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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/97962 since 2023-01-23T08:04:53Z

Published version:

DOI:10.1111/j.1476-4431.2012.00816.x

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08 July 2024

In vitro evaluation of canine hemostasis following dilution with hydroxyethyl starch (130/0.4) via thromboelastometry

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Abstract

Objective

To assess the effects of in vitro hemodilution of canine blood with a low molecular weight hydroxyethyl starch (HES 130/0.4) by means of thromboelastometry (TEM).

Design

In vitro experimental study.

Setting

University Teaching Hospital.

Animals

Ten healthy adult staff-owned dogs were sampled for the purposes of the study. Samples were also collected from 45 clinically normal dogs to establish thromboelastometric reference intervals.

Interventions

For each dog whole blood was collected by jugular venipuncture and placed into tubes containing 3.8% trisodium citrate and stored at 37°C. Two standard dilutions (1:4 and 1:10) from canine blood specimens were prepared with HES 130/0.4 and saline 0.9%.

Measurements and Main Results

The effects of dilution were studied by TEM. No statistically significant differences were observed between the TEM values of the control samples and the samples diluted with saline solution (ratio 1:10 and 1:4). In contrast, hypocoagulability was observed in the samples treated with HES 130/0.4, with more severe effects at 1:4 dilution than at 1:10 dilution. The 1:4 dilution with HES 130/0.4 produced marked alterations: CT (P = 0.035) and CFT (P = 0.0007) on the ex-TEM profile, CT (P = 0.0005) and ML (P = 0.0017) on the fib-TEM profile and CFT (P = 0.0043) on the in-TEM, were all significantly increased (P < 0.05), whereas MCF (P = 0.0042) and alpha angle (P = 0.002) on the in-TEM and MCF (P = 0.011) and alpha angle (P = 0.001) ex-TEM profiles and MCF (P = 0.0001) on the fib-TEM profile were significantly decreased (P < 0.05).

Conclusions

Dilution of canine blood with HES 130/0.4 results in a thromboelastometric pattern consistent with hypocoagulation and this effect appears to result from a dose-dependent alteration in fibrinogen concentration and inhibition of platelet function. As this is an in vitro study, further in vivo investigations are necessary to confirm the results.

Keywords: coagulation, colloid, viscoelastic techniques, ROTEM, TEM

Introduction

In critical care medicine, a wide range of fluids such as crystalloids, natural colloids, and synthetic colloids (eg, dextrans, gelatins, and starches) are available for intravascular volume expansion and support.[1] In veterinary medicine, synthetic colloids are more often used compared with natural colloids. Following gelatin and dextran, hydroxyethyl starches (HES) are the latest generation of artificial colloids. Their physicochemical and pharmacological properties depend on their mean molecular weight, molar substitution, and C2/C6 ratio.[2] Molar substitution and the C2/C6 ratio

determine the rate of HES degradation by plasma α-amylase and intravascular retention time.[3, 4] These features of HES may produce some side effects such as hypocoagulability and impairment of renal function.[5-8] The etiology of hypocoagulability is multifactorial: there is a dilutional effect typified a decrease in blood components or plasma factors, and a nondilutional effect. In vivo studies have demonstrated a decrease in factor VIII, von Willebrand factor, decreased expression of integrin $\alpha_{IIb}\beta_3$ on activated platelets, acquired fibrinogen deficiency, and impairment of polymerization of fibrin monomers.[9-11]

HES 130/0.4, a corn-derived synthetic colloid commercially available in Europe, Australia, and recently approved in the United States, has a low mean molecular weight (130 kD) and a low degree of substitution (0.4).[6] Unlike high molecular weight HES (ranging from 450 to 670 kD), HES 130/0.4 is rapidly degradable and does not accumulate in plasma following repeated administration. It has a shorter intravascular retention time and has minor effects on coagulation and renal function, as demonstrated in studies in people.[4, 12, 13] The enhanced safety margin of HES 130/0.4 allows higher daily doses of up to 70 mL/kg in human medicine compared with other colloids.[7]

The interaction between colloids and hemostasis has been extensively examined in people.[2, 4, 5, 7, 14-20] Some studies investigating the effect of HES 130/0.4 have demonstrated an impairment of several plasmatic factors. Fenger-Eriksen et al[5] found a decrease in fibrinogen concentration, decrease activities for factor II, factor X, and factor XIII to values lower than expected from the dilutional effect alone.[5] In this study, as in that by Casutt et al,[20] hypocoagulation was characterized by means of thromboelastometry (TEM).[20] By measuring the viscoelastic properties of whole blood, TEM provides a complete, dynamic view of hemostasis and fibrinolysis.[21] The use of TEM in dogs was recently described by Smith et al[22] in 2010.

In veterinary medicine, few studies have investigated the interaction between colloids and hemostasis. Two recent studies have evaluated the effects of slowly degradable synthetic colloids (eg, HES 600/0.7, calcium-containing HES 670/0.75) on platelet function, while some early studies assessed clotting times following administration of various synthetic colloids (eg, oxypolygelatin, dextran 70, hetastarch 600/0.75).[23-29]

Considering the lack of information regarding the effect of newer synthetic colloids (ie, HES 130/0/4) on hemostasis in dogs, the aim of the current study was to develop a canine reference interval for TEM at our institution, and secondly, to assess the effects of in vitro hemodilution of canine blood with HES 130/0.4 by means of TEM. Our hypothesis was that in vitro dilution of canine blood with HES 130/0.4 would impair hemostasis despite its low molecular weight and low degree of substitution.

Materials and Methods

Animals

The study protocol was approved by the Bioethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Turin, Italy. Informed owner consent was obtained before enrollment into study. For the determination of the institutional reference interval, 45 adult dogs were recruited. Dogs admitted to the Veterinary Teaching Hospital for routine neutering or being used for blood donation that had routine hematological screening were eligible for enrollment.

For inclusion into study all dogs were deemed healthy on the basis of a complete history and physical exam, hematological[1] and biochemical[2] evaluation, coagulation profile,[3] and serological titers. Serum biochemistry included albumin, total protein, blood urea nitrogen, creatinine, alkaline phosphatase, alanine aminotransferase, γ-glutamyl transpeptidase, glucose, and cholesterol. The coagulation profile included prothrombin time and activated partial thromboplastin time (aPTT), fibrinogen concentration, antithrombin, and D-dimer[4] concentrations. All dogs had to have a serological titer for *Leishmania infantum* <1:40 (assessed via immunofluorescence antibody test) negative serology for *Ehrlichia canis*,[5] *Borrelia burgdorferi*,[e] and a negative antigenic test for *Dirofilaria immitis*.[e] Exclusion criteria included abnormal blood parameters, a history of spontaneous bleeding, and administration of steroidal or nonsteroidal anti-inflammatory medications, propofol, synthetic colloids, blood products,

antimicrobials, or heparin during the 3 weeks prior to the beginning of the study.

In the experimental part of the study, 10 adult staff-owned dogs were recruited for routine hematological testing. All dogs were selected according the inclusion and exclusion criteria listed above.

Determination of reference interval

For each dog, whole blood was collected by jugular venipuncture (20-Ga needle) using minimum vessel occlusion. The blood was collected into tubes containing 3.8% trisodium citrate (1 part citrate: 9 parts blood)[6] and stored at 37°C. TEM[7] was performed within 30 minutes after blood collection according to the manufacturer's instructions, and the analyses were run for 60 minutes. Intrinsic TEM (in-TEM), extrinsic TEM (ex-TEM), and fibrinogen function TEM (fib-TEM) profiles for each sample were performed to evaluate the intrinsic pathway (with activation by ellagic acid), the extrinsic pathway (with tissue factor activation), and fibrinogen function (platelets inactivated with cytochalasin D), respectively. The following parameters were assessed for each profile: clotting time ([CT], s); clot formation time ([CFT], s); maximum clot firmness ([MCF], mm); α angle (α, °); and maximum lysis ([ML],%). For the first 10 animals recruited in the study, a simultaneous TEM analysis of 2 duplicate samples was conducted in order to evaluate the analy-tical imprecision of the instrument for each profile.

Experimental study

For the experimental part, the investigation, blood collection, and the thromboelastometric analysis were performed described above. This part of the study was conducted in 2 consecutive days: on day 1, baseline TEM was measured from the citrated undiluted whole blood (control group). On day 2, a new citrated sample was obtained and divided into 4 equal parts. The first part was diluted with saline solution (0.9% NaCl)[8] at a ratio of 1:10 (1 part of diluent and 9 parts of blood). The second part was diluted with saline solution at a ratio of 1:4 (1 part of diluent and 3 parts of blood). The third and the fourth part were diluted with HES 130/0.4[9] at a ratio, respectively, of 1:10 and 1:4.

Statistical analysis

Data were analyzed using commercial statistical software.[10] Data distribution was tested using the Skewness and Kurtosis test for normality. Homogeneity of variance was evaluated using Levene's robust test. The reference intervals were determined as 5th–95th percentile with 95% confidence interval. The intraassay coefficients of variation (CV) of TEM values were assessed by the arithmetic mean and standard deviation, based on the differences in the duplicate determination.

The effect of the dilution and that of HES 130/0.4 were assessed using analysis of variance for paired data with Bonferroni correction if the data were normally distributed. When the data did not fulfill the assumptions of the parametric method, Friedman's two-way analysis of variance was performed. Values obtained from the simultaneous analysis of 2 duplicate samples were averaged. A value of $P < 0.05$ was considered significant.

Results

Forty-five adult dogs were included for the determination of the institutional reference interval for TEM. Twenty-three were males (22 intact and 1 neutered) and 22 females (15 intact and 7 neutered), aged 1 to 9 years, body weight 23 ± 7.2 kg; age 4.6 ± 3.4 years). Thirty-four dogs were crossbreeds and, among the remaining dogs, the most represented breeds were Golden Retriever, Cocker Spaniel, and Italian Hound (2 dogs each). No abnormalities were detected on hematological, biochemical, or hemostatic screening.

The reference intervals generated for this population of dogs for in-TEM, ex-TEM, and fib-TEM profiles are listed in Table 1. The intraassay CVs are shown in Table 2. In the experimental part of the study, consisting of 10 crossbreed healthy dogs, 5 males and 5 females, aged 1–6 years (body weight 22.6 ± 8.0 Kg; age 4.0 ±2.6 years), all baseline TEM tracings were within our reference intervals.

Table 1. Canine reference intervals generated for various ROTEM assays

Assays	CT (s)	CFT (s)	MCF (mm)	α (°)	ML (%)
In-TEM	126– 363	47–224	50–75	55–81	0–40
Ex-TEM	29–92	54–275	36–73	47–79	0–56
Fib-TEM	14–102	N/A	6–26	40–78	7–52

. Values are expressed as 95% confidence intervals.

. In-TEM, intrinsic thromboelastometry; Ex-TEM, extrinsic thromboelastometry; Fib-TEM, functional fibrinogen thromboelastometry; N/A, not applicable; °, degrees.

Table 2. Coefficients of variation for different ROTEM assays

Assay	CT	CFT	MCF	α	ML
In-TEM	7.93%	8.23%	1.75%	3.41%	28.53%
Ex-TEM	9.08%	5.38%	1.31%	2.81%	23.34%
Fib-TEM	14.19%	N/A	10.77%	5.21%	31.04%

. In-TEM, intrinsic thromboelastometry; Ex-TEM, extrinsic thromboelastometry; Fib-TEM, functional fibrinogen thromboelastometry; N/A, not applicable; °, degrees.

No statistically significant differences were observed between baseline TEM values for control dogs and the samples diluted with saline solution (ratio 1:10 and 1:4). When compared to baseline values, the 1:10 dilution with HES 130/0.4 produced a moderate increase in CFT on the ex-TEM profile and a moderate decrease in MCF on the fib-TEM profile (Table 3).

Table 3. Comparison of thromboelastometry values among baseline, saline dilutions, and hydroxyethyl starch (HES) dilutions in the experimental group (*n* = 10)

Table 3. Comparison of thromboelastometry values among baseline, saline dilutions, and hydroxyethyl starch (HES) dilutions in the experimental group (*n* = 10)

	Variable	Baseline	Saline 1:10	Saline 1:4	HES 1:10	HES 1:4
In-TEM	CT (s)	235 (144–219)	205 (142–260)	200 (161–243)	201 (136–276)	236 (161–298)
	CFT (s)	136 (76–224)	107 (78–240)	143 (80–347)	171 (101–328)	↑ 261[a] (125–581)
	MCF (mm)	56 (50–67)	58 (47–65)	52.5 (43–63)	54 (41–64)	↓ 49[a] (37–59)
	α (°)	66 (55–74)	69 (54–75)	65 (47–74)	60 (47–70)	51[a] (35–66)
	ML (%)	3 (0–30)	2 (0–21)	7 (0–16)	1 (0–73)	12 (0–31)
Ex-TEM	CT (s)	61 (37–86)	62 (11–113)	63 (32–134)	75 (41–153)	↑ 144[a] (36–244)
	CFT (s)	150 (106–275)	177 (110–265)	181.5 (124–360)	194[a] (160–324)	↑ 296[a] (196–625)
	MCF (mm)	53 (36–65)	50.5 (42–63)	48 (35–59)	49 (37–60)	44[a] (29–55)
	α (°)	62 (47–69)	58.5 (51–68)	56.5 (45–77)	55 (47–79)	47[a] (42–56)
	ML (%)	18 (1–48)	16.5 (2–57)	23 (0–55)	19 (1–83)	30 (0–94)
Fib-TEM	CT (s)	50 (38–86)	55 (36–111)	38.5 (28–283)	85 (42–419)	↑ 366[a] (46–760)
	MCF (mm)	10 (6–13)	9 (5–13)	8 (2–12)	6[a] (3–11)	↓ 3.5[a] (3–5)
	ML (%)	28 (20–45)	32 (21–52)	28 (4–45)	33 (23–90)	↑ 62.5[a] (25–98)

. *a* The variables where a significant difference ($P < 0.05$) between the controls and the diluted samples was found.

. Values are expressed as median (minimum-maximum); °, degrees; ↑↓, median value post-dilution that was outside of the reference interval.

In contrast, the 1:4 dilution with HES 130/0.4 produced marked alterations: CT and CFT on the ex-TEM profile, CT and ML on the fib-TEM profile, and CFT on the in-TEM profile (Table 3) were significantly increased, whereas MCF and α on both the in-TEM and ex-TEM profiles and MCF on the fib-TEM profile were significantly decreased (Table 3).

Discussion

Coagulation abnormalities are often present in critical care medicine and therefore knowledge of the potential hemostatic interactions with therapeutic products administered is essential. Several studies in human medicine have investigated the hemostatic changes associated with the administration of synthetic colloids.[2-5, 14-20] Viscoelastic coagulation tests such as TEM and thromboelastography (TEG) are useful diagnostic tools to help identify hypo- and hypercoagulable conditions in dogs. [30-34] In the TEM profiles, CT represents the first phase of fibrin formation from activation of the test to a

clot amplitude of 2 mm; this parameter is mainly affected by the concentration of plasma coagulation factors and coagulation inhibitors (eg, antithrombin or drugs).[11] CFT depicts the velocity of clot formation and is affected predominantly by platelet number and function and by fibrinogen activity. MCF, the maximum firmness reached by the clot, is determined by both platelet number and function and fibrin formation in the presence of factor XIII.[k] The α angle corresponds to the slope of the tangent on the elasticity curve and indicates a tendency toward a hypo- or hypercoagulable condition. Finally, ML indicates the degree of fibrinolysis.[k]

The reference intervals for in-TEM and ex-TEM of the present study are similar to those reported by Smith et al,[22] although greater variation is noted. This might be explained by differences in sample size and geographic origin of the population. In addition, reference intervals for the fib-TEM and the ML are provided. In regards to the fib-TEM assay, we were unable to calculate the CFT range because the tracing never reached the amplitude of 20 mm. The α value was also not measurable following dilution with both saline and HES 130/0.4 and therefore it was not included in the analyses.

As reported in a previous study, TEM analyses showed high precision in the in-TEM and ex-TEM profiles, as demonstrated by low intraassay CV.[22] The fib-TEM profile also showed good precision (Table 2). The only variable to demonstrate low precision was ML, with a CV > 20%. This may be related to the fact that this parameter is not influenced by the use of the activators, which has been suggested to reduce interindividual variability.[22]

In the experimental part of the study, saline was chosen to investigate the dilutional effects of HES 130/0.4. Unlike other crystalloids (eg, lactated Ringers), saline does not contain calcium, which could induce coagulation. The 2 dilutions employed in the present study (ie, 1:10, 1:4) were selected to simulate bolus administration of a fluid dose of 10 and 30 mL/kg, respectively, which correspond to an in vitro dilution of 10% and 25%, respectively.[23]

Dilution with saline (1:10 and 1:4) did not induce statistically significant alterations in TEM tracings; therefore, the hypocoagulable effects seen with hemodilution with HES could not solely be attributed to a dilutional effect.

In some animals (especially at the 1:4 saline dilution), however, we noted a reduced clot consistency (ie, low MCF) and slower CT and CFT as compared with our established reference interval. In contrast to studies in people using TEG (using native whole blood), we did not observe hypercoagulability following mild-to-moderate hemodilution (20–40%) with crystalloid.[35, 36] This phenomenon could be explained by disturbances resulting from the interaction between thrombin and antithrombin due to hemodilution.[37] The lack of changes compatible with hypercoagulability when the analysis is performed on citrated blood (as is done with TEM) indicates that hemodilution exerts its major effects on the early parts of the pathway that are activated during citrate storage.[35, 37, 38]

In our study, the effect of the 1:10 HES 130/0.4 dilution on TEM was modest and limited to CFT on the ex-TEM and MCF on the fib-TEM thromboelastometric profiles. Significant hypocoagulability was observed after 1:4 hemodilution with HES 130/0.4, and this effect was greater when the dilution was increased, as demonstrated by the alterations in ex-TEM thrombo-elastometric parameters (Table 3). The thromboelastometric profiles showed hypocoagulability characterized by longer plasma activation time (ie, increased CT), longer clot formation and propagation time (increased CFT and decreased α), and lower clot stability and firmness (decrease in MCF and increase in ML). In the 1:4 HES 130/0.4 dilution, significant alterations were observed in additional variables when compared with 1:10 dilution. Coagulation times were significantly prolonged on the ex-TEM and fib-TEM profiles, in line with results from recent human studies.[19, 39] According to Weiss et al,[40] CT prolongation by colloid solution could be related to a delayed initiation of clot formation rather than an impaired function of clotting factors.

In our study, the 1:4 dilution also produced a decrease in the α angle and an increase in CFT on the in-TEM and ex-TEM profiles. These alterations indicate a delayed interaction between fibrinogen,

platelet, and plasma factors, as reported by Casutt et al[20] and Godier et al.[39] Reduced clot firmness was indicated by a decrease in MCF on all profiles, probably due to interference between the colloid molecules, factor XIII, and fibrinogen. Fibrinogen deficiency and fibrinogenopathy are thought to be the major mechanisms underlying coagulopathy in people.[6, 17, 40]

Finally, we observed a significant increase in ML on the fib-TEM profile. Generally, an increase in ML reflects either a deficiency/alteration of factor XIII (which interferes with clot firmness) or an increase in fibrinolysis. In this case, hypocoagulability is suggested mainly by decreased clot consistency (MCF) and increased fibrinolysis (ML) in relation to the increase in the dose employed.

One of the limitation of this study is that an ap-TEM profile (strong extrinsic activation of coagulation in the presence of aprotinin, a fibrinolysis inhibitor), which is useful to differentiate between a decreased clot consistency and hyperfibrinolysis, was not carried out in the study and other fibrinolysis markers were also not investigated.

Another limitation of this study is that as this was an in vitro study, it may not necessarily reflect real-world in vivo conditions, owing to the absence of blood flow, endothelium, metabolic degradation, and compensatory mechanisms. Since it has been suggested that in vivo, molecular weight, molar substitution, and the C2/C6 ratio are the main factors compromising blood coagulation in HES, in vivo studies are needed to evaluate how different infusions of HES can modify the hemostatic process.[3, 7] In addition, it will be necessary to study how TEM alterations correlate with clinical signs of bleeding, as done in a previous study for tissue factor-activated TEG in dogs.[34] The small study population also represents a limitation of this study.

Based on several studies in people, platelet function is known to be affected by the presence of colloids, especially slowly degradable HES. [8, 20, 37] It is also known that HES 130/0.4 interferes platelet function to a lesser degree than does slowly degradable HES. Wierenga et al[23] and Smart et al[24] both confirmed that high molecular weight HES affects platelet aggregation in dogs. However, the degree of interference on canine platelet function caused by low molecular weight HES is presently unknown. Since TEM is not sensitive for the study of platelet function, it would be interesting to assess primary hemostasis with other techniques.

In conclusion, in vitro hemodilution at 1:10 and 1:4 with HES 130/0.4 in dogs is associated with changes compatible with hypocoagulability as assessed by TEM. Further in vivo studies are warranted to confirm these results.

Acknowledgments
The authors are grateful to Dr. Vincenzo Scala (TEM INNOVATION 23 GmbH) for the technical support provided for this study.

Footnotes
. 1 ADVIA 120 Hematology, System Siemens Diagnostics, Milano, Italy.
. 2 ILAB 300 plus, Clinical Chemistry System, Instrumentation Laboratories, Milano, Italy.
. 3 Coagulometer StART, Diagnostica Stago, Roche, Monza (MB), Italy.
. 4 NycoCard D-dimer test, AXIS-SHIELD PoC AS, Oslo, Norway.
. 5 Snap 3 DX, IDEXX Laboratories, Westbrook, ME.
. 6 Venosafe 3.8% buffered sodium citrated, Terumo, Leuven, Belgium.
. 7 ROTEM, TEM innovation GmbH, Munich, Germany.
. 8 NaCl 0.9%, Baxter s.p.a., Roma, Italy.
. 9 Voluven, Fresenius Kabi Italia srl., Isola della Scala (VR), Italy.
. 10 Stata Statistical Software: Release 10, StataCorp LP, College Station, TX.
11 Pentapharm GmbH. Manual ROTEM; ROTEM gamma.

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