Investigation of the effect of kaolin and tissue factor–activated citrated whole blood, on clot forming variables, as evaluated by thromboelastography

Pär I. Johansson, Louise Bochsen, Søren Andersen, and Dorthe Viuff

BACKGROUND: The Thrombelastograph (TEG; Haemoscope Corp.) analyzes clot formation in whole blood (WB) and treatment based on this analysis has been shown to reduce transfusion requirements in liver and cardiac surgery when compared to conventional coagulation analysis. Implementing TEG as a routine laboratory-based analysis, however, requires validation of the activators employed and the effect of storage of the WB sample in citrate before analysis.

STUDY DESIGN AND METHODS: The effect of kaolin, tissue factor (TF) 1:17,000, or TF 1:42,500 on TEG clotting time (R), Angle (velocity of clot formation), and maximum clot strength (amplitude [MA]) were evaluated, together with day-to-day variation, the coefficient of variance (CV%), and the effect of citrate storage time.

RESULTS: Clot formation variables were equally affected by TF 1:17,000 and kaolin activation, whereas R was significantly longer when TF 1:42,500 was used. The CV for the different variables varied from 3 to 13 percent with no significant differences between assays. Storage of citrated WB significantly affected the TEG variables in a hypercoagulable direction. Only the R, however, was significantly affected (12%) when samples rested for 0 and 30 minutes were evaluated with kaolin as the activator.

CONCLUSION: The TEG assays evaluated were reproducible and present with an acceptable CV% for routine clinical practice. Kaolin and TF 1:17,000 equally affected the clot formation variables. Storage of WB for up to 30 minutes in citrate did not, except for R, affect clot formation variables when kaolin was used as activator allowing for immediate analysis when the sample arrives in the laboratory.

ABBREVIATIONS: Angle = clot forming kinetics; LysisAUC = the area under the fibrinolysis curve calculated from maximum amplitude; MA = maximum amplitude; R = clotting time; TEG = Thrombelastograph; TF = tissue factor; tPA = tissue plasminogen activator; WB = whole blood.

Assessment of hemostasis with the Thrombelastograph (TEG; Haemoscope Corp., Skokie, IL) system has been used clinically for more than 40 years to identify, monitor, and guide treatment of coagulopathies.¹ Transfusion therapy based on the result of the TEG analysis has been shown to reduce transfusion requirements in patients undergoing liver² and cardiac³ surgery and to better predict the need for blood transfusion than conventional coagulation assays.⁴⁻⁵ In clinical practice, this point-of-care analysis has typically been performed outside the laboratory setting, in the operating room, or in the intensive care unit on native whole blood (WB).⁶

With the introduction of the cell-based model of hemostasis, which emphasizes the role of thrombin generation and the cellular elements, that is, the platelets (PLTs) for intact hemostasis⁷ together with the finding that thrombin generation, as measured by thrombin-antithrombin complexes,⁸ correlates with TEG thrombus generation, the interest in this WB assay has further increased. Before implementing the TEG as a routine
analysis in the blood bank laboratory to monitor and guide transfusion therapy, however, certain questions regarding the utility of this technology needed to be addressed. First, in clinical practice, kaolin is employed to initiate the coagulation process, whereas laboratory investigations favor recombinant tissue factor (TF) as the activator based on the observation that the TF pathway provides the major stimulus of initiation of clot formation in vivo, whereas kaolin, also containing buffered stabilizers and a blend of phospholipids, initiates coagulation via contact activation. Utilizing activators with potentially different effects on the clot formation could make interpretation of the results difficult to translate into a clinical setting. Second, performing the TEG analysis in a laboratory setting often requires transportation of the blood sample for more than 5 minutes, being the upper limit for running native samples and, hence, storage of the blood sample in citrate becomes necessary. Citrate storage has been reported to influence the result of the TEG analysis and it has been suggested that the blood sample should be rested for a fixed number of times before the analysis is performed to standardize the results. Such an approach would seriously hamper the usefulness of the technology, relying on displaying the result in real-time from start of analysis and thereby allow for earlier interventions than when relying on the conventional coagulation assays. Furthermore, we also added tissue plasminogen activator (tPA) to the blood samples to evaluate its effect on clot formation variables and to investigate the ability of TEG to identify increased fibrinolysis in a reproducible manner.

MATERIALS AND METHODS

The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethics committee in Copenhagen, Denmark. Written informed consent was obtained from each participant prior to trial entry.

Study design

TEG analyses were performed on blood from healthy donors employing six different assays to investigate 1) normal values, 2) day-to-day variation, 3) variations between sexes, 4) the coefficient of variance (CV%), 5) differences between and within assays with and without addition of tPA, and 6) variation over time, when citrate stabilized blood was stored at room temperature for up to 90 minutes.

Participants

Danish blood donors, 27 male and 32 female, participated in the generation of normal values for the various assays. Additionally, in a repeated blood sampling study, blood samples were taken from nine donors (2 male, 7 female), on each of 3 different days. For the blood storage study, blood samples were taken from 7 additional donors (3 male, 4 female).

Blood sampling

Normal values were generated with blood collected from a sample bag attached to the blood collection bag. This blood was immediately transferred into a vacutainer tube containing 1/10 of 3.2 percent citrate (BD Biosciences, Plymouth, UK). For the remainder of the experiments, blood was obtained by a smooth cubital venipuncture, employing minimal stasis and a 21-gauge needle into vacutainers containing 3.2 percent citrate.

TEG coagulation analysis

Before any of the assays were investigated, the operational integrity of the TEG system was secured by performing the recommended quality controls from the manufacturer, and these were found within the recommended ranges. Six different TEG assays were performed with citrated fresh WB using kaolin or two different solutions of TF (1:42,500 or 1:17,000; Innovin, Dade Behring, Deerfield, IL) in the absence or presence of 0.75 nmol per L tPA (American Diagnostica, Stamford, CT). Kaolin or TF with or without tPA was carefully mixed with WB by inverting the tube five times before adding it to the TEG cup, which was preloaded with CaCl for recalcification (15 mmol/L, free CaCl approx. 2 to 3 mmol/L). The hemostatic process was recorded with a TEG coagulation analyzer (5000 series, Haemoscope Corp.). Three TEG variables (clotting time [R], clot forming kinetics [Angle], and maximum amplitude [MA]) were analyzed for all assays. The clotting time, R, denotes the latency from mixing until the clot starts to form (2-mm amplitude), the Angle (α) represents velocity of clot formation, and MA represents the maximum strength of the clot. The lysis variable area under the fibrinolysis curve calculated from MA (Lysis-AUC) was recorded in the assays including tPA (Fig. 1). The same technician performed all analyses. Data analysis was processed in computer software (Version 4.1.73, Database Version 1.0.16, Haemoscope Corp.).

Normal value study

Analysis of TEG variables measured in WB from 59 donors using six different sample preparation protocols was performed and data were summarized as mean and standard deviation (SD) for each assay and sex. Data from males and females were compared for each assay by a t test on log-transformed values. Agreement between pairs of assays was investigated from the paired difference, d (ln scale), generated for each donor. Limits of agreement (ln scale) were established as $d \pm 2 \times s_d$ and transformed...
to ratios on the original scale by the exp function \( \exp(d \pm 2 \cdot s_d) \).

Repeated blood sampling study
In this study (9 donors \( \times \) 3 separate days), all variables (R, Angle, MA, and LysisAUC) were log-transformed (natural logarithm, ln) before the statistical analysis, and the standard error on the ln scale was interpreted as CV\% on the original scale. Initially data from each assay preparation and variable were analyzed separately in a two-way analysis of variance (ANOVA) with day (three levels) as a fixed effect and person (nine levels) as a random effect. Systematic differences between the 3 days in this study, that is, differences common for all donors, were interpreted as technical day-to-day variation.

Subsequently a model without an effect of day and with person as a random effect was fitted. From this model CV\% within person was estimated and limits of agreement between two measurements from the same person but from different days was calculated.

Systematic differences between assays were investigated in analyses that included data from all assays, but separate analyses were undertaken for different variables. The applied model was a three-way ANOVA with factors day (three levels), person (nine levels), and assay (three or six levels depending on variable). Interaction between day and person and between day and assay was included. After removal of the day \( \times \) person interaction and of the main effect of day, the effect of addition of tPA was further investigated.

Storage study
The results were analyzed in a two-way ANOVA model with time (0, 30, 60, and 90 min) as a fixed factor and person (seven persons, of whom four were females) as a random factor. The variables were log-transformed before the statistical analysis and the difference between Time 0 minute and the other time points were back-transformed to the original scale and expressed as a ratio to the result at Time 0.

RESULTS

Normal values for TEG assays employing different activators
Results are presented in Table 1. The data show significant differences between males and females for the Angle and MA variables for all assays investigated. For the R variable, no significant sex differences were found. Estimated means of ratios in level between assays are presented in Table 2. Only the R was significantly different for both sexes when comparing the TF 1:42,500 with either of the other two activators (TF 1:17,000 or kaolin, \( p < 0.0001 \) for both activators).

Day-to-day variation
In the two-way ANOVA with day as a fixed effect and person as a random effect, the effect of day was only significant (at the 5% level) in 2 of the 36 combinations of variable and assay. This is considered a chance finding and the technical day-to-day variation was not significant for any of the assays investigated. The estimated CV\% within person from the model without an effect of day is presented in Table 3. When comparing the CV\% in the different assays investigated this varied for R between 10.3 and 13.0, for Angle from 3.2 to 5.7, for MA from 5.1 to 7.4, and for LysisAUC from 11.7 to 13.7 with no significant difference between assays.

Effect of blood sample storage in citrate
When kaolin activation was used, only R was significantly affected by resting at the 30-minute time point whereas all the TEG variables, except MA, were significantly changed when TF 1:17,000 was used. When TF 1:42,500 was employed as activator, all variables were significantly changed (Table 4).

DISCUSSION
The principal finding of this study was that no significant difference between any of the TEG clot forming variables was found when WB was activated by kaolin or TF (1:17,000). Recombinant TF has been suggested as the preferred activator based on the observation that it is the TF pathway that provides the major stimulus for clot formation. In vitro, however, the activators investigated here appear to merely accelerate the initiation phase of
coagulation process not influencing the amplification or propagation phases. In concert with this, using TF at a lower concentration (1:42,500 dilution) only resulted in a significantly delayed initiation (increased R), without alterations in the other clot forming variables. Clinical studies reporting a decrease in blood product utilization in patients treated according to the result of the TEG analysis compared to conventional coagulation assays have been performed using both TF and kaolin activation and the results of this study supports that both these assays can be employed. Furthermore, the results of in vitro studies, mainly relying on TF-activated TEG analysis, to evaluate the effects of hypothermia, acidosis, crystalloids and colloids, and prohemostatic and anti-fibrinolytic agents on the coagulation process are relevant for the clinical setting.

The ability to employ kaolin as activator in routine laboratory practice is important because this allows WB to be added to a ready-to-use vial containing the activator and thereafter ready for analysis. Contrary to this, to employ TF as the activator requires diluting it from a stock to the required concentration with buffers that should be prepared in the laboratory in advance, increasing preanalytical factors, which may adversely influence the test result.

The result of this study demonstrated that the CV% varied between 4 and 14, not significantly different from what Jennings and colleagues reported when investigating external quality control of the TEG presenting CV%
between 8 and 17 for the different TEG variables, and in alignment with our results also Jennings and colleagues found the R variable to present with the highest CV%.

WB storage in citrate for up to 90 minutes significantly affected the TEG variables in a hypercoagulable direction in alignment with previous reports. When kaolin was used as the activator, however, only the R was significantly altered toward a hypercoagulable state when the nonrested and blood rested for 30 minutes was compared and the difference was merely 12 percent, which in the clinical situation would rarely affect the decision in how to treat a given patient. For the TEG analysis to be performed in a laboratory setting, transportation of the blood sample often requires more than 5 minutes, which is the time frame of analyzing a native blood sample and, hence, citrate stabilized samples are necessary. It has previously been suggested that blood samples stored in citrate should rest for a fixed time before analyzing to previously been suggested that blood samples stored in citrate for up to 90 minutes significantly affected the TEG variables in a hypercoagulable direction in alignment with previous reports. When kaolin was used as the activator, however, only the R was significantly altered toward a hypercoagulable state when the nonrested and blood rested for 30 minutes was compared and the difference was merely 12 percent, which in the clinical situation would rarely affect the decision in how to treat a given patient. For the TEG analysis to be performed in a laboratory setting, transportation of the blood sample often requires more than 5 minutes, which is the time frame of analyzing a native blood sample and, hence, citrate stabilized samples are necessary. It has previously been suggested that blood samples stored in citrate should rest for a fixed time before analyzing to secure standardized conditions but the results of the present study indicate that, if kaolin is used as activator, no resting of the blood sample is necessary. This aspect is important, because one of the most valuable features of the TEGs is the speed of the analysis and thereby the ability to start therapeutic interventions within 15 minutes from the start of the analysis.

Consistent for all assays investigated, females presented with a more hypercoagulable TEG profile with regard to R and Angle compared to males in alignment with the findings of Bochsen and coworkers. In contrast, Schreiber and coworkers reported that only the R was significantly different between sexes, being shorter in female trauma patients during the first 4 days after injury. A possible explanation for these divergent findings may be the patient population studied. Schreiber and coworkers studied trauma patients with substantial tissue injury, which may result in maximal activation of the PLTs and thrombin generation, thereby masking differences between the genders.

This study confirms the utility of TEG analysis to identify enhanced fibrinolysis in a reproducible manner. This finding is in alignment with Nielsen and coworkers who reported that the degree of fibrinolysis demonstrated by TEG analysis is dose-dependently related to the in vitro addition of tPA. This finding suggested that TEG analysis can be used to quantify the extent of fibrinolysis in clinical and laboratory setting and may potentially guide antifibrinolytic therapy. This is in alignment with Ghosh and colleagues who demonstrated that the classification of severe hemophilia patients based on TEG patterns, including fibrinolysis, correlated well with the severity of bleeding and the hemostatic effect of antifibrinolytics agents.

Important limitations of the present study must be considered. First, WB from Danish blood donors was used and hence the normal values generated in this predominantly Caucasian population may not apply universally. Furthermore, only citrated blood was investigated, and hence, the results presented may not apply when native WB is employed although a current technical report, by Wasowicz and colleagues demonstrated that when kaolin, as opposed to TF, was used to activate citrated blood, samples yielded results similar to those obtained from noncitrated, fresh blood samples.

Also, only standard TEG assays were investigated and the TEG PLT mapping assay, enabling evaluation of the effect of antithrombotics on clot strength, was not investigated. Furthermore, in the generation of normal values for the different assays blood was sampled from a collection bag and it cannot be excluded that this procedure resulted in a more pronounced activation of the blood than had it been sampled by venipuncture. For this study, however, this may be of less importance because the aim was not to establish reference values but to compare the effect of different activators and these were all evaluated on blood collected as described above and hence with the same degree of activation. In addition, the blood samples were not run in duplicate in the repeated blood sampling study and, therefore, the comparisons of the TEG clot forming variables in the different assays were made from the results of blood samples run with and without the addition of tPA and this was considered comparable to a duplicate comparison. This assumption was based on the observation that addition of tPA did not significantly alter the clot forming variables for any of the assays investigated.

In conclusion, the current study supports the routine use of TEG technology in a laboratory setting by demonstrating an acceptable technical day-to-day and intraassay

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<th>Activator</th>
<th>Time (min)</th>
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<th>Angle MA</th>
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<td>89</td>
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<tr>
<td></td>
<td>90</td>
<td>81†</td>
<td>117†</td>
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<td>84*</td>
<td>112*</td>
</tr>
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<td></td>
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* p < 0.05. † p < 0.001. ‡ p < 0.01.
variation. Based on these results, we suggest that the TEG analysis in a laboratory setting may be performed by kaolin activation of citrated WB.

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REFERENCES


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