UNIVERSITÉ DE LAUSANNE - FACULTÉ DE BIOLOGIE ET DE MÉDECINE
DEPARTEMENT DE MEDECINE
DIVISION SPECIALISEE DE PHARMACOLOGIE ET TOXICOLOGIE CLINIQUES

Impact of the C2/C6 Ratio of High-Molecular-Weight Hydroxyethyl Starch on Pharmacokinetics and Blood Coagulation in Pigs

THESE

préparée sous la direction du Professeur Donat R. Spahn jusqu'en octobre 2006,
puis sous la direction du Docteur Thierry Buclin, Privat Docent
et présentée à la Faculté de biologie et de médecine de
l'Université de Lausanne pour l'obtention du grade de

DOCTEUR EN MEDECINE

par

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Lausanne

2008

Rapport de synthèse

Contexte: l'hydroxyéthylamidon (HEA) est largement utilisé comme expanseur volémique en anesthésiologie et réanimation. Cependant, cette classe de produits perturbe le system de la coagulation. Des améliorations restent possibles dans le choix de la combinaison optimale de poids moléculaire, de degré de substitution en radicaux éthyle et de localisation de ces radicaux sur le squelette glucidique des polymères, afin d'optimiser leur efficacité et leur tolérance. L'HEA de poids moléculaire élevé et faiblement substitué n'affecte pas plus la coagulation sanguine que de l'HEA de bas poids moléculaire faiblement substitué. Nous examinons *in vivo* l'effet d'un abaissement du rapport C2/C6 sur les caractéristiques pharmacocinétiques et l'impact sur la coagulation sanguine d'un HEA de haut poids moléculaire faiblement substitué.

Matériels et méthode: nous comparons dans une étude prospective, randomisée et parallèle l'HEA 650/0.42/2.8 avec l'HEA 650/0.42/5.6 auprès de 30 cochons. Avant, pendant et jusqu'à 630 minutes après une perfusion de 30 ml/kg d'HEA, des échantillons sanguins ont été collectés pour mesurer les concentrations d'HEA, les tests de coagulation plasmatique classiques et la coagulation sanguine par thrombélastographie (TEG®, Haemoscope Corporation, Niles, Il, U.S.). Les paramètres pharmacocinétiques ont été estimés en adaptant un modèle à deux compartiments.

Résultats: la constante d'élimination est de 0.009 ± 0.001 (min^{-1}) pour l'HEA 650/0.42/2.8 et 0.007 ± 0.001 (min^{-1}) pour l'HEA 650/0.42/5.6 ($p<0.001$); la surface sous la courbe de concentration est de 1374 ± 340 min*g/L pour l'HEA 650/0.42/2.8 et 1697 ± 411 min*g/L pour l'HEA 650/0.42/5.6 ($p=0.026$). Les concentrations mesurées d'HEA ne montrent pas de différence entre l'HEA 650/0.42/2.8 et l'HEA 650/0.42/5.6. Les deux solutions d'HEA affectent de façon identique la coagulation sanguine: l'index de coagulation thrombélastographique diminue pareillement à la fin de la perfusion d'HEA 650/0.42/2.8 et d'HEA 650/0.42/5.6 ($p=0.29$). De même, le temps de thromboplastine partielle activée et le temps de prothrombine augmentent de manière similaire pour l'HEA 650/0.42/2.8 et l'HEA 650/0.42/5.6 ($p=0.83$).

Conclusion: la réduction du rapport C2/C6 de l'HEA de poids moléculaire élevé et faiblement substitué aboutit à une élimination légèrement accélérée d'HEA. Cependant, elle ne modifie pas l'effet perturbateur sur la coagulation.

Anesthesiology 2007; 107:442–51

Impact of the C2/C6 Ratio of High-molecular-weight Hydroxyethyl Starch on Pharmacokinetics and Blood Coagulation in Pigs

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Background: **High-molecular-weight, low-substituted hydroxyethyl starch (HES) may not affect blood coagulation more than low-molecular-weight, low-substituted HES. The authors assessed *in vivo* the effect of a lowered C2/C6 ratio on pharmacokinetic characteristics and the impact on blood coagulation of high-molecular-weight, low-substituted HES.**

Methods: **A prospective, randomized, parallel study in 30 pigs compared HES 650/0.42/2.8 with HES 650/0.42/5.6. Before, during, and after infusion of 30 ml/kg body weight HES, blood samples were collected over 630 min to measure HES concentrations and plasmatic coagulation and to assess blood coagulation in whole blood by Thrombelastography® (TEG®; Haemoscope Corporation, Niles, IL). Pharmacokinetic parameters were estimated using a two-compartment model.**

Results: **The elimination constant was 0.009 ± 0.001 min^{-1} for HES 650/0.42/2.8 and 0.007 ± 0.001 min^{-1} for HES 650/0.42/5.6 ($P < 0.001$); the area under the plasma concentration–time curve was 1,374 ± 340 min · g/l for HES 650/0.42/2.8 and 1,697 ± 411 min · g/l for HES 650/0.42/5.6 ($P = 0.026$). The measured plasma HES concentrations were not different between HES 650/0.42/2.8 and HES 650/0.42/5.6. Both HES solutions equally affected blood coagulation: Thrombelastographic coagulation index decreased similarly at the end of infusion of HES 650/0.42/2.8 and at the end of infusion of HES 650/0.42/5.6 ($P = 0.293$). Also, activated partial thromboplastin and prothrombin times increased similarly for HES 650/0.42/2.8 and HES 650/0.42/5.6 ($P = 0.831$).**

Conclusion: **Reducing the C2/C6 ratio in high-molecular, low-substituted HES solutions results in a slightly faster HES elimination. However, the blood coagulation compromising effect was unaffected.**

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Received from the Department of Anesthesiology, University Hospital Lausanne and University of Lausanne, Lausanne, Switzerland. Submitted for publication November 15, 2006. Accepted for publication May 9, 2007. Supported by departmental funds and B. Braun Medical SA, Crissier, Vaud, Switzerland. The funding by B. Braun was exclusively for the experiment and not personal. The publication of the current results did not require a formal authorization of the manufacturer but was discussed with all coauthors, including those being employees of B. Braun (Drs. Fisch and Burmeister). Drs. Burmeister and Fisch are employees of B. Braun AG, Melsungen, Germany, and Crissier, Switzerland. Donat R. Spahn is a paid consultant for B. Braun Medical SA, Crissier, Switzerland. The Department of Anesthesiology of the University Hospital of Lausanne has done other research projects in collaboration with B. Braun AG, Melsungen, Germany, and Crissier, Switzerland, and has thereby received other funding in the past. In the past, the Department of Anesthesiology of the University Hospital of Lausanne also received educational funds from competitor companies.

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FOR almost four decades, hydroxyethyl starches (HESs) are known as effective plasma volume expanders and have therefore found widespread clinical application as plasma substitutes.[1–3] HESs are high polymeric glucose compounds obtained by hydrolysis and subsequent hydroxyethylation from the waxy maize starch amylopectin.[4] The physicochemical characteristics of HESs are determined by their mean molecular weight; by their molar substitution ratio, which expresses the average number of hydroxyethyl groups per unit of glucose; and by their C2/C6 ratio, which refers to the preferential hydroxyethylation site at the glucose subunit carbon atoms.[5]

The clinical use of HES is limited mainly by their affection of hemostasis, which is detectable by impaired platelet function[6] as well as a type I von Willebrand–like syndrome with reduced levels of factor VIII (FVIII) and von Willebrand factor (vWF), observed to a greater extent than expected from the hemodilution effect.[4,7–9] In addition, altered coagulation was reported by Thrombelastography® (TEG®; Haemoscope Corporation, Niles, IL).[10,11] The extent of such alterations has classically been related to the molecular weight or molar substitution of the used HES solutions.[4,8,9,12] But in most studies, a reduction of molecular weight was followed by a concomitant reduction of molar substitution and was therefore not assessed specifically. In contrast, a recent study has shown that the coagulation compromising effects of high-molecular-weight, low-substituted HES was not greater than those of low-molecular-weight, low-substituted HES 130/0.42.[13] Such findings question the assumption that molecular weight may be the only factor determining coagulation-compromising effects of HES.

The C2/C6 ratio of HES solutions is often not specified in published studies or varies between tested solutions. A study in healthy volunteers showed that medium-molecular-weight HES with a high C2/C6 ratio was eliminated more slowly than HES with a low C2/C6 ratio, but the impact on blood coagulation was not assessed.[14] In a recent *in vitro* study, a reduction of the C2/C6 ratio to 2.7 of high-molecular-weight, low-substituted HES (HES 700/0.42/2.7) was shown to minimize the effect on human blood coagulation as assessed by TEG®.[15] However, a low C2/C6 ratio may shorten the elimination half-life, and could therefore be disadvantageous in terms of diminished volume-expanding effect.[14]

This *in vivo* study in pigs was performed to define the

pharmacokinetics and the impact on blood coagulation as well as hemoglobin and albumin concentrations as surrogates for hemodilutional effect of a high-molecular-weight (650 kd), low-substituted (0.42) HES with a low C2/C6 ratio of 2.8 (HES 650/0.42/2.8). We compared this solution with HES 650/0.42/5.6 to assess the specific effect of C2/C6 ratio.

Materials and Methods

The animal experiments were performed according to the guidelines of the Swiss Federal Veterinary Office. The protocol was approved by the Veterinary Office of the Canton de Vaud, Switzerland. The study group consisted of 30 pigs with a mean body weight of 41.2 ± 3.6 (SD) kg. The pigs were fasted overnight but allowed free access to water.

Animal Preparation

The evening before the study, the pigs were separated from their group and premedicated with 150 μg clonidine (Boehringer Ingelheim [Schweiz] GmbH, Basel, Switzerland) *via* intramuscular injection. Before the transportation, a dose of 0.5 mg/kg body weight (BW) midazolam (Roche Pharma AG, Reinach, Switzerland) was administered intramuscularly. Upon arrival in the laboratory, the animals received intramuscular premedication of 1 mg/kg BW midazolam (Roche Pharma AG) and 20 mg/kg BW ketamine (Veterinaria AG, Zurich, Switzerland). Once sedation was obtained, anesthesia was induced by administration of 3% halothane (Dräger, Luebeck, Germany) by mask, followed by tracheal intubation.

Controlled ventilation was performed. An end-tidal volume of 10–13 ml/kg BW and a ventilation rate of 13–18/min were adjusted to maintain partial pressure of carbon dioxide at 35–40 mmHg before the onset of the protocol (Ventilator Dräger Sulla 909 V; Dräger). The inspired oxygen fraction was monitored and maintained at 1.0 during surgical instrumentation and decreased to 0.4 (air–oxygen mixture) during the experimental protocol. The ear vein was cannulated (18-gauge cannula). Anesthesia was now switched to a total intravenous anesthesia using 5–10 mg · kg BW^{-1} · h^{-1} propofol (B. Braun Medical AG, Emmenbruecke, Switzerland), 0.6 ml · kg^{-1} · h^{-1} midazolam (Roche Pharma [Schweiz] AG, Reinach, Switzerland), and 25 μg · kg BW^{-1} · h^{-1} fentanyl (Sintetica S.A., Mendrisio, Switzerland) during surgical instrumentation. Fentanyl dosing was lowered after the surgical instrumentation to a dose of 3–7 μg · kg BW^{-1} · h^{-1}, according to the requirements of each individual pig. NaCl 0.9% solution was infused in a dosage of 10 ml · kg^{-1} · h^{-1} during the entire period of anesthesia. Glucose 20% solution was administered according to the requirements of each individual pig to keep the blood glucose concentration in a range of 3.7–5.2 mM.

A catheter was placed into the right internal jugular vein for administration of the intravenous anesthesia. Another catheter was placed in the ipsilateral carotid artery for blood withdrawal for laboratory measurements, arterial blood gas analyses, and continuous measurement of arterial blood pressure. In the contralateral internal jugular vein, a catheter was inserted for HES administration and later on for 20% glucose administration. Further monitoring of the animals included continuous three-lead electrocardiogram, heart rate reading, and continuous body temperature monitoring by a probe inserted in the esophagus. In addition, a transmural bladder catheter was inserted performing a minilaparotomy. Sheets, a ventilator, and ice were used if necessary to keep body temperature at 38° ± 1°C.

HES Synthase

Thin-boiling waxy maize starch was suspended in water, activated by means of sodium hydroxide, and allowed to react with ethylene oxide for 2 h at 40°C. The amounts of waxy maize starch and ethylene oxide were chosen to yield HES with a molar substitution of 0.42. HES C2/C6 ratios of 2.8 and 5.6 were obtained by changing the quantities of sodium hydroxide during hydroxyethylation. The raw HES species were hydrolyzed by hydrochloric acid to a molecular weight of 650 kd, treated with activated carbon, purified by ultrafiltration, and diluted to a final concentration of 6% (wt/vol) in isotonic saline, filled in glass bottles of 500 ml each and heat-sterilized at 121°C for 20 min.

HES Characterization

The mean weight average molecular weight (MWw) of HES was determined by gel permeation chromatography/multiangle laser light scattering (GPC-MALLS; Wyatt Technology, Woldert, Germany) at a flow rate of 1 ml/min in a 70 mM phosphate buffer pH 7.0 using serial GPC columns HEMA Bio 40, 100, and 1,000 (PSS, Mainz, Germany). Molecular weight was calculated using ASTRA Software (Wyatt Technology). The weight averaged molecular weight, calculated as $MWw = \Sigma_I (M_i^2 N_i)/\Sigma_i (M_i N_i)$, gives higher statistical weight to larger molecules. In contrast, the number averaged molecular weight, calculated as $MWn = \Sigma_I (M_i N_i)/\Sigma_i N_i$, gives equal statistical weight to each molecule. The polydispersity index MWw/MWn describes the distribution of the molecular weights, with values near 1 for a narrow distribution.

Molar substitution was determined by gas chromatography after transforming hydroxyethyl groups into ethyl iodine by hydriodic acid in the presence of adipic acid using a gas chromatograph Perkin Elmer Autosystem (Boston, MA). The C2/C6 ratio was determined after hydrolysis of HES by sulfuric acid and gas chromato-

Table 1. HES Characteristics

	HES 650/0.42/2.8	HES 650/0.42/5.6
In vitro molecular weight, kd	641	647
Molar substitution	0.42	0.42
C2/C6 ratio	2.8	5.6
Concentration, %	6	6

Physiochemical characteristics of the two hydroxyethyl starches (HESs).

graphic separation of the sialylated hydroxyethylated glucose derivatives using a gas chromatograph Carlo Erba Mega 5300 (Milano, Italy). In table 1, the physiochemical parameters of the investigated HES solutions are shown.

Experimental Protocol

The animals were randomized into two groups of 15 pigs each, receiving either 6% HES 650/0.42/2.8 (test group) or 6% HES 650/0.42/5.6 (reference solution). The treatments were strictly blinded over the whole study period. Unblinding was delayed until the data analysis was finalized. After completion of all surgical preparations, the first of 13 blood samples were taken for baseline values using citrated blood containers. Directly after baseline blood sampling, the administration of the solution started. A top-load dose of 30 ml/kg BW was infused over exactly 30 min. During and after the infusion, blood withdrawals were performed at 10, 20, 30, 40, 50, 60, 90, 120, 150, 270, 390, 510, and 630 min after the start of the infusion.

Laboratory Measurements

Hemoglobin Concentration. Arterial blood samples were collected using heparinized syringes (BD Preset; BD Vacutainer Systems, Plymouth, United Kingdom). Hemoglobin concentration was determined immediately after collection using the Rapidlab 865 analyzer (Bayer Vital GmbH, Fernwald, Germany).

Albumin Concentration. Albumin concentration was measured by means of enzyme-linked immunosorbent assay (ELISA) using the sandwich technique. ELISA kit starter accessory package E 101 (Bethyl, Montgomery, TX) was combined with the Pig Albumin ELISA Quantification Kit E100-110 (Bethyl). Briefly, coating of the wells with the capture antibody and blocking was effected before the increasingly diluted plasma samples were incubated for 60 min. These were linked with antibody conjugate for another 60 min. In a last step, enzyme-substrate solution was added and quantification was measured by means of calorimetry at 490 nm (ELX808; BioWhittaker Inc., Walkersville, MD).

Thrombelastography® Analysis. Citrated whole blood was used to issue a Thrombelastogram®. An initial incubation for 1 h in a 37°C water bath was performed with the citrated whole blood samples,[16] followed by blood recalcification and TEG® measurements using two computerized Thrombelastography® coagulation analyzers 5000 (Haemoscope Corporation) with two channels for each analyzer. Four samples were analyzed at a time with a randomly chosen channel sequence. The following TEG® parameters are reported: reaction time (r time), coagulation time (k time), maximal amplitude, angle α, elastic shear modulus, and coagulation index.[16,17]

For further laboratory measurements, blood samples were immediately centrifuged at 3,000 rpm for 15 min at 4°C for separation of plasma and blood cellular components (Rotanta/RP; Hettich, Bäch, Switzerland).

Plasma Coagulation. Prothrombin time and activated partial thromboplastin time (aPTT) were determined on an automated coagulation analyzer (BCS; Dade Behring, Marburg, Germany) using a prothrombin time reagent containing recombinant tissue factor (Innovin; Dade Behring) and an aPTT reagent containing ellagic acid (Actin FS; Dade Behring), respectively. Functional activity of vWF was determined in a commercial ristocetin-cofactor assay (vWF RCA; Dade Behring) on an automated coagulation analyzer (BCS). Briefly, vWF activity was assessed by the ability to agglutinate fixed human platelets in the presence of ristocetin. Agglutination was measured turbidimetrically using the coagulation analyzer. FVIII was assessed functionally using FVIII-deficient plasma according to the manufacturer's instructions (Dade Behring).

HES Concentration. Hydroxyethyl starch concentration was quantified after extraction from plasma and hydrolysis to glucose monomers. Briefly, plasma samples (1 ml) were incubated at 100°C for 60 min after addition of 0.5 ml KOH solution 35% (wt/wt) (Fluka, Buchs, Switzerland). HES was precipitated by adding of 10 ml ice-cold absolute ethanol (Fluka) to the supernatant of the reaction mixture and acid hydrolyzed in 2N HCl (Fluka) for 60 min at 100°C. Glucose determination was performed using an enzymatic test kit based on hexokinase/glucose 6-phosphatase (Boehringer Mannheim, Darmstadt, Germany). For determination of HES molecular weight, plasma proteins were eliminated by trichloroacetic acid precipitation (6.4% [wt/wt] end concentration), and neutralized supernatants were analyzed by GPC-MALLS at a flow rate of 1 ml/min in 70 mM phosphate buffer, pH 7.0, using serial GPC columns HEMA Bio 40, 100, and 1,000.

Pharmacokinetic Modeling

Pharmacokinetic parameters were obtained through individual fitting of the plasma concentration data with a biexponential model using the software Kinetica, version 4.3 (InnaPhase Corp., Philadelphia, PA). Curve fitting was performed by nonlinear regression, using weights of $1/Y_{obs}^2$ to accommodate for data heteroscedasticity. The use of a two-compartment model seemed appropriate to describe the concentration curves:

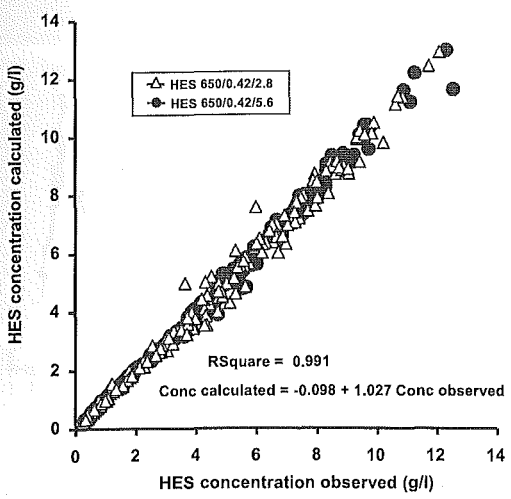

Fig. 1. Pharmacokinetic modeling. The quality of fit between observed hydroxyethyl starch (HES) concentration data and calculated data was analyzed by linear regression analysis.

$$\text{Concentration during infusion} = \frac{R_0}{V_1} \cdot \left[\frac{c_1}{\lambda_1}\left(1 - e^{-\lambda_1 t}\right) + \frac{(1 - c_1)}{\lambda_z}\left(1 - e^{-\lambda_z t}\right)\right]$$

$$\text{Concentration after infusion} = \frac{R_0}{V_1} \cdot \left[\frac{c_1}{\lambda_1} e^{-\lambda_1 t} + \frac{(1 - c_1)}{\lambda_z} e^{-\lambda_z t}\right],$$

where c_1 = relative coefficient for the central compartment, λ_1 = slope of the distribution phase, λ_z = slope of the elimination phase, V_1 = volume of the central compartment, and R_0 = rate of HES administration, *i.e.*, the dose divided by the infusion duration. A one-compartment model showed significant misfit, whereas a three-compartment model seemed overparameterized, with high SE and strong intercorrelation values of the estimates. In addition, a visual inspection of diagnostic plots such as observed *versus* calculated values (fig. 1) and residuals *versus* time (not shown) did not reveal any systematic trend suggestive of model misfit. Further macroconstants (area under the curve; CL = systemic clearance = ratio of dose over area under the curve; $t_{1/2}$ = terminal half-life = $\log 2/\lambda_z$, C_{max} = end-of-infusion level) and microconstants of the model (k_{12}, k_{21}, and k_{el}, *i.e.*, transfer constants between compartments and to elimination) were derived by the Kinetica software using standard formulae.

Statistical Analysis

Sample size has been determined by a power analysis based on a previous *in vivo* study.[13] To obtain a power of 80% with an estimated difference between groups of 7.5% using TEG® parameters angle α and maximal amplitude and a SD of 5%, a total sample size of 30 pigs has been determined with a type I error of 0.05. The high-molecular-weight HES solution with the low C2/C6 ratio of 2.8 (HES 650/0.42/2.8) was compared with the high-molecular-weight HES solution with the C2/C6 ratio of 5.6 (HES 650/0.42/5.6) using the JMP 5.1 statistical package (SAS Institute, Inc, Cary, NC). Data (with and without baseline correction) were analyzed using the Shapiro-Wilk test for normality and a two-way analysis of variance for repeated measures on one way (time) with the Greenhouse-Geisser correction for assessing solution and time effects. Pharmacokinetic parameters obtained from model fitting were thereafter compared between HES 650/0.42/2.8 and HES 650/0.42/5.6 by way of the unpaired Student *t* test. No data transformation was applied. Results are expressed as mean ± SD.

Table 2. Baseline Values

	HES 650/0.42/2.8 (n = 15)	HES 650/0.42/5.6 (n = 15)
Body weight, kg	41.3 ± 3.8	41 ± 3.7*
Mean arterial blood pressure, mmHg	93 ± 19	81 ± 17*
Heart rate, beats/min	79 ± 13	84 ± 18*
Body temperature, °C	37.8 ± 0.2	38.2 ± 0.2*
Hemoglobin concentration, g/dl	8.6 ± 1.1	8.1 ± 1*
Albumin concentration, g/l	22.08 ± 3.95	20.82 ± 5.19*

Baseline values of the two groups.

* No statistical significant difference when compared with hydroxyethyl starch (HES) 650/0.42/2.8.

Results

In total, 31 animals had to undergo the study protocol to complete the investigation in 30 because of erroneous HES infusion in one individual. There were no differences between groups at baseline (table 2).

HES Pharmacokinetics

The HES concentrations increased progressively in both groups during infusion and decreased from the end of infusion until 630 min, following a biexponential profile. There were no significant differences between the solutions (figs. 2A and B).

A two-compartment model for the calculation of the HES concentration was able to fit well the concentration curves of the solutions, producing an excellent correlation between measured and calculated values (R^2 = 0.991) (fig. 1).

Hydroxyethyl starch 650/0.42/2.8 had a 29% higher elimination constant k_{el} than HES 650/0.42/5.6 ($P < 0.001$). Also compared with this solution, a significantly smaller area under the plasma concentration–time curve

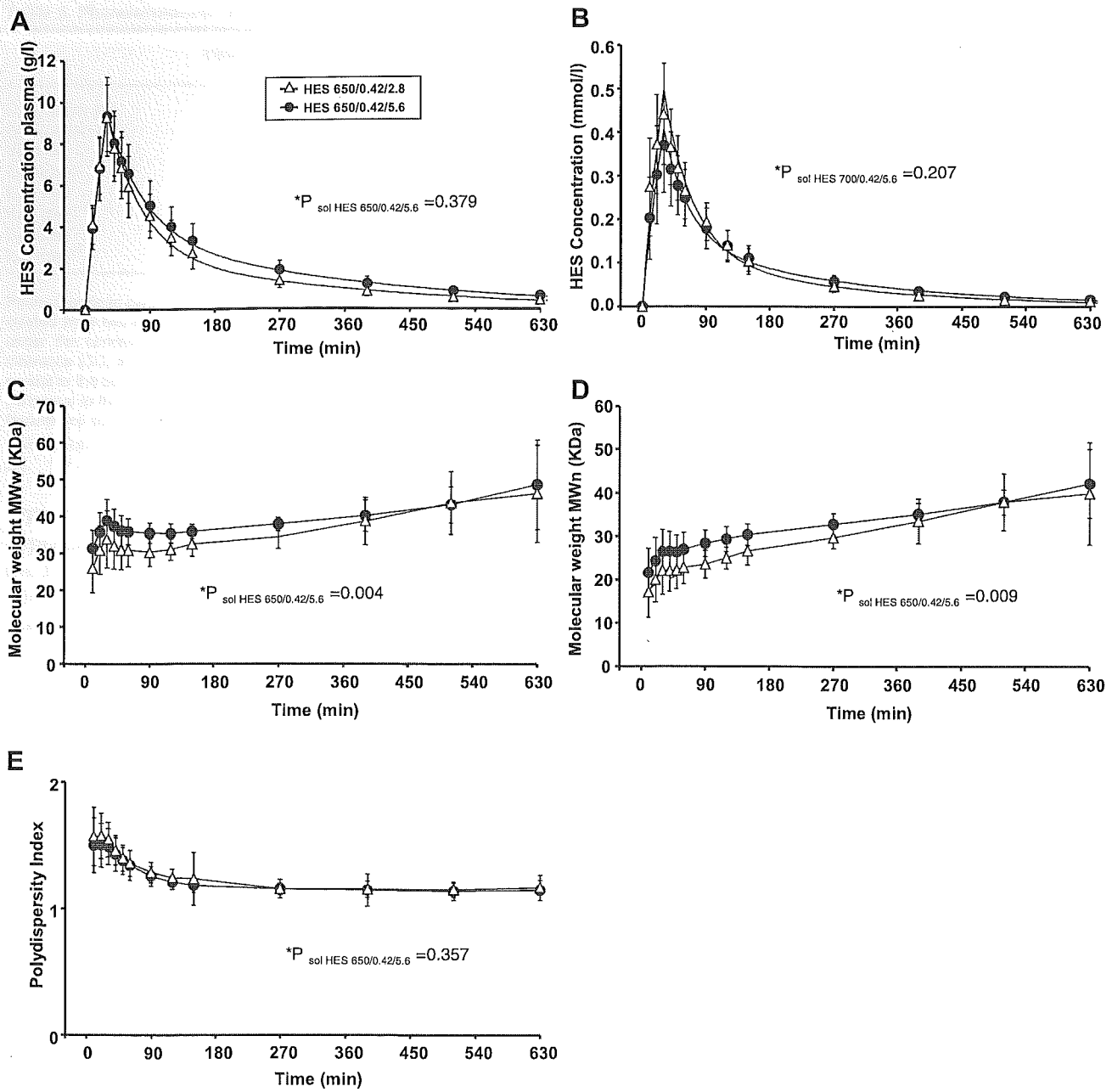

Fig. 2. Time course of hydroxyethyl starch (HES) 650/0.42/2.8 and HES 650/0.42/5.6 plasma concentration in g/l (*A*), plasma concentration in mM (*B*), weight averaged molecular weight (MWw) in kd (*C*), and number averaged molecular weight (MWn) in kd (*D*). In *A* and *B*, the absolute measured values are displayed by the *symbols*, whereas the *lines* represent the calculated values obtained by the used pharmacokinetic model. *E* shows the time course of the polydispersity index of the solutions. Concentrations and molecular weights were determined directly before infusion and 10, 20, 30, 40, 50, 60, 90, 120, 150, 270, 390, 510, and 630 min after. Results are shown as mean ± SD. * Solution effects of HES 650/0.42/2.8 *versus* HES 650/0.42/5.6 are determined by two-way analysis of variance.

was found (19%; $P = 0.026$). All other pharmacokinetic parameters showed no significant differences between the groups (table 3).

In vivo, the MWw ($P = 0.004$) and the MWn ($P = 0.009$) were smaller for HES 650/0.42/2.8, compared with HES 650/0.42/5.6 (figs. 2C and D). The index of polydispersity MWw/MWn revealed no significant differences between the HES solutions (fig. 2E).

Coagulation Analysis

Plasma Coagulation Tests. Parameters of plasma coagulation such as prothrombin time (seconds), aPTT (seconds), and functional FVIII and vWF activity (%) changed significantly over time ($P < 0.001$ for all), but no significant between-group differences were found. The maximum effect was reached immediately after the end of infusion at 30 min (figs. 3A–D).

Table 3. Pharmacokinetic Parameters

	HES 650/0.42/2.8 (n = 15)	HES 650/0.42/5.6 (n = 15)
C_{max} calc, g/l	9.50 ± 1.82	9.50 ± 2.01*
AUC, min · g/l	1,374 ± 340	1,697 ± 411†
Volume, l	6.54 ± 1.77	6.77 ± 1.81*
CL, l/min	0.057 ± 0.016	0.047 ± 0.015*
$t_{1/2}$, min	221 ± 37	238 ± 33*
k_{12}, min^{-1}	0.007 ± 0.005	0.007 ± 0.002*
k_{21}, min^{-1}	0.007 ± 0.003	0.008 ± 0.003*
k_{el}, min^{-1}	0.009 ± 0.001	0.007 ± 0.001‡

Pharmacokinetic parameters of the hydroxyethyl starch (HES) solutions, calculated by a two-compartment model: maximal concentration (C_{max}), area under the concentration–time curve (AUC), distribution volume (Volume), clearance (CL), elimination half-time ($t_{1/2}$), rate constant of transfer from the central to the peripheral compartment (k_{12}), rate constant of transfer from the peripheral to the central compartment (k_{21}), rate constant of elimination (k_{el}). Results are shown as mean ± SD.

* No statistical significant differences, when compared with HES 650/0.42/2.8. † $P = 0.026$, ‡ $P < 0.001$, when compared with HES 650/0.42/2.8.

TEG®. All TEG® parameters showed significant time-dependent alterations after the infusion irrespective of the HES solution used ($P < 0.001$ for all). The compromising effect on Thrombelastography® parameters reached its maximum at the end of infusion (30 min for k time, maximal amplitude, angle α, coagulation index, and shear elastic modulus), only for r time the maximum was reached after 20 min, during the infusion. No significant between-group differences were found in TEG® (figs. 4A–F).

Hemoglobin Concentration

During the HES administration, the hemoglobin concentration decreased continuously from 8.1 ± 1 g/dl to 5.9 ± 0.6 g/dl (HES 650/0.42/5.6) at the end of the infusion (30 min), respectively, and from 8.6 ± 1.1 g/dl to 6.2 ± 0.6 g/dl (HES 650/0.42/2.8) ($P < 0.001$ for all). Thereafter, hemoglobin concentration increased progressively to values close to the baseline values. After 630 min, the hemoglobin was 7.2 ± 0.9 g/dl for HES (650/0.42/5.6) and 7.2 ± 0.7 g/dl for HES (650/0.42/2.8). No significant differences were found between the tested solutions (fig. 5A).

Albumin Concentration

The albumin concentration showed a similar behavior with an initial decrease until the end of infusion followed by a subsequent increase without reaching the baseline values. No significant differences were found between the tested solutions (fig. 5B).

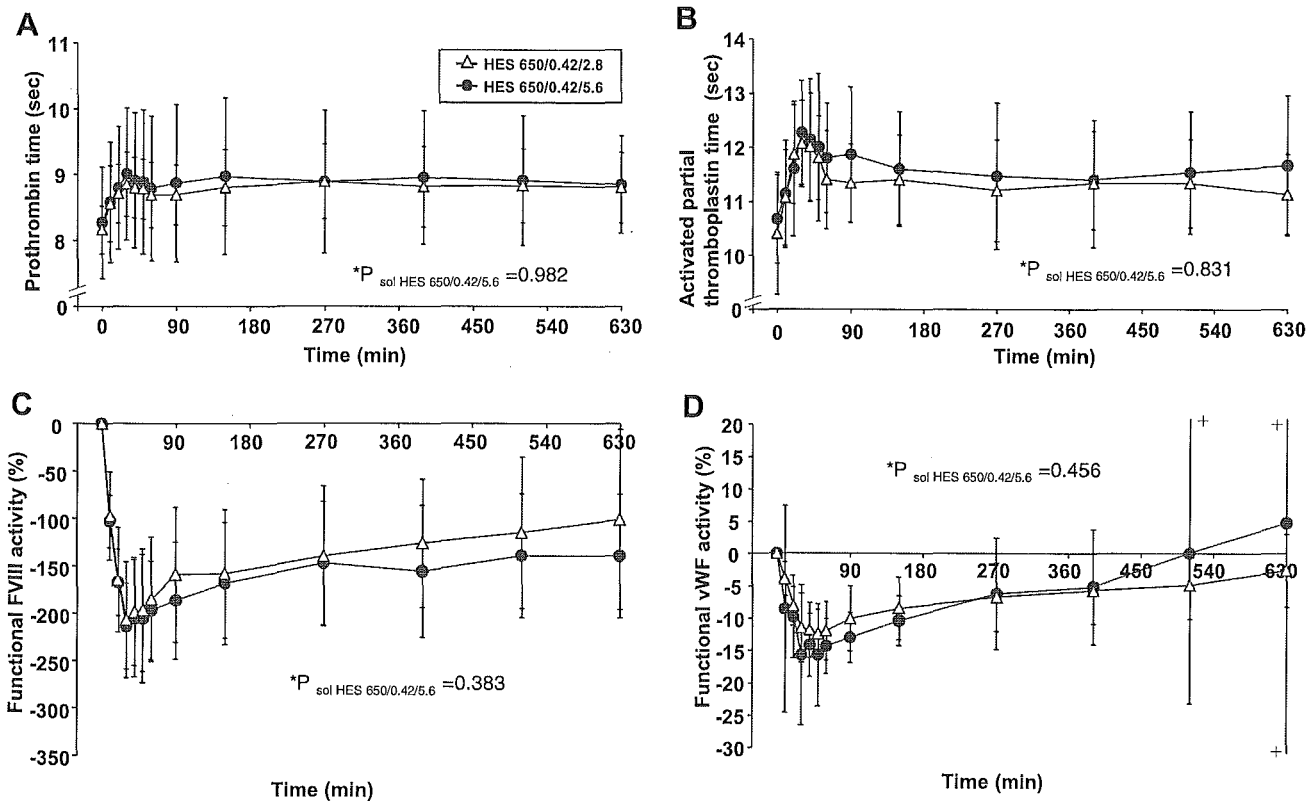

Fig. 3. Time course of coagulation parameters: prothrombin time in seconds (*A*) and activated partial thromboplastin time in seconds (*B*) were displayed with absolute values. *C* is functional factor VIII (FVIII) activity in % (human) and *D* is functional von Willebrand factor (vWF) activity in % (human) displayed as absolute changes. Results are shown as mean ± SD. * Solution effects of hydroxyethyl starch (HES) 650/0.42/2.8 *versus* HES 650/0.42/5.6 are determined by two-way analysis of variance. + SD not shown entirely.

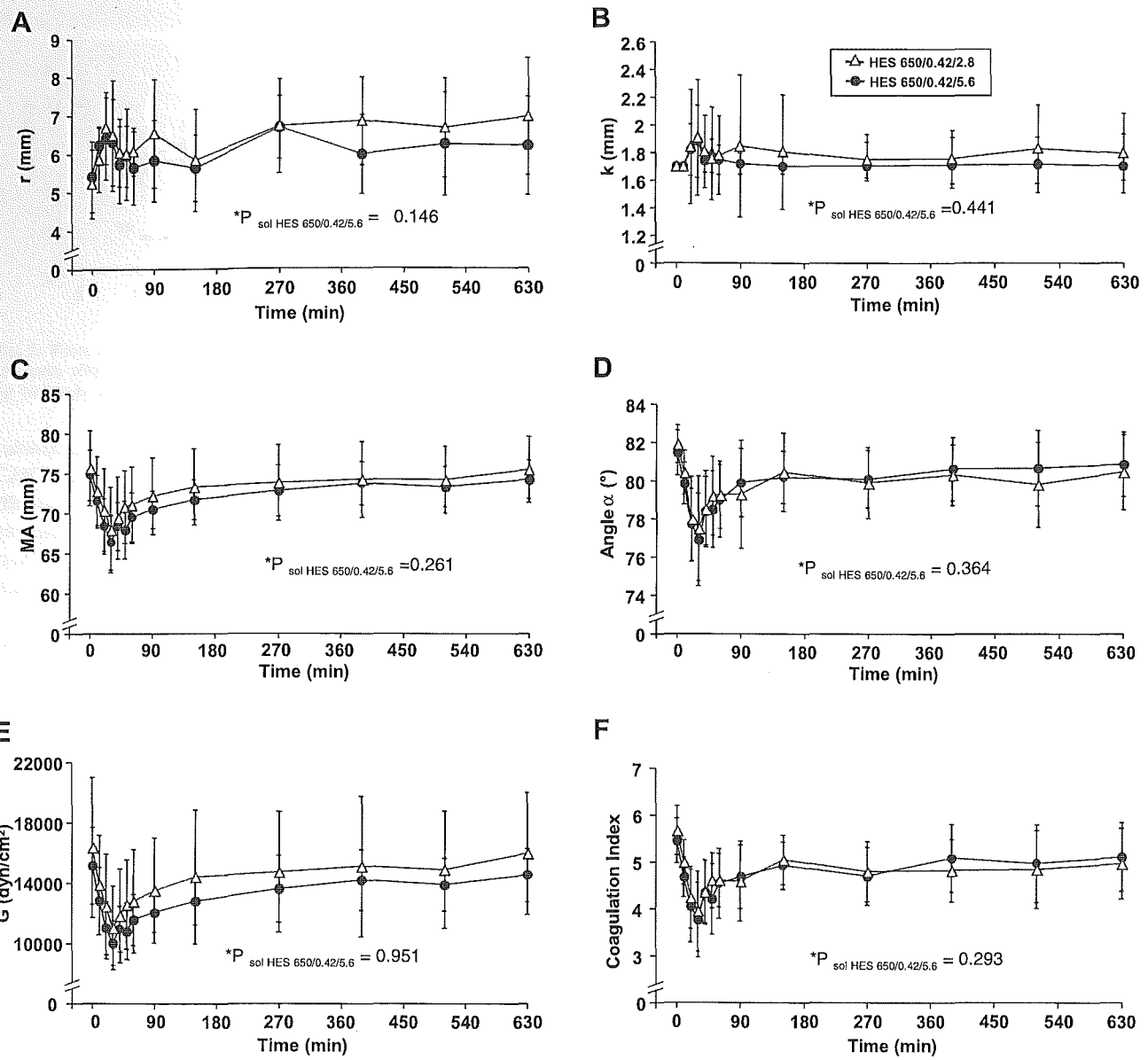

Fig. 4. Time course of TEG® parameters: reaction time (r) in mm (*A*), coagulation time (k) in mm (*B*), maximal amplitude (MA) in mm (*C*), angle α in degrees (*D*), elastic shear modulus (G) in dyn/cm² (*E*), and coagulation index (*F*). TEG® parameters were measured directly before the start of infusion and 10, 20, 30, 40, 50, 60, 90, 150, 270, 390, 510, and 630 min after the start. Results are shown as mean ± SD. * Solution effects of hydroxyethyl starch (HES) 650/0.42/2.8 *versus* HES 650/0.42/5.6 are determined by two-way analysis of variance.

Discussion

The primary aim of this study was to examine the single-dose administration of a new high-molecular-weight, low-substituted HES with a low C2/C6 ratio regarding its pharmacokinetic profile, compared with a reference solution. We found that a reduction of the C2/C6 ratio from HES 650/0.42/5.6 to HES 650/0.42/2.8 modifies to some extent the pharmacokinetic profile, resulting in lower area under the curve surface and higher elimination constant for HES 650/0.42/2.8. Regarding the global effects, HES 650/0.42/2.8 and HES 650/0.42/5.6 reach similar levels of absolute concentrations, hemodilutional effect, and alteration of blood coagulation, without differences between the groups.

The higher elimination constant found for HES 650/0.42/2.8 and the resulting smaller area under the curve, when compared with HES 650/0.42/5.6, confirm the fact that the C2/C6 ratio influences the HES degradation: The higher the C2/C6 ratio, the slower the HES is hydrolyzed due to a relative resistance toward α-amylolytic attack of the C2-linked side chains of the molecule.[18,19] Jung *et al.*[20] found a prolongation of the elimination half time when increasing the C2/C6 ratio from 4.6 to 10.8, while keeping the same molecular weight of 200 kd and molar

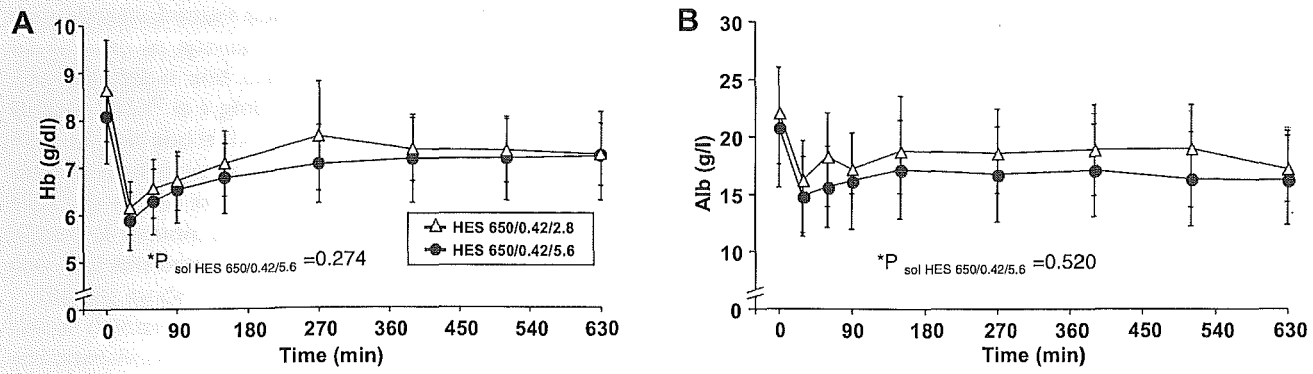

Fig. 5. Time course of hemoglobin (Hb) in g/dl (*A*) and albumin (Alb) in g/l (*B*) directly before and 30, 60, 90, 150, 270, 390, 510, and 630 min after start of infusion. * Solution effects of hydroxyethyl starch (HES) 650/0.42/2.8 *versus* HES 650/0.42/5.6 are determined by two-way analysis of variance.

substitution of 0.5. We found a similar influence of the C2/C6 ratio on high-molecular-weight, low-substituted HES in the current study, resulting in a decreased blood exposure. This indicates that the influence of the C2/C6 ratio on HES pharmacokinetics is consistent over a considerable range of molecular weight. However, the range of C2/C6 ratio assessed in the current study is limited. To verify a general impact of the C2/C6 ratio on HES pharmacokinetics, a wider range of molar substitution including higher-substituted HES with molar substitutions of up to 0.6 or 0.7 and a wider range of C2/C6 ratios must be studied.

Treib *et al.*[18] showed a significant difference regarding the *in vivo* molecular weight after repeated infusion of HES 200/0.5/5.7 and HES 200/0.5/13.4 in patients. In that study, the HES with the higher C2/C6 ratio accumulated and had a higher *in vivo* molecular weight, indicating a slower enzymatic breakdown. Contrarily to this, we did not observe higher concentrations 600 min after stop of infusion, possibly because of the lower substitution of the HES used.[21] Also, the lower level of C2/C6 ratio of the HES used in the current study may have contributed to a stronger cleavage by the α amylase. Moreover, we administered one single top-load dose without any redosing. In another crossover study with six patients who received either HES 200/0.5/10.8 or HES 200/0.5/5.8, the HES with the higher C2/C6 ratio had a higher plasma concentration and a higher *in vivo* molecular weight after single-dose administration.[14] These results are partly confirmed by our study, as we also found significantly higher *in vivo* molecular weights in the HES 650/0.42/5.6 than in the HES 650/0.42/2.8 group (figs. 2C and D).

In numerous studies, molecular weight is measured for the first time at the end of infusion. In these studies *in vivo* molecular weight is at its maximum at the end of infusion and decreases subsequently.[7,14,18,22] We therefore expected during infusion an *in vivo* molecular weight relatively close to the *in vitro* molecular weight or at least between the *in vitro* molecular weight and the *in vivo* molecular weight at the end of infusion (30 min). However, we found that the *in vivo* molecular weight increased during infusion to follow the expected decrease thereafter, *i.e.*, after the end of infusion at 30 min (figs. 2C and D). This phenomenon may suggest a metabolism mainly directed by α-amylase activity, which is increasingly saturated during HES infusion.

It remains to be elucidated whether the smaller area under the concentration–time curve for HES 650/0.42/2.8 compared with HES 650/0.42/5.6 is only due to higher intravascular metabolism and elimination rates or whether HES with a lower C2/C6 ratio may extravasate through the endothelial barrier[23] and subsequently be degraded by lysosomal α glycosidase[24] or other extravascular mechanisms.

Modifying the C2/C6 ratio did not affect the impact on blood coagulation (figs. 3 and 4). The major side effect of HES is the alteration of blood coagulation, which may be a serious limitation to its clinical use, especially in hemorrhagic patients or in those with coexisting coagulation disorders.[25] This was confirmed by our study, as several of the coagulation assays reflected the significant alteration of blood coagulation at the end of the HES infusion, which might become clinically relevant when used in humans. But, as mentioned above, molecular weight is not the only or the most important determinant of this side effect of HES. The isolated effect of molecular weight on blood coagulation has been assessed in a study using a top-load dose pig model, showing that high-molecular-weight, low-substituted HES (HES 900/0.4 and HES 500/0.4) influence plasma blood coagulation similarly as low-molecular, low-substituted-weight HES (HES 130/0.4).[13] The C2/C6 ratio was identical for these three solutions, as confirmed recently by the manufacturer. Also, the polydispersity index seems not to have a major influence on blood coagulation, because it does not represent molar substitution and or C2/C6 ratio, but only the distribution of molecular weight.

In combination with standard coagulation assays, TEG® is suitable to get a comprehensive assessment of

coagulation changes during hemodilution (*in vivo* as *in vitro*) and in special clinical situations such as liver transplantation and cardiac surgery.[9-11,13,26-33] Nevertheless, TEG® is a global measure of the entire coagulation cascade and is therefore not suited to detect specific defects or deficits of the coagulation factors. However, TEG® is useful as a complementary measure to assess effects of volume expanders such as HES on clot formation and firmness.

The pig model is considered suitable for blood coagulation research.[34] Coagulation changes under various circumstances can be detected in the porcine model, such as in supraceliac aortic cross clamping, normovolemic hemodilution, and hemorrhagic shock.[35-37] There are differences as compared with the human coagulation system: elevated activity of FV, FVIII, FIX, FXI, and FXII resulting in a shortened aPTT indicating a slight hypercoagulability,[34,38-40] which was confirmed in our study. This hypercoagulability of the pig may have masked a part of the negative impact on blood coagulation of the HES solutions tested, and this may be a limitation of our study. However, we were capable of detecting a highly significant compromise of blood coagulation due to the infusion of HES. The absence of differences between the groups thus is not due to a lack of sensitive measures but due to the fact that there is no relevant difference between the HES solutions tested. In addition, McLoughlin *et al.*[36] have shown that the response of prothrombin time and aPTT to profound hemodilution is similar in humans and in pigs. Therefore, we may assume that the porcine coagulation reacts relatively similar to hemodilution with HES solutions as compared with the human coagulation system.

Hemodilutional effects, as measured by the indirect markers hemoglobin and albumin, were not different between the study group and the control group (fig. 5). This is not a surprising finding, despite the fact that the area under the time-concentration curves and the elimination constants showed significant differences between the solutions. In fact, water binding capacity is related to the number of the osmotically active particles.[3,5] The number and size of HES molecules *in vivo* is changing constantly over time from the beginning of infusion.[3,4] In our study, we confirmed these findings: HES 650/0.42/2.8 has *in vivo* significantly lower molecular weights than HES 650/0.42/5.6 because of the facilitated cleavage by α amylase, which results in a decreased area under the curve. Because of the equal number of osmotically active particles, the same volume-expanding effect is mediated by the two HESs (figs. 2B and 5). Also, the molar substitution and C2/C6 ratio of the HES solution change constantly in the organism and could therefore influence the volume-expanding properties.[4]

Our findings regarding hemodilution are limited by the fact that the study was not specifically designed to directly detect plasma volume-expanding effects. To determine exactly volume changes in the organism, more sophisticated methods must be applied, such as double-label measurements of blood volume.[23] Beyond this, HES 650/0.42/2.8 must prove its volume-expanding effect in clinical more relevant models, such as redosing studies[7,18,41] or animal models of controlled hemorrhage, which have been shown to be adequate for the comparison of the effectiveness of different resuscitation fluids.[42-46]

We conclude that lowering of the C2/C6 ratio of high-molecular-weight, low-substituted HES 650/0.42/5.6 to HES 650/0.42/2.8 results in a faster cleavage and elimination from the intravascular space, without any evidence for a reduction of the volume-expanding effect. Because the blood coagulation compromising effect of the two solutions was similar, the specific impact of the C2/C6 ratio of high-molecular-weight, low-substituted starches on blood coagulation seems to be relatively small. More research remains to be conducted with HES 650/0.42/2.8 to evaluate its clinical benefit.

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