHES, c = significant in comparison to balCry. Abbreviations: aPTT: activated partial thromboplastin time, bal: balanced, Ca2+: ionized calcium, Cry: crystalloid, HES: hydroxyethyl starch, nbal: non-balanced.

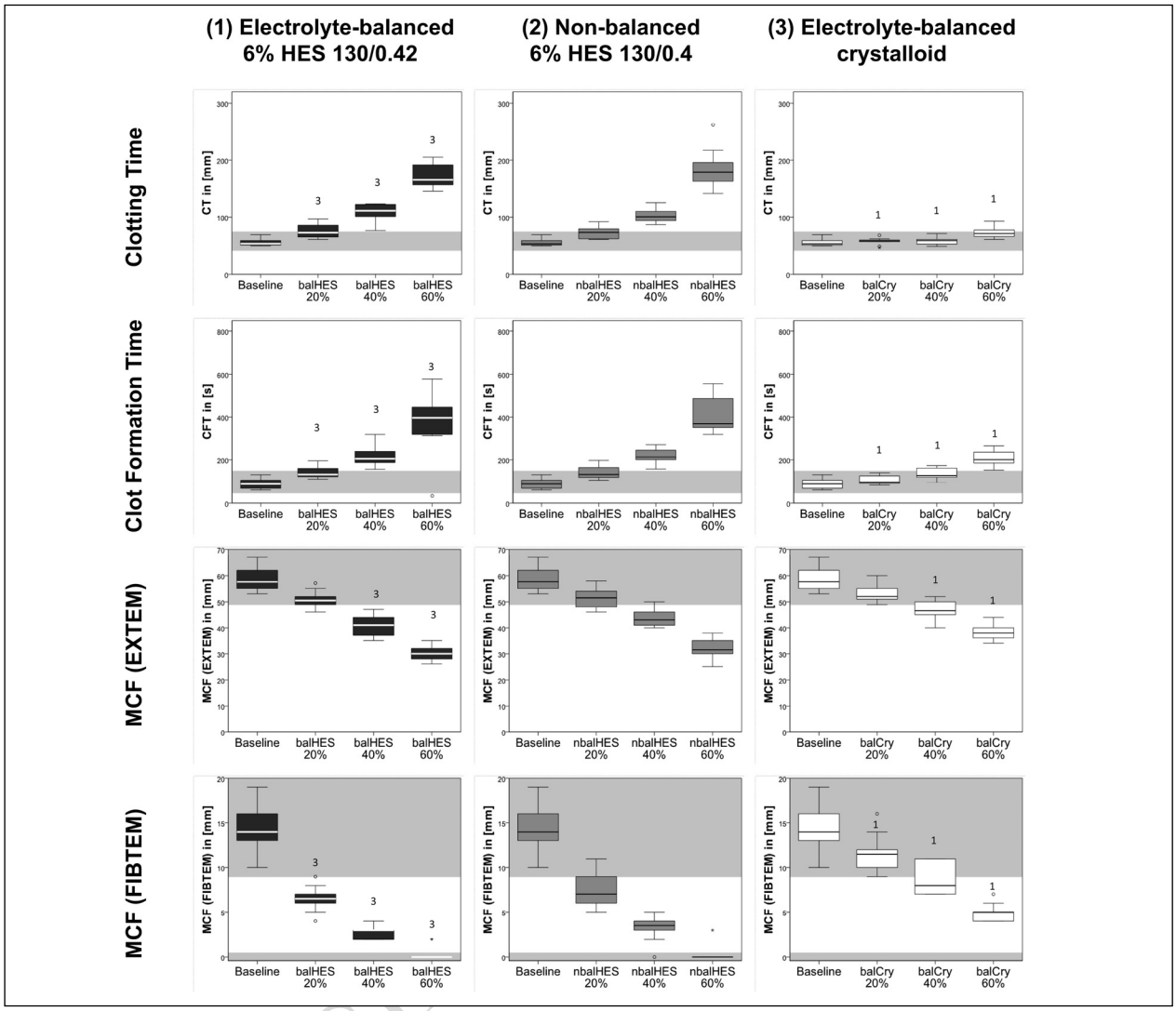

Figure 1 - Effect of *in vitro* haemodilution on thromboelastometry parameters.
CT, CFT and MCF of the EXTEM-test, and MCF of the FIBTEM-test of activated rotational thromboelastometry before and after progressive *in vitro* haemodilution with balHES, nbalHES, and balCry. Reference ranges for thromboelastometry parameters (shaded area) were taken from a multi-centre investigation on reference ranges for ROTEM® [15]. Results are presented as box-and-whisker plots. The superscripts show the statistical significance ($p \leq 0.008$) of any differences if applicable: 1 = significant in comparison to balHES, 2 = significant in comparison to nbalHES, 3 = significant in comparison to balCry. Abbreviations: bal: balanced; Cry: crystalloid; CFT: clot formation time, CT: clotting time, HES: hydroxyethyl starch, MCF: maximum clot firmness, nbal: non-balanced.

$p_{FVIII} \leq 0.007$) (Tables III and IV). Furthermore, vWF:Ag ($p<0.001$), and vWF:Act ($p=0.005$) were higher in balHES in 40% dilution, while vWF:CBA was lower at 60% dilution ($p=0.007$) (Table IV). In thromboelastometry, CT_{EXTEM} and CFT_{EXTEM} were prolonged in balHES ($p \leq 0.007$) at all degrees of dilution. MCF_{EXTEM} was lower for balHES at 40% and 60% dilution ($p \leq 0.004$) compared to balCry. MCF_{FIBTEM} was decreased ($p<0.001$) in balHES at all degrees of dilution (Figure 1).

Discussion

This study assessed the impact of a calcium-containing balanced carrier solution *vs* a non-balanced (sodium chloride) carrier solution of modern third-generation HES (130/0.4-0.42) on coagulation in a model of dilutional coagulopathy *in vitro*. The balanced HES was also compared to a balanced crystalloid. The main findings of this investigation were higher PTR and FVIII levels, and increased ionized calcium levels after haemodilution using a calcium-containing balanced carrier solution compared to a non-balanced one. Another finding was that parameters derived from rotational thromboelastometry did not reflect the above listed changes that were detected by conventional coagulation tests.

Table IV - Effect of in vitro haemodilution on relative values for factor VIII and von Willebrand factor parameters.

	(1) Electrolyte-balanced 6% HES 130/0.42	(2) Non-balanced 6% HES 130/0.4	(3) Electrolyte-balanced crystalloid
Factor $VIII_{rel}$ [%]			
Undiluted samples	100	100	100
Diluted by 20 %	79 (75-88)	74 (71-77)	72 (68-75)
Diluted by 40 %	64 (60-67)[b,c]	49 (43-52)[a]	43 (40-49)[a]
Diluted by 60 %	39 (35-40)[b,c]	31 (26-31)[a]	33 (27-35)[a]
$vWF:Ag_{rel}$ [%]			
Undiluted samples	100	100	100
Diluted by 20 %	70 (67-73)	69 (68-72)	69 (67-71)
Diluted by 40 %	49 (46-51)[c]	47 (44-49)	42 (38-44)[a]
Diluted by 60 %	29 (28-30)	28 (26-31)	28 (27-29)
$vWF:Act_{rel}$ [%]			
Undiluted samples	100	100	100
Diluted by 20 %	70 (68-75)	71 (69-74)	72 (68-77)
Diluted by 40 %	49 (46-52)[c]	46 (44-49)	44 (36-46)[a]
Diluted by 60 %	33 (30-34)	29 (25-33)	29 (28-31)
$vWF:CBA_{rel}$ [%]			
Undiluted samples	100	100	100
Diluted by 20 %	71 (60-77)	73 (69-79)	73 (69-84)
Diluted by 40 %	43 (40-46)[b]	50 (50-56)[a]	44 (37-50)
Diluted by 60 %	24 (21-29)[c]	27 (22-30)	29 (28-31)[a]

Factor VIII and the vWF parameters vWF antigen (vWF:Ag), vWF activity (vWF:Act), and vWF collagen-binding activity (vWF:CBA) before and after progressive *in vitro* haemodilution with nbalHES, balHES, and balCry. Results are given as percentage of undiluted values, presented as median (IQR). The superscripts show statistically significant differences ($p \leq 0.008$) if applicable: a = significant in comparison to balHES, b = significant in comparison to nbalHES, c = significant in comparison to balCry. Abbreviations: bal: balanced, Cry: crystalloid, HES: hydroxyethyl starch, nbal: non-balanced, rel: relative, vWF: von Willebrand factor.

Effect of the carrier solution (balHES *vs* nbalHES)

Conventional coagulation assays. In this *in vitro* study, the calcium-containing balHES preserved coagulation better than did nbalHES, based on assessments of PTR and FVIII. These results are inconsistent with previous *in vivo* data[18], but not contradicted by other prior *in vitro* data on 30% haemodilution[19]. In those *in vitro* and *in vivo* studies using first-generation HES, no significant differences in conventional coagulation results, i.e. aPTT only[19] or aPTT, prothrombin time, fibrinogen, FVIII and vWF[18], were observed between calcium-containing balanced or non-balanced HES solutions[18,19]. The present study could not show significant differences in aPTT between samples diluted with balanced or non-balanced carrier solution of modern HES preparations.

The Ca^{2+} content of the carrier solutions differed. In our study the balanced HES solution contained 2.5 mmol/L Ca^{2+} while the nbalHES contained no Ca^{2+}. The physiology of coagulation and laboratory coagulation assays are influenced by Ca^{2+} [20] and the structural integrity of factors VIII and V depends on Ca^{2+} [21]. PTR is directly influenced by Ca^{2+} concentrations and, among others, by factor V levels[22]. In the *in vivo* study of non-balanced *vs* balanced first-generation HES, in which no difference regarding conventional laboratory data was seen, any changes in calcium ion levels were counteracted by substitution of calcium[18]. It is possible that the better preservation of coagulation assay parameters by balHES in our study was due to higher Ca^{2+} levels *in vitro*.

Additionally, our study assessed the impact of the carrier solutions of low-molecular weight HES on the von Willebrand type 1-like syndrome, which has also been described in association with medium- and low-molecular weight HES[8]. In this analysis, vWF:CBA was better preserved in 40% dilution in nbalHES. vWF antigen and vWF activity did not differ between the two HES groups. Our results confirm a decrease of all three investigated vWF parameters that exceeds the degree of dilution, which might be interpreted as a criterion for the vWF type 1-like syndrome. Nonetheless, as vWF:CBA differed between the calcium-containing balanced and non-balanced HES at only one dilutional level in our study, it could be concluded that there is no relevant impact of the carrier solution on the vWF system, which is in line with previous *in vivo* data[18].

Thromboelastometry. Contrary to the findings of conventional coagulation assays, we found no differences

between the two carrier solutions of HES with regard to clot initiation (CT) and formation (CFT) times or maximum clot firmness (MCF) measured by activated thromboelastometry. This is in agreement with the findings of previous *in vitro* studies on third-generation HES using activated rotational thromboelastometry[13,14]. The study by Casutt and colleagues compared the effects on coagulation of both 6% balanced HES 130/0.42 and non-balanced HES 130/0.4 in 33% and 66% dilution, and reported no significant differences in NATEM, INTEM, or EXTEM in the parameters CT, CFT, alpha-angle, and MCF[13]. These and our results could indicate either that there is really no impact of the carrier solution of the HES or that there is no impact that can be measured by activated thromboelastometry. Concerning the latter possibility, standard coagulation tests have slightly better imprecision than have CT and CFT of activated rotational thromboelastometry[16]. However, there seems to be a measurable effect of the carrier solution in conventional coagulation assays *in vitro*. This finding requires further investigation in clinical studies of the effects of dilution with modern balanced HES solutions.

Crystalloid *vs* colloid

Conventional coagulation assays. For the first time this study analysed the impact of escalating dilution with modern low-molecular HES on conventional coagulation assays, the von Willebrand system and viscoelastic coagulation monitored by rotational thromboelastometry. At first glance, the results of conventional coagulation assays may indicate a more compromised coagulation with crystalloids, since FVIII, vWF antigen and vWF activity were lower in balCry than in balHES at some degrees of dilution. These results support findings from a previous *in vitro* study, which stated that coagulation was impaired more by crystalloids than by HES[23]. On the other hand, conflicting results had been published earlier, with thromboelastography being affected even more by colloids than by crystalloids[6]. The authors concluded that conventional laboratory assays, such as aPTT and coagulation factor activities, should not be relied upon in HES-induced coagulopathy[23]. This view is supported by the fact that turbidimetric measurements are vulnerable to artefacts caused by a well-known physicochemical phenomenon: HES solutions are polydispersed colloids, and colloids scatter light[24]. Scattering of light increases turbidity and HES was shown to increase turbidity[25]. This phenomenon has already been described to result in falsely elevated values when measuring fibrinogen levels[25]. Coagulation assays for aPTT, PTR, and FVIII in this study used turbidimetric methods. Although the methods of determination for these parameters differ from fibrinogen measurements, HES-induced increase of turbidity cannot be excluded to have interfered with the measurements, and this should raise caution in the interpretation of conventional coagulation results when high degrees of dilution with colloid solutions are present.

Moreover, concerning the vWF system, dilution with balHES yielded higher vWF antigen levels and vWF activity than balCry. This finding is not consistent with the results of a previous *in vivo* study on cardiopulmonary bypass priming solutions, in which plasma levels of vWF antigen were further depressed after priming with HES compared to a balanced crystalloid[26]. As vWF is stored in endothelium[27], which our model is lacking, and the mechanisms involved in HES-induced von Willebrand-like syndromes are supposed to be inhibited release of endothelial vWF[27] and increased elimination rates of the factor[8], these results should be interpreted with caution and re-assessed *in vivo*.

Thromboelastometry. In the present study, we found less impaired coagulation in activated thromboelastometry tests after *in vitro* haemodilution with a balanced crystalloid compared to either third-generation HES. The lesser impairment of coagulation by crystalloids compared to third-generation HES supports results from previous studies[13,28]. In addition, our results support the view that the carrier solution of the HES molecule -calcium-containing or not- does not influence coagulation measurements of activated thromboelastometry *in vitro* compared to crystalloid solutions indicating that the HES molecule itself is responsible for impairment of coagulation as measured by thromboelastometry.

Study limitations

Our study does have a few limitations. First of all, the *in vitro* design of this study inevitably excludes effects on coagulation from the endothelium as well as metabolic compensatory mechanisms, e.g. buffering, pH control, electrolyte homoeostasis, or metabolic degradation and renal elimination of HES[29]. Secondly, the use of turbidimetric methods to compare HES and crystalloid solutions may lead to incorrect conclusions being drawn[25], as discussed above. Thirdly, the influence of Ca^{2+} levels should be interpreted with caution as the measurements were performed in citrated blood subjected to different degrees of haemodilution. Haemodilution itself influences the levels of Ca^{2+}. Nevertheless, the changes of Ca^{2+} concentrations were consistent with the degree of dilution and the type of the carrier solution used for haemodilution. Fourthly, the use of sodium citrate for anticoagulation prior to coagulation measurements has been described to alter the coagulation process itself[20]; nevertheless, it is standard practice to use citrated blood samples for most coagulation assays[30].

Conclusions

In conventional coagulation assays, *in vitro* haemodilution with calcium-containing balanced HES yields better preserved PTR, and FVIII values than does non-balanced HES, possibly because of the higher calcium levels, whereas no difference between the two different carrier solutions of HES could be detected by ROTEM measurements. Our results may provide the basis for further clinical studies investigating the effects of the carrier solutions of modern HES on coagulation and haemostasis *in vivo*.

Acknowledgements

The Authors thank Stephen Richey for his assistance in proofing this paper for grammar and phrasing.

Balanced HES solutions were provided by B. Braun Melsungen.

Funding

This study was funded by institutional grants from Charité of University Medicine Berlin, Berlin, Germany.

Conflict of interest disclosure

In the past 5 years, Juliane Rau has received travel grants from B. Braun, Melsungen, Germany. Michael Sander has received lecture fees from Edwards Lifesciences, and Pulsion Medical Systems. Christoph Rosenthal, Elisabeth Langer, Michael Schuster and Erika Schulte declare no conflict of interest. Christian von Heymann has received a research grant and lecture fees from B. Braun Melsungen. No organization had any role in the design, collection, analysis or interpretation of data, in writing the manuscript, or in the decision to submit this manuscript for publication.

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Arrived: 19 March 2013 - Revision accepted: 18 June 2013
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