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Molar substitution and C2/C6 ratio of hydroxyethyl starch : influence on blood coagulation

THESE

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Molar substitution and C2/C6 ratio of hydroxyethyl starch: influence on blood coagulation†

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Background. Development of hydroxyethyl starches (HES) with a low impact on blood coagulation but a long intravascular persistence is of clinical interest. A previous *in vitro* study showed that low substituted high molecular weight HES does not compromise blood coagulation more than medium molecular weight HES. In the present study we assessed the individual effects on blood coagulation of molar substitution and C2/C6 ratio of a high molecular weight HES.

Methods. Blood was obtained from 30 healthy patients undergoing elective surgery and mixed with six high molecular weight (700 kDa) HES solutions differing in their molar substitution (0.42 and 0.51) and C2/C6 ratio (2.7, 7 and 14) to achieve 20, 40 and 60% dilution. Blood coagulation was assessed by Thrombelastograph® analysis (TEG) and plasma coagulation tests. Data were compared using a three-way analysis of variance model with repeated measures on the three factors.

Results. Higher molar substitution compromised blood coagulation most (for all TEG parameters, $P<0.05$). The lowest C2/C6 ratio was associated with the lowest effect on blood coagulation; r ($P<0.001$), angle α ($P=0.003$) and coagulation index ($P<0.001$). No effect on k and maximum amplitude was observed (P for both >0.50). The higher molar substitution was associated with a lesser increase in PT ($P=0.007$) and a greater decrease in factor VIII ($P=0.010$). PTT, functional and antigenic von Willebrand factors were not significantly influenced by molar substitution (P for all >0.20). No significant differences between solutions with the same molar substitution but different C2/C6 ratios were found in plasma coagulation parameters (P for all >0.05).

Conclusions. TEG analysis indicates that high molecular HES with a molar substitution of 0.42 and a C2/C6 ratio of 2.7 has the lowest effect on *in vitro* human blood coagulation.

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Hydroxyethyl starches (HES) are widely used plasma substitutes.[1–4] The clinical use of HES is limited by its impact on blood coagulation associated with a reduction of circulating levels of factor VIII and von Willebrand Factor (vWF) in a greater proportion than expected by plasma dilution.[5–7] Direct inhibition of platelet function through binding of HES to the platelet surface may also contribute to the blood coagulation compromising effects of HES.[4 8]

HES is a modified branched natural polymer of amylopectin. Besides its molecular weight, HES is characterized

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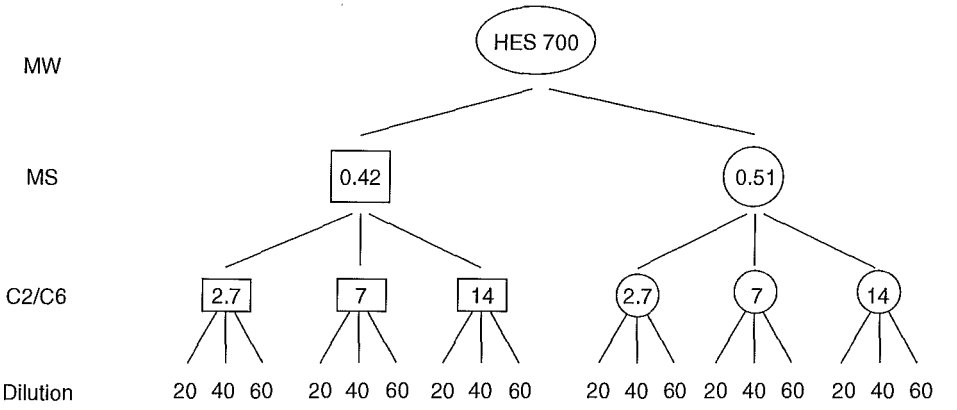

Fig 1 Design of the trial: the blood was diluted at 20, 40 and 60% with each of the six blinded solutions (A, B, C, D, E and F) in polystyrene tubes yielding 5 ml of blood–HES mixtures.

by its molar substitution, which expresses the average number of hydroxethyl groups per unit of glucose, and by the C2/C6 ratio, which describes more precisely the substitution pattern: indeed, the hydroxyethyl units can be introduced at positions 2, 3 and 6 of the glucose unit but most frequently at positions C2 and C6, so that the C2/C6 ratio is commonly indicated.[2 4]

There was a generally held view that low molecular weight HES affects blood coagulation to a lesser extent than high molecular HES. Indeed, studies comparing high *vs* medium and low molecular weight HES confirmed this advantage of medium and low molecular weight HES.[5 9] However, in these studies, a reduction in molecular weight has always been associated with a reduction in molar substitution.

A preliminary *in vitro* study[10] showed that two high molecular HES solutions with a molar substitution of 0.4, HES 500/0.4 and HES 800/0.4 (molecular weight/molar substitution), had a slightly reduced effect on blood coagulation compared with HES 200/0.5. In addition, an *in vivo* study showed that, at low substitution, high molecular weight HES did not compromise blood coagulation to a greater extent than low molecular weight HES.[11] We tested the hypothesis that molar substitution might be a key factor in determining the effect of a HES solution on blood coagulation.

The present study assesses systematically the individual effects of molar substitution and C2/C6 ratio and the possibility of an interaction between these two parameters on the reduced coagulation produced by high molecular weight HES.

Materials and methods

This study of 30 patients was approved by our local ethics committee and each patient gave written informed consent. Patients between 18 and 80 yr of age undergoing various surgical procedures were enrolled. Exclusion criteria were emergency surgery, a history of preoperative coagulation disorders, anticoagulation, treatment with acetylsalicylic acid or nonsteroidal anti-inflammatory drugs within 5 days before surgery, history of renal dysfunction or increased serum creatinine levels (>120 μmol litre^{-1}), history of liver dysfunction or increased plasma levels of aspartate aminotransferase (>50 u litre^{-1}) or alanine aminotransferase (> 50 u litre^{-1}), treatment with HES solutions within 30 days before surgery and ASA III or IV classification as judged by the responsible anaesthesiologist. A standard prophylaxis of 3000 u of s.c. low molecular weight heparin the day before surgery was allowed.

Eighty millilitres of blood was removed from an antecubital vein by direct venipuncture using a 19 gauge butterfly connected to 10 ml monovettes containing 3.13% trisodium citrate (S-Monovette 10 ml 9 NC, Sarstedt, Nümbrecht, Germany). The first 10 ml of blood was discarded to avoid activation of blood coagulation.

The blood from eight citrated monovettes was added into one polystyrene tube to produce one homogenous sample, and the whole blood was then incubated at 37°C for 1 h.[12] The blood was then diluted. All 18 different possible dilutions (six different HES solutions at three dilutions; see Fig. 1) were initially made with one blood sample using blinded HES solutions labelled A, B, C, D, E and F. The diluted samples were again maintained for 1 h at 37°C in an incubator.[12]

TEG analysis was performed using two computerized Thrombelastograph® coagulation analysers 5000 (TEG, Haemoscope Corporation, Niles, IL, USA). Four samples were analysed at a time and the sequences of analysis and the TEG channels were randomly chosen for each patient using the randomly permuted blocks method. One millilitre of diluted blood was added to a tube containing 1% kaolin. The tube was mixed and an aliquot of 340 μl kaolin activated blood was then placed into the TEG cup containing 20 μl of 2 M $CaCl_2$. TEG analysis started immediately. Haemoglobin concentration was determined with a blood gas analyser (Bayer Diagnostic Ltd, model 865, Bayer,

Zürich, Switzerland) using arterial blood samplers containing lyophilized lithium heparin (Marquest Quik A.B.G., Englewood, CO, USA).

For prothrombin time (PT), activated partial thromboplastin time (aPTT), factor VIII and vWF measurements, samples were centrifuged at 3000 rpm for 15 min at 4°C (Rotanta/RP, Hettich, Bäch, Switzerland). PT and aPTT were determined on an automated coagulation analyser (BCS, Dade Behring, Marburg, Germany) using a PT reagent containing recombinant tissue factor (Innovin®, Dade Behring) and an aPTT reagent containing ellagic acid (Actin FS®, Dade Behring), respectively. Factor VIII was assessed functionally using factor VIII-deficient plasma (Helena Haemostasis Systems Ltd, Sunderland, Tyne and Wear, UK). Functional activity of vWF was determined in a commercial ristocetin-cofactor assay (vWF RCA, Dade Behring) on an automated coagulation analyser (BCS, Dade Behring). Briefly, vWF activity was assessed by the ability to agglutinate fixed human platelets in the presence of ristocetin. Agglutination was turbidimetrically measured by the coagulation analyser. Antigenic vWF levels were assayed by a commercial ELISA kit (Asserachrom vWF antigenic, Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's instructions.

HES syntheses

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 molar substitutions of 0.42 and 0.51. HES C2/C6 ratios of 2.7, 7 and 14 were obtained by changing the quantities of sodium hydroxide during hydroxyethylation. All raw HES species were hydrolysed by hydrochloric acid to molecular weights of 700 kDa, treated with activated carbon, purified by ultra-filtration and diluted to a final concentration of 6% (w/v) in isotonic saline, filled in glass bottles of 500 ml each, and heat-sterilized at 121°C for 20 min. HES synthesis was performed in the laboratories of B. Braun Medical SA, Crissier, Switzerland.

HES characterization

Mean molecular weight (Mw) of HES was determined by GPC-MALLS (Wyatt Technology, Woldert, Germany) at a flow rate of 1 ml min^{-1} in a 70 mM phosphate buffer pH 7.0 using serial GPC columns HEMA Bio 40, 100 and 1000 (PSS, Mainz, Germany). Mw was calculated using ASTRA Software (Wyatt Technology, Woldert, Germany).

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, USA). C2/C6 ratio was determined after hydrolysis of HES by sulphuric acid and gas chromatographic separation of the silylated hydroxyethylated glucose derivatives using a gas chromatograph (Carlo Erba Mega 5300; Milan, Italy).

Statistical analysis

Sample size has been determined by a power analysis based on a previous *in vitro* study.[10] To obtain a power of 80% with an estimated difference between groups of 10% and a SD of 15% a total sample size of 30 patients has been determined with a type I error of 0.05.

All data were calculated as changes *vs* baseline and reported as mean (SD). A three-way analysis of variance model with repeated measures on three ways was constructed using the JMP 5.1 statistical package (SAS Institute, Inc., Cary, NC) for assessing the effect of molar substitution, C2/C6 ratio and dilution, respectively, and all possible interaction between these factors. Thus, the variability was partitioned into the following components: molar substitution, C2/C6 ratio, interaction between molar substitution and C2/C6 ratio, dilution, interaction between dilution and C2/C6 ratio, interaction between dilution and molar substitution, and interaction between all three factors.

Results

Plasma coagulation and TEG parameters were normal at baseline (Table 1).

Dilution effect

Haemoglobin decreased from 12.2 (1.3) to 9.8 (1.2) g dl^{-1} (20% dilution), 7.6 (1.0) g dl^{-1} (40% dilution) and 5.2 (0.8) g dl^{-1} (60% dilution) because of *in vitro* haemodilution ($P<0.001$). No difference was found between the different HES solutions investigated. Dilution alone had a significant effect on all TEG parameters and on all plasma coagulation parameters ($P<0.001$ for all parameters): the more the blood was diluted, the more the coagulation was compromised (see Figs 2–5).

Table 1 Baseline values. Data are mean (SD). The following parameters were measured: TEG: *r*-time, *k*-time, angle α, MA, shear elastic modulus (G) and CI; plasma coagulation: PT, aPTT, functional vWF and antigenic vWF (AG vWF) and factor VIII (F VIII)

			Normal range
TEG parameters			
r-time (mm)	9.9	(1.1)	5–15
k-time (mm)	3.1	(0.7)	2–6
Angle α (°)	67.6	(4.2)	55–78
MA (mm)	62.0	(7.4)	51–69
G (Kd s^{-1})	9.0	(2.4)	4.6–10.9
CI	1.2	(1.4)	−3 to 3
Plasma coagulation			
PT (s)	10.1	(0.7)	7.7–11
PTT (s)	32.4	(3.2)	26–36
Functional vWF (%)	97.2	(39.9)	50–200
AG vWF (%)	139.8	(57.9)	50–200
F VIII (%)	89.5	(27.9)	50–200

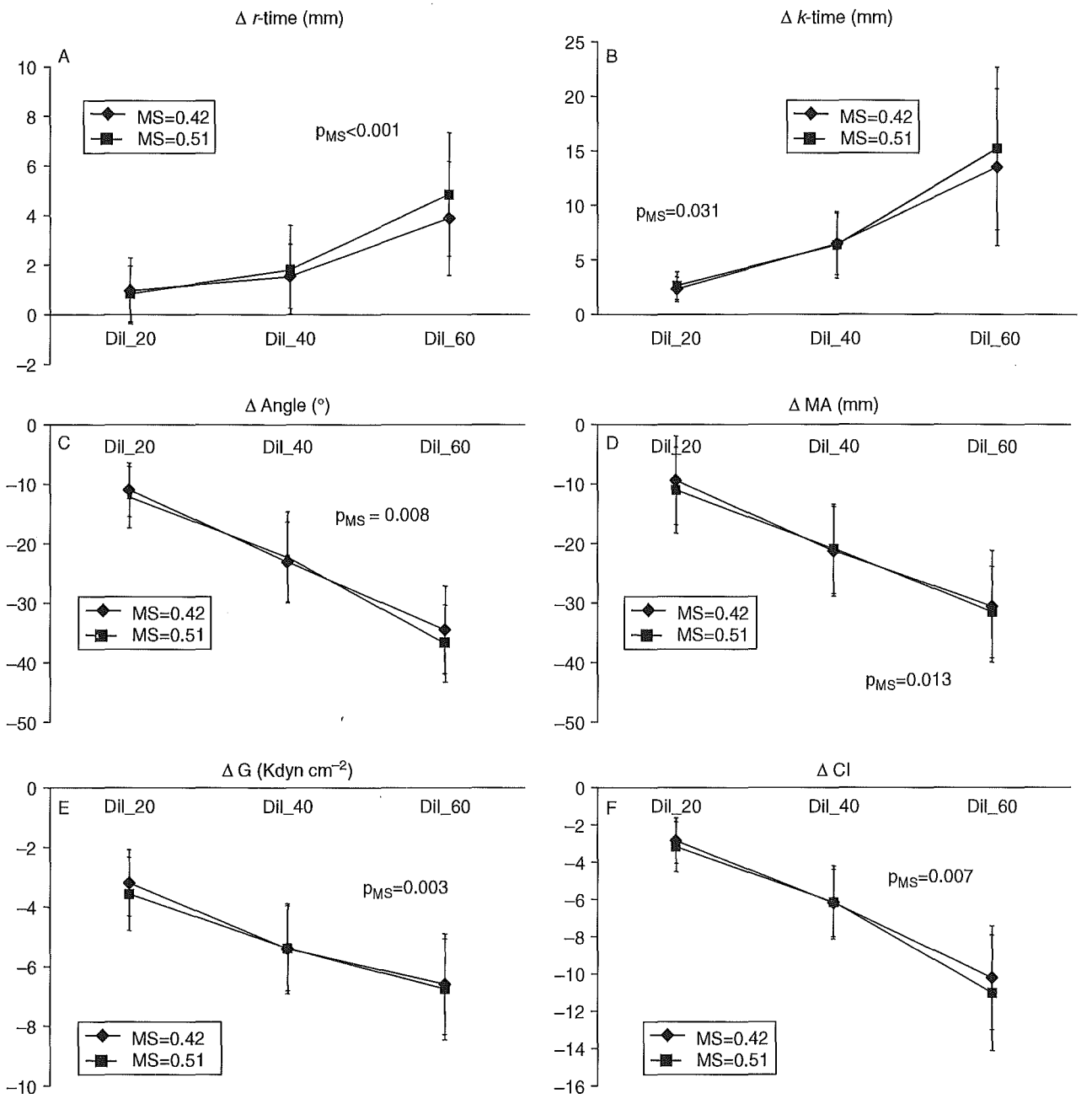

Fig 2 Effects of molar substitution (MS) on TEG analysis. Blood samples were diluted with HES solutions with a MS 0.42 or 0.51 at dilutions of 20% (Dil_20), 40% (Dil_40) and 60% (Dil_60). The following TEG parameters were measured: *r*-time (A), *k*-time (B), angle α (C), MA (D), shear elastic modulus (G) (E) and CI (F). Mean (SD) changes *vs* baseline are depicted (n=30).

Molar substitution effect

TEG

The higher molar substitution of 0.51 resulted in a greater compromise in blood coagulation for all TEG parameters: *r*-time (P<0.001) and *k*-time (P=0.031) were prolonged more when diluted using a HES solution with a molar substitution of 0.51 than when diluted using a HES solution with a molar substitution of 0.42 (Fig. 2A and B). Angle α (P=0.008), maximal amplitude (MA) (P=0.013), G (P=0.003) and coagulation index (CI) (P=0.007) showed a stronger decrease when diluted with a HES solution with a molar substitution of 0.51 than when diluted with a HES solution with a molar substitution of 0.42 (Fig. 2C–F).

Plasma coagulation

PT (P=0.007) increase was lower and factor VIII (P=0.010) decrease was greater when diluted using a HES solution with a molar substitution of 0.51 than when diluted using

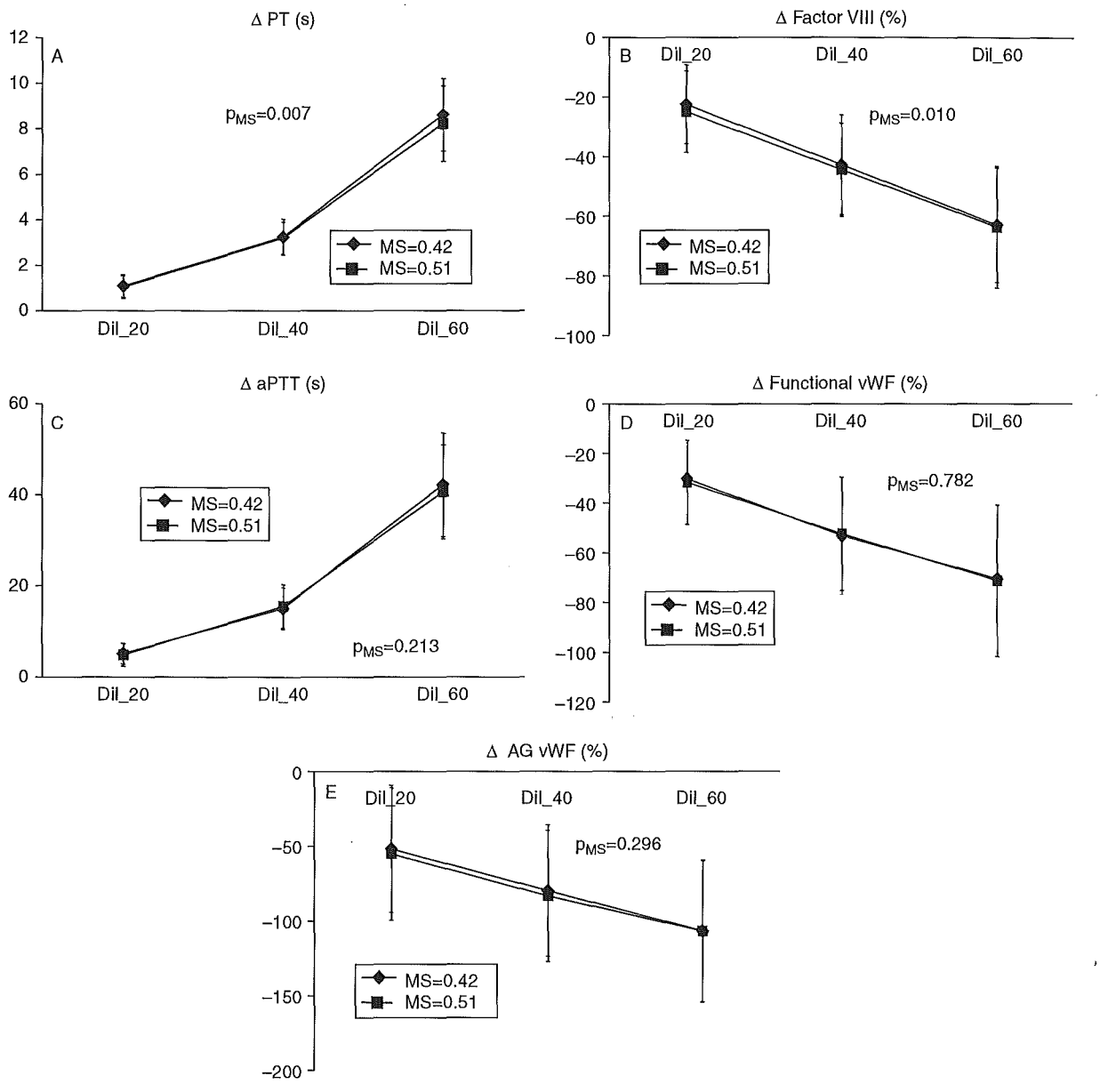

Fig 3 Effects of molar substitution (MS) on plasma coagulation. Blood samples were diluted with HES solutions with a MS 0.42 or 0.51 at dilutions of 20% (Dil_20), 40% (Dil_40) and 60% (Dil_60). The following plasma coagulation parameters were measured: PT (A), factor VIII (B), aPTT (C), functional vWF (D) and antigenic vWF (E). Mean (SD) changes *vs* baseline are depicted (n=30).

a HES solution with a molar substitution of 0.42 (Fig. 3A and B) while aPTT (P=0.213), functional and antigenic vWFs (P=0.782 and 0.296, respectively) showed no significant differences when diluted using HES solutions with a molar substitution of 0.51 or 0.42 (Fig. 3C–E).

C2/C6 ratio effect

TEG

HES solutions with different C2/C6 ratios affected r-time (P<0.001), angle α (P=0.003) and CI (P<0.001) (Fig. 4A–C), while there were no significant differences between these solutions for k-time (P=0.513), MA (P=0.699) and G (P=0.246) (Fig. 4D–F).

Plasma coagulation

No significant differences between solutions with different C2/C6 ratios was found concerning PT (P=0.061), factor VIII (P=0.099), aPTT (P=0.086), functional and antigenic vWFs (P=0.916 and 0.064, respectively) (Fig. 5).

Interactions

A significant interaction between molar substitution and C2/C6 ratio was found for r-time, angle α and CI (all P<0.001).

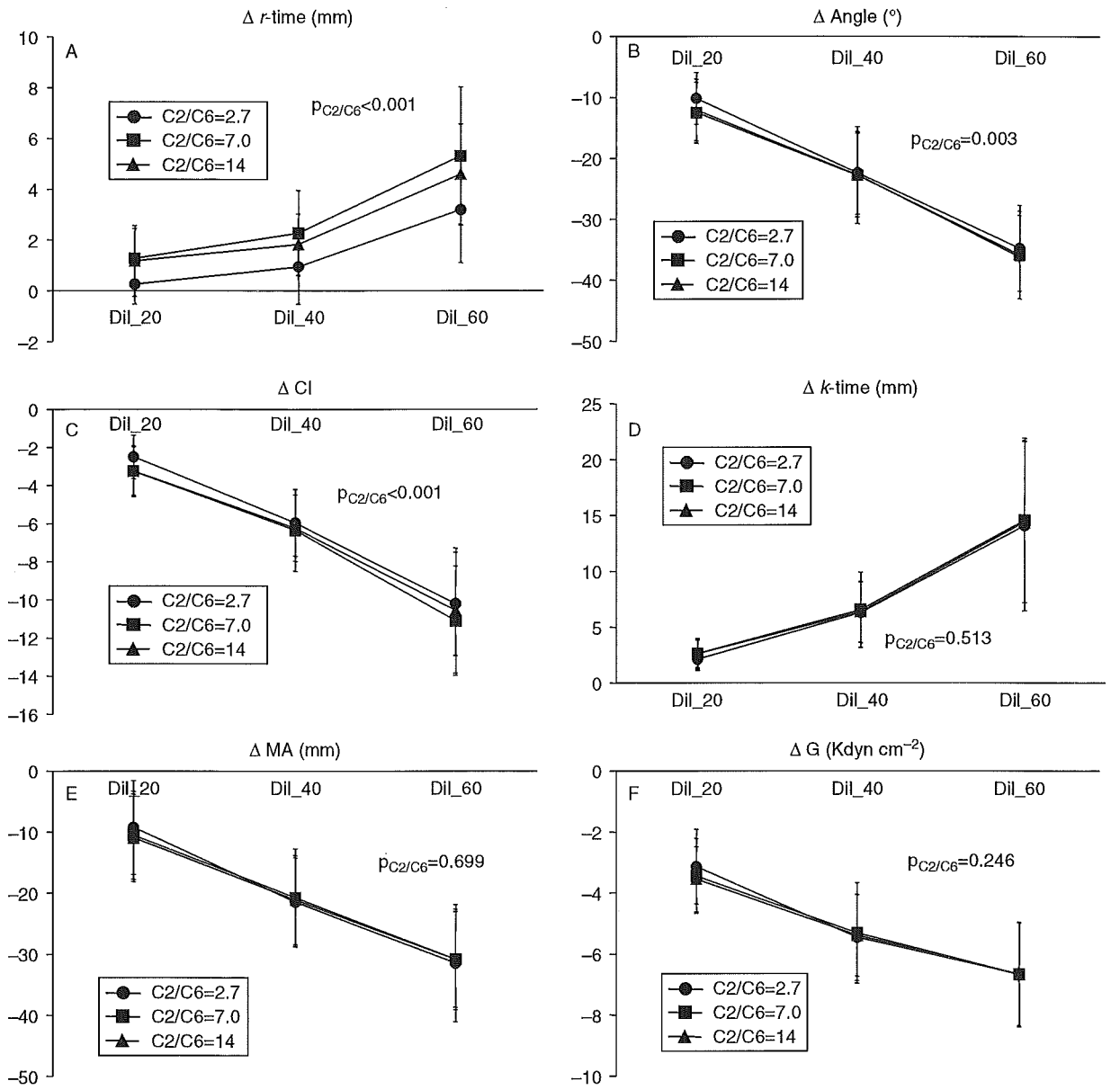

Fig 4 Effects of C2/C6 ratio on TEG analysis. Blood samples were diluted with HES solutions with C2/C6 ratio of 2.7, 7 or 14 at dilutions of 20% (Dil_20), 40% (Dil_40) and 60% (Dil_60). The following TEG parameters were measured: *r*-time (A), angle α (B), CI (C), *k*-time (D), MA (E) and shear elastic modulus (G) (F). Mean (SD) changes *vs* baseline are depicted (n=30).

At a molar substitution of 0.42, *k*-time was progressively prolonged and angle α and CI were progressively reduced with increasing C2/C6 ratios (Fig. 6). In contrast, at a molar substitution of 0.51, a C2/C6 ratio of 7 resulted in a maximum compromise of blood coagulation (Fig. 6). No significant interaction was found for *k*-time (P=0.786), MA (P=0.696), G (P=0.489), PT (P=0.343), factor VIII (P=0.874), aPTT (P=0.237), functional and antigenic vWFs (P=0.865 and 0.821, respectively).

Discussion

This study indicates that HES 700/0.42/2.7 (molecular weight/molar substitution/C2/C6 ratio) has a less pronounced effect on *in vitro* human blood coagulation than HES solutions of the same molecular weight with a higher degree of molar substitution and a higher C2/C6 ratio.

Molecular weight has been considered for a long time as a key factor in determining the compromising effects of HES on blood coagulation.[1 2] However, in recent years the development of HES was always characterized by a concomitant reduction in molecular weight and molar substitution whereas the isolated effect of the molecular weight has not been systematically examined. Only recently Madjdpour and colleagues[11] tested HES molecules with different molecular weights but identical molar substitution. They found that, at a low molar substitution (0.42), high

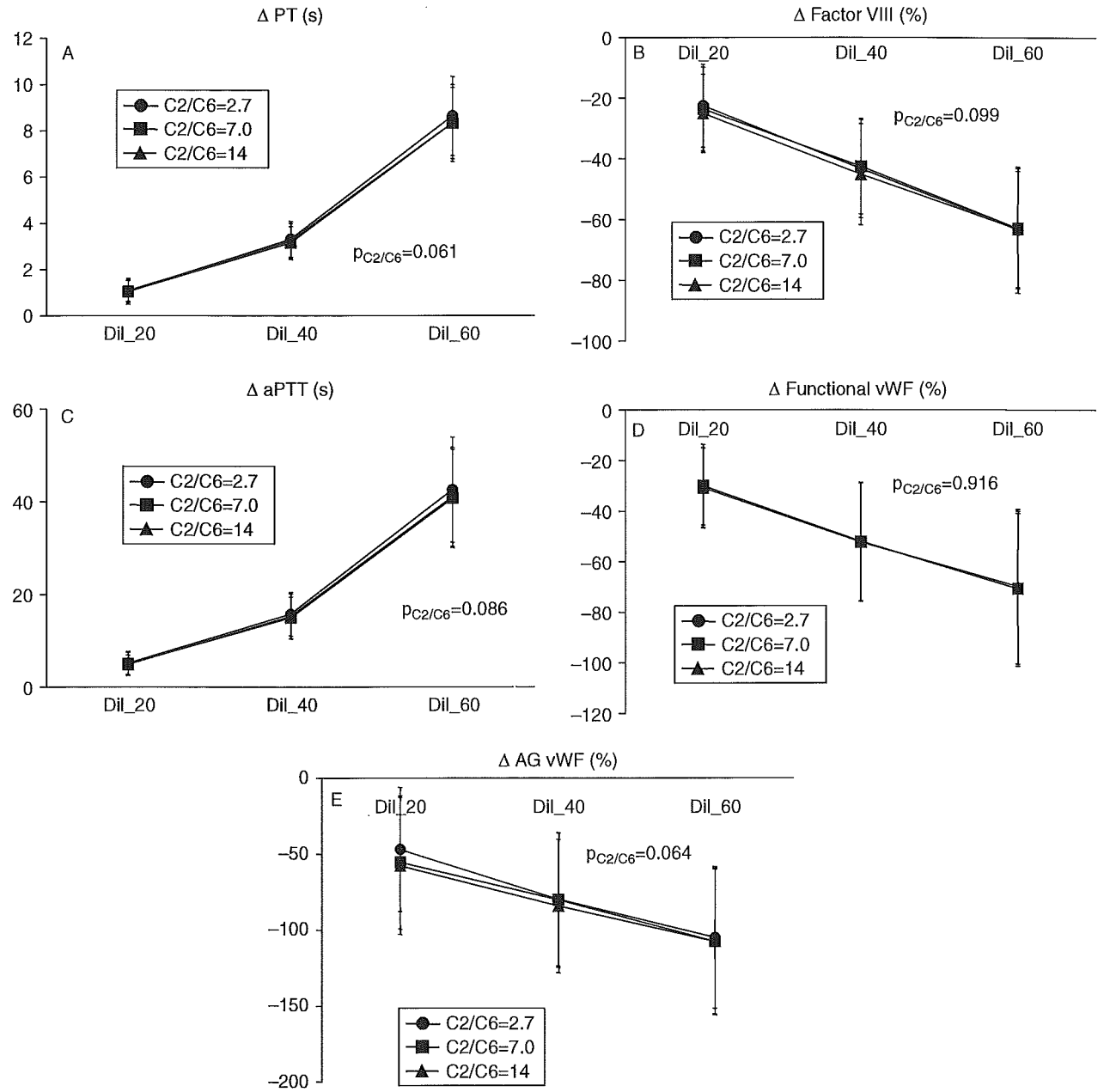

Fig 5 Effects of C2/C6 ratio on plasma coagulation. Blood samples were diluted with HES solutions with C2/C6 ratio of 2.7, 7 or 14 at dilutions of 20% (Dil_20), 40% (Dil_40) and 60 % (Dil_60). The following plasma coagulation parameters were measured: PT (A), factor VIII (B), aPTT (C), functional vWF (D) and antigenic vWF (E). Mean (SD) changes *vs* baseline are depicted (n=30).

molecular weight HES does not compromise blood coagulation to a greater extent than low molecular weight HES with the same molar substitution.

Treib and colleagues[13] studied the effect of molar substitution on blood coagulation. They showed that a HES 200/0.62 solution affected coagulation of human blood more than HES 200/0.5. Unfortunately, the C2/C6 ratio of the solutions used was not indicated. Furthermore, only plasma coagulation but not TEG analyses have been performed with the latter providing a complementary view on the process of blood coagulation by testing the kinetics of the whole coagulation process.[14]

An *in vitro* study investigated the effect of HES 70/0.5/3.2, HES 130/0.4/11.2 and HES 200/0.5/4.6 solutions on blood coagulation and found a reduced effect on blood coagulation of HES 130/0.4/11.2.[15] The authors postulated that the smaller molar substitution might be responsible for this beneficial effect. However, in this study, molecular weight, molar substitution and C2/C6 ratio varied considerably. Thus, it is difficult to allocate precisely an effect to

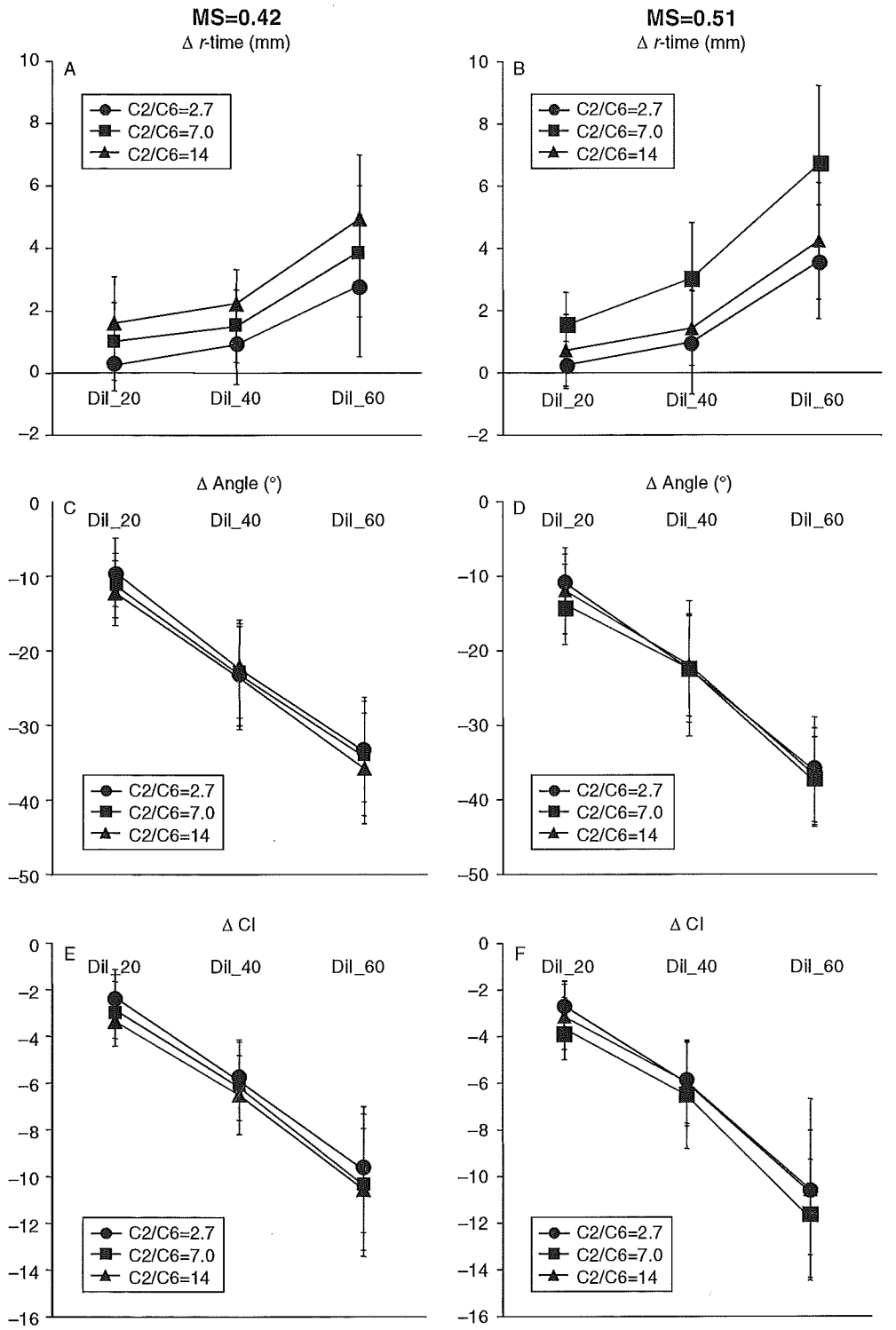

Fig 6 TEG parameters for which a significant interaction between molar substitution (MS) and C2/C6 ratio was found for HES solutions with a MS of 0.42 (left) or a MS of 0.51 (right) at dilutions of 20% (Dil_20), 40% (Dil_40) and 60% (Dil_60): *r*-time (A and B), angle α (C and D) and CI (E and F). Mean (SD) changes *vs* baseline are depicted (n=30).

one parameter. Interestingly, Niemi and Kuitunen[16] recently found in an *in vitro* study that HES 120/0.7 compromised blood coagulation less than HES 130/0.4. Unfortunately, the C2/C6 ratio was not described.

This illustrates the need for systematic studies, which may only be possible *in vitro*, elucidating the complex interplay between molecular weight, molar substitution and the C2/C6 ratio on the effect of HES on blood coagulation. In such a

study, we have now found that in high molecular HES a molar substitution of 0.42 and a C2/C6 ratio of 2.7 results in slightly reduced *in vitro* blood coagulation compromising effects. The observed differences are obviously small but the study design allowed paired comparisons and had thus the power to detect even minor differences. Extrapolating this finding to clinical practice is fraught with difficulties and certainly requires confirmatory *in vivo* studies. Also the effect of the relatively low C2/C6 ratio on pharmacokinetics needs to be equally assessed *in vivo*.

Prophylactic s.c. low-molecular weight heparin does not modify TEG parameters.[17] This is in keeping with the finding that baseline TEG parameters were in the normal range in this study (Table 1), as they were in several previous studies.[12 18 19]

The ideal properties of a HES solution may differ between the intraoperative and the postoperative period. Intraoperatively, a minimal effect on blood coagulation is certainly important. A longer intravascular retention time may be desirable after operation, whereas a certain blood coagulation compromising effect may not necessarily be deleterious. In fact, McCrath and colleagues[20] have recently shown that patients already with a minimal hypercoagulability at the end of surgery had a higher incidence of major thromboembolic complications such as ischaemic strokes, myocardial infarctions, deep venous thrombosis and venous thromboembolism after operation despite standard thromboembolic prophylaxis.

In conclusion, a molar substitution of 0.42 and a C2/C6 ratio of 2.7 were found to be associated with the least compromise on blood coagulation in high molecular (700 kDa) HES. Further studies are necessary to assess whether such a HES also compromises *in vivo* blood coagulation minimally and whether its intravascular retention is indeed higher than a low molecular, low substituted HES such as a HES 130/0.42.

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