Evaluation of contact activation of citrated equine whole blood during storage and effects of contact activation on results of recalcification-initiated thromboelastometry

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OBJECTIVE
To evaluate the degree of activation of the contact pathway in citrated equine whole blood over holding times ≤ 30 minutes and assess effects of contact activation on recalcification-initiated thromboelastometry.

ANIMALS
11 healthy adult mixed-breed horses.

PROCEDURES
Blood was collected by atraumatic jugular venipuncture into prewarmed evacuated siliconized glass tubes containing citrate anticoagulant and held at 37°C for ≤ 30 minutes. Thromboelastometry was performed with an in vitro viscoelasticity (thromboelastometry) monitoring system. Factor XII and factor XI procoagulant activities were determined in contemporaneously collected platelet-poor plasma samples by assessing changes in turbidity for 1 hour at approximately 25°C, with clotting times calculated by fitting a line to the steepest segment of the absorbance curve and determining its intersection with baseline. Effect of holding time on thromboelastometry parameters and plasma enzyme activity was evaluated by repeated-measures ANOVA on ranks. Association of procoagulant activities with coagulation time was determined by Spearman rank-order correlation analysis.

RESULTS
Thromboelastometry parameters (coagulation time, clot formation time, α angle, and maximum clot firmness) reflected significant increases in coagulability during the holding period. Factor XII and factor XI procoagulant activities were significantly increased at 30 minutes, compared with 2 or 10 minutes (indicating contact activation of samples), and had significant negative correlation with coagulation time.

CONCLUSIONS AND CLINICAL RELEVANCE
Ex vivo activation of the contact system in equine whole blood was evident, suggesting that recalcification of blood in the absence of a trigger is not an acceptable method of assessing the hemostatic system in horses. (Am J Vet Res 2015;76:122–128)
30 minutes is common,\textsuperscript{6,7,12,15,16} and holding for 2 to 20 hours prior to initiation of thromboelastography or thromboelastometry has also been described.\textsuperscript{11,17} Initiation of coagulation in thromboelastography or thromboelastometry of equine whole blood samples has included use of tissue factor\textsuperscript{11,12,19,16,18} or contact activators\textsuperscript{11,18} and simple recalcification of citrated blood.\textsuperscript{3,10,15,16} Recalcification in the absence of a trigger for coagulation allows for thrombin formation as a downstream effect of activation of the contact system that occurs during sample collection and processing. Experiments with human and canine blood have indicated that continued contact activation during sample holding adversely impacts the results obtained when thromboelastography or thromboelastometry is performed in the absence of a strong coagulation trigger.\textsuperscript{19} Furthermore, multiple studies\textsuperscript{16,18} have indicated that lack of an initiating trigger results in imprecision issues and wider variability in reference ranges for thromboelastometry or thromboelastography parameters of equine blood. The objective of the study reported here was to evaluate the degree of contact activation that occurs ex vivo in citrated equine whole blood and to determine the impact, if any, of such contact activation on results obtained with recalcification-initiated thromboelastometry. We hypothesized that results of thromboelastometry would be impacted by ex vivo activation of the contact system.

Materials and Methods

ANIMALS

Eleven apparently healthy research horses (5 mares and 6 geldings of mixed breeds) were included in the study. Horses were determined to be healthy on the basis of lack of clinical signs of illness, normal physical examination results, and clinically acceptable results of a CBC\textsuperscript{c} and serum biochemical analysis\textsuperscript{c} performed in the clinical laboratory at the veterinary teaching hospital where the study was performed. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Illinois College of Veterinary Medicine.

BLOOD COLLECTION AND PROCESSING

Blood was collected by atraumatic venipuncture of a jugular vein with 21-gauge, 1.25-inch blood collection needles.\textsuperscript{3} Samples were collected directly into evacuated tubes to expose collected blood to the citrate anticoagulant as rapidly as possible. A nonactivated sample (5 mL) was collected first and discarded, and then the citrated sample (4.5 mL) was collected. Citrated samples were collected at a 9:1 ratio in a 3.2% citrate solution in prewarmed (37°C) siliconized glass tubes,\textsuperscript{3} and then mixed thoroughly by gentle repeated inversion. The citrated samples were then held at 37°C for ≤30 minutes.

By use of the supplied electronic pipette, whole blood aliquots (300 µL) were removed from the sample collection tube and added to 20 µL of the manufacturer-supplied concentrated calcium chloride reagent in the thromboelastometry cup at 1, 10, 20, and 30 minutes after collection. Additional aliquots (0.5 mL) for plasma production were removed immediately after collection (0 minutes) and at 8, 18, and 28 minutes after collection, then centrifuged for 1.5 minutes at 5,000 × g. Platelet-poor plasma was transferred at 2, 10, 20, and 30 minutes after collection to tubes containing corn trypsin inhibitor\textsuperscript{f} (a factor XIIa inhibitor) to achieve 50 µg/mL plus SBTI\textsuperscript{f} (a kallikrein inhibitor) at 50 µg/mL, or to tubes containing SBTI alone. Plasma samples were then immediately flash frozen in a 100% ethanol–dry ice bath and stored at −80°C until batch analysis. Owing to the time requirement for centrifugation of blood samples to obtain plasma, the initial sample aliquots for thromboelastometry had coagulation initiated 1 minute after venipuncture, whereas plasma was added to the inhibitor and frozen at 2 minutes after venipuncture.

THROMBOELASTOMETRY

Thromboelastometry was performed with an in vitro viscoelasticity monitoring system\textsuperscript{c} in accordance with the manufacturer’s instructions. Citrated whole blood (300 µL) was added at each designated time to the thromboelastometry cup as described. Variables measured with the manufacturer’s modeling software included coagulation time (the lag period from initiation of the reaction until an amplitude of 2 mm was recorded), clot formation time (the duration necessary for amplitude to increase from 2 to 20 mm), α angle (the angle of change of amplitude during the clot formation time), and maximum clot firmness (the greatest measured amplitude). These thromboelastometry parameters are comparable to R (reaction time), K (clot formation time), α angle, and maximum amplitude, respectively, for thromboelastography. Clotting reactions were followed until maximum clot firmness was achieved.

MEASUREMENT OF PLASMA CONTACT ENZYME ACTIVITY

Plasma samples containing SBTI were thawed at 37°C for 5 minutes, then diluted 10-fold into 125mM imidazole (pH, 7.0). Diluted samples (60 µL) were mixed with 60 µL of human factor XII-deficient plasma,\textsuperscript{1} and clotting was initiated with 60 µL of 25mM CaCl\textsubscript{2}, containing 75µM sonicated phospholipid\textsuperscript{f} (10% phosphatidylserine, 40% phosphatidylethanolamine, and 40% phosphatidylcholine) vesicles. Plasma clotting times were quantified in 96-well ultralow-binding polystyrene microplates.\textsuperscript{4} Clotting was evaluated by monitoring the change in turbidity (light absorbance at 405 nm) for 1 hour at room temperature (approx 25°C) with a microplate reader.\textsuperscript{1} Clotting times were calculated through use of analytical software\textsuperscript{m} by fitting a line to the steepest segment of the absorbance curve and determining its intersection with the initial baseline light absorbance at 405 nm (representing the
Factor XII procoagulant activity was calculated in reference to a standard curve for purified human factor XIIa. Factor XI procoagulant activity was determined in a similar manner for equine plasma samples that contained SBTI and corn trypsin inhibitor, but with human factor XI-deficient plasma and a standard curve for purified human factor XIa used as the basis for calculations.

**STATISTICAL ANALYSIS**

The effect of holding time on thromboelastometry variables and plasma enzyme activity was evaluated by 1-way repeated-measures ANOVA on ranks, with the Tukey test used to determine P values when a significant difference was found. Correlation between factor XII or factor XI procoagulant activity and coagulation time was determined with Spearman rank-order correlation analysis. Analyses were performed with statistical software. Values of P ≤ 0.05 were considered significant.

**Results**

**THROMBOELASTOMETRY PARAMETERS AND FIBRINOGEN**

All 4 measured thromboelastometry parameters were affected by sample holding prior to initiation of coagulation with recalcification of the citrated sample, although significant differences were not found for maximum clot firmness at all time points.
and effects on this variable were less substantial overall (Figure 1). Samples became progressively more coagulable over the 30-minute holding period, as indicated by significant decreases in coagulation time and clot formation time and increased α angle at each time point, compared with earlier time points, and increased maximum clot firmness at 10 and 20 minutes, compared with that at 1 minute. A representative set of thromboelastometry curves from a single sample is provided (Figure 2). Fibrinogen concentrations in samples were within the reference range and did not change over time.

PLASMA CONTACT ENZYME ACTIVITY

Factor XII procoagulant activity (Figure 3) and factor XI procoagulant activity (Figure 4) increased significantly in equine plasma during the whole blood sample holding period. Factor XII procoagulant activity was significantly increased at 20 minutes, compared with 2 minutes, and at 30 minutes, compared with 2 and 10 minutes. Factor XII procoagulant activity in the plasma sample was linearly related to whole blood coagulation time on a log-log scale and had a significant (P < 0.001) negative correlation (r = -0.662) with coagulation time. Factor XI procoagulant activity was significantly increased at 30 minutes, compared with 2 or 10 minutes, and similarly had a significant (P < 0.001) negative correlation (r = -0.642) with coagulation time.

Discussion

Previous reports of use of whole blood thromboelastography and thromboelastometry in veterinary patients have included a wide variety of methodological approaches, including different blood collection methods (evacuated glass tubes and collection needles, syringes, or a combination of syringes and evacuated tubes), types of sample (citrated vs native), coagulation triggers (tissue factor, contact activators, or recalcification alone), and different handling methods after collection. Early reports19,20 of the use of thromboelastography or thromboelastometry for assessment of canine and equine blood indicated that whole blood samples became progressively hypercoagulable during sample holding. This change was initially attributed to ex vivo platelet activation secondary to venipuncture21 or to continued thrombin generation during storage.20,22 These early reports resulted in widespread adoption of a 30-minute holding period for animal blood prior to initiation of thromboelastography or thromboelastometry. However, results of our previous study20 to evaluate canine and human whole blood samples with thromboelastometry found that the shift toward hypercoagulability during sample holding is primarily attributable to activation of the contact system, which is more efficient in canine blood than in human blood. On the basis of data collected in the present study, it appears that equine blood undergoes an even more profound ex vivo activation of the contact pathway during sample holding. Coagulation kinetics for whole blood thromboelastometry were markedly affected by sample holding, and evaluation of the plasma obtained from whole blood samples of healthy horses indicated that both factor XII and factor XI were activated ex vivo to a marked degree. Furthermore, the significant negative correlations found between coagulation time...
and factor XII and factor XI procoagulant activities indicated that the observed hypercoagulability was a function of contact pathway activation.

Although activation of the contact pathway detected in the present study occurred ex vivo as a result of exposure of the blood to artificial surfaces, it is interesting to speculate as to whether horses may have a highly responsive contact pathway in vivo. Although contact activation plays no part in hemostatic responses, recent evidence suggests that the contact pathway may contribute to pathological conditions. Further exploration of contact activation as it occurs in horses in vivo may be indicated.

Assembly of the contact factors factor XII, prekallikrein, and high molecular-weight kininogen readily occurs on artificial surfaces. The resulting production of factor Xa and its subsequent cleavage of factor XI to factor Xla is not prevented by citrate anticoagulation because these enzymatic reactions are not calcium dependent. The amount of factor Xla available for generation of downstream coagulation enzymes once a sample is recalcified is a function of the concentration of these factors in a given blood sample, nature of the surfaces to which the blood is exposed, temperature of the sample, duration of exposure to artificial surfaces, and rate of inhibition of enzymes generated in the sample. Removing blood from the vasculature is always associated with some contact activation because blood is exposed to the metal, glass, and plastic of blood collection supplies. Siliconization of citrate-containing glass tubes and use of minimally charged plastics reduce the degree of, but do not completely eliminate, contact activation. Maximal activation of the contact system in equine blood stored in siliconized tubes was subjectively slow over the 30-minute evaluation period, with maximal procoagulant activity detected at the 30-minute time point, which is a commonly reported sample holding period. In comparison, strongly anionic surfaces such as kaolin or ellagic acid can be expected to maximally activate the contact pathway within 3 to 5 minutes, a time frame generally used for aPTT assays. Considering that factor Xla is slowly inhibited by factors present in plasma (primarily C1 inhibitor, less so α2-antiplasmin, α2-macroglobulin, and antithrombin), holding periods > 30 minutes would presumably lead to a gradual decrease in coagulability in the whole blood sample. Reports of longer holding times for equine blood support this notion.

Maximal clot strength (maximum clot firmness) was less profoundly affected by the ex vivo contact activation than were other thromboelastometry parameters. This finding is consistent with previous reports that maximum clot firmness is the measure that is least sensitive to the kinetics of thrombin formation because it is primarily a function of fibrinogen concentration and platelet numbers.

Although the technology of the in vitro viscoelasticity monitoring system used in the present study is not identical to thromboelastography systems, it is quite similar. The sample and reagent volumes used for thrombelastography and thromboelastometry are slightly different, and the available reagents, cups, and pins are quite dissimilar. It is therefore likely that, had the study reported here been performed with thromboelastography, the raw data obtained would have been somewhat different. However, we have no reason to expect that different conclusions would have been reached had the alternate system been used. A recent report comparing results for human plasma obtained with the 2 systems did suggest that the cups and pins supplied for the thromboelastometry system used in the present study might be more charged than those used with thrombelastography, causing slightly greater contact activation in the sample during generation of the thromboelastometry tracing.

The materials used for blood collection could also possibly contribute to contact activation during sample holding. All of our samples were collected in an identical manner with a blood collection system that allowed samples to be collected directly into evacuated glass tubes containing citrate anticoagulant, although a previous report of variables affecting thromboelastography results for canine blood samples indicated minimal to no influence of sample collection method on the results obtained in the study.

To the authors’ knowledge, the influence of holding temperature on thromboelastometry or thrombelastography results has not been specifically addressed in previous studies of viscoelastic testing of equine blood. In a study of sample handling effects on thromboelastometry results for canine blood samples, collection of blood into room temperature (approx 25°C) tubes followed by holding at room temperature rapidly decreased the sample temperature to that of the ambient environment, and this may vary considerably. When a sample at room temperature is added to a thromboelastometry or thrombelastography cup at 37°C, it takes time for the sample to warm to this operating temperature, the time being dependent on sample volume and the ambient temperature. During this initial period, the speed of enzymatic reactions changes with the sample temperature. To eliminate this source of inconsistency, we elected to collect our samples into prewarmed tubes and to hold the samples at 37°C prior to initiation of thromboelastometry. The warmer temperature of storage may have impacted the rate of contact activation in these blood samples, potentially exaggerating the impact of storage as compared with storage at room temperature (which is more commonly described). As a general rule, enzymatic activity doubles for every 10°C increase in temperature, so we would expect that, had we stored the blood samples at a temperature of 25°C, the degree of contact activation would have been decreased by approximately 50%. Storage at cooler temperatures such as 4°C would be expected to delay contact activation, but would not be recommended because of the well-described ex vivo activation of platelets under these conditions. Regardless of tem-
perature used, contact activation would be expected to occur in equine blood and consequently would influence the results obtained with recalcification for thromboelastometry and thromboelastography. Therefore, establishment of protocols for sample collection, preanalytic handling, and storage is essential for test precision and accuracy.

Aliquots of whole blood from each horse were obtained from 1 sample collected into a single evacuated tube. This may represent a study limitation in that the tubes may have been exposed to a greater degree of manipulation than in usual laboratory protocol. In addition, the study population was a convenience sample of 11 healthy horses, which may not have been representative of the equine population in our veterinary hospital and should not be extrapolated to different populations. However, selecting a true random sample of horses for this study was not logistically possible. Future studies, with larger study populations, should allow for evaluation of the effects of age, sex, and other variables on thromboelastometry results.

Although it seems rational that, to most closely resemble in vivo conditions, in vitro testing should minimally manipulate the sample, it must be recognized that clot formation in vivo is usually initiated by exposure of extravascular tissue factor; the contribution of which cannot be assessed by any in vitro methodology. Further, in the absence of any coagulation activator, recalcification of citrated blood allows for thrombin generation only via the contact pathway, which is irrelevant for in vivo hemostasis (although it may possibly have relevance to pathological thrombus development). Consequently, lack of application of an appropriate trigger means that the kinetics of clot formation are primarily dependent on ex vivo factors that have little association with the inherent hemostatic capacity of the animal from which the blood sample was collected. Recalcification of blood in the absence of a trigger is therefore not an acceptable method of assessing the hemostatic system in equine patients. Implementation of a consistent holding time prior to recalcification does not rectify the problem, considering that such an approach would affect the variable magnitude of the artifact and not eliminate the fact that the assay result was largely dependent on changes that occurred ex vivo. The approach most likely to mimic in vivo conditions would likely be use of a tissue factor trigger. The difficulty comes in determining the optimal concentration of tissue factor to use in an assay; the amount needs to be adequate to maximally trigger thrombin formation and minimize the contribution of any ex vivo contact activation. The minimal amount of tissue factor required is likely to vary, depending on the species source of the tissue factor and of the blood being evaluated. Veterinary thromboelastography assays often use a prothrombin time reagent that contains recombinant human tissue factor. When this reagent was diluted, a much higher concentration of the tissue factor was required to minimize the influence of factor XII activation in canine blood (approx 3,500-fold diluted) than in human blood (approx 17,000-fold diluted), for which contact activation ex vivo is much less profound.

A previous report describing use of the same reagent diluted 3,600-fold for use with equine blood samples indicated an influence of ex vivo changes on thromboelastography results with holding times $\leq$ 1 hour. We would consequently expect that for equine blood, a higher concentration of tissue factor would be required to avoid the influence of ex vivo contact activation. The amount needed would be similar to, or greater than, that reported in some recent studies describing thromboelastography results for equine whole blood. Alternatively, there may be specific circumstances under which contact activation with a strong activator (eg, ellagic acid or kaolin) would be indicated. This approach, like the plasma-based aPTT, could potentially be more sensitive to deficiencies in intrinsic factors (eg, with hemophilia) and may be more sensitive for monitoring effects of anticoagulant treatment. The optimal trigger to be used for both thromboelastometry and thromboelastography testing of equine whole blood samples needs to be further evaluated in outcome-based studies of equine patients. However, our results suggested that use of simple recalcification should be eliminated for both thromboelastometry and thromboelastography performed on equine blood.

Acknowledgments

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Footnotes

c. Hitachi 917 Automatic Analyzer, Roche Ltd, Basel, Switzerland.
d. BD Eclipse, Becton-Dickson, Franklin Lakes, NJ.
e. BD Vacutainer Citrate tube, 4.5 mL, Becton-Dickson, Franklin Lakes, NJ.
f. Corn trypsin inhibitor, Haematologic Technologies, Essex Junction, VT.
g. SBTI, Sigma-Aldrich, St Louis, MO.
h. ROTEM, Pentapharm GmbH, Munich, Germany.
i. Human factor XII–deficient plasma, Haematologic Technologies, Essex Junction, VT.
j. Phospholipids, Avanti Polar Lipids, Alabaster, Al.
k. Ultralow cluster microplates, Corning Inc, Corning, NY.
l. Spectrmax Microplate Reader, Molecular Devices Corp, Sunnyvale, Calif.
m. SigmaPlot, version 7.101, SPSS Inc, Chicago, Ill.
n. Human Factor XIIa, Haematologic Technologies, Essex Junction, VT.
o. Human factor XI–deficient plasma, Haematologic Technologies, Essex Junction, VT.
p. Human factor XIa, Haematologic Technologies, Essex Junction, VT.
q. Sigma Stat, version 2.03, SPSS Inc, Chicago, Ill.
r. Innovin, Dade Behring Hochs, Liederbach, Germany.
References


