Effect of whole blood viscosity and red cell mass on canine thromboelastographic tracings

THESIS

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Abstract

Abnormalities in hemostasis can result in life-threatening bleeding or thrombotic disorders. Whole blood viscoelastic methodologies including thromboelastography (TEG) have become increasingly popular in human and veterinary research and clinical medicine as a means of assessing coagulation. However, the properties of blood that influence these methodologies must be understood to accurately interpret the results, especially when in vitro findings do not match what is expected in vivo.

It is well established that the hematocrit (Hct) of a sample influences the TEG tracing. While, clinically, anemia has been shown to result in an increased risk of bleeding, anemic TEG tracings typically have a hypercoagulable appearance. The opposite is also seen, as TEG tracings of samples with high Hct appear hypocoagulable. The reason for this effect of Hct on TEG tracings is unknown. However, as Hct is the main determinant of whole blood viscosity, it is possible the influence of red blood cells on the TEG is truly the effect of viscosity. In order to elucidate this difference, whole blood viscosity must be manipulated separately from Hct. We hypothesized that the effect of Hct on TEG tracings may be due to a mechanical bias of the methodology due to the effect of Hct on blood viscosity, rather than the red blood cells themselves.
To test this hypothesis, three experiments were performed. The first used alginate (ALG) to normalize blood viscosity independent of red cell mass at varying hematocrits (45%, 20%, and 10%) and compared to control samples diluted in saline. The second experiment had a similar design, but used a different agent, carboxymethylcellulose (CMC) to modify viscosity at Hcts of 20% and 10% compared to saline-diluted controls. Finally, TEG tracings and whole blood viscosity of naturally anemic dogs were compared immediately before and after red blood cell transfusion.

These three experiments demonstrate that increasing the whole blood viscosity of a sample does make TEG tracings appear less hypercoagulable than anemic saline controls, independent of Hct. However, this effect appears to be relatively small, and the Hct has additional effects shifting the TEG tracing towards decreased hypercoagulability, independent of viscosity. The influence of the agent used to alter viscosity demonstrates that ALG may have additional properties that alter coagulation that have not been previously reported. To rule out an artifact of calcium binding, we demonstrate that the effect of CMC-altered viscosity on TEG tracings is preserved even with the addition of extra calcium. Finally, increasing Hct with RBC transfusion in clinically anemic dogs correlates with mildly decreased MA and G TEG values and increased whole blood viscosity. However, these alterations towards decreased hypercoagulability are relatively small compared to the global coagulation status of the animal. Overall, this data indicates that whole blood viscosity does have a small impact on TEG tracings independent of Hct, the effect of RBC mass on TEG tracings is greater than what can be explained by the
impact of viscosity alone, and that these changes are relatively small in dogs with clinically significant anemia requiring red blood cell transfusion.
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Chapter 1: INTRODUCTION

1.1 The cell based model of coagulation

Hemostasis is an extremely complex system that is essential for life in any organism with a circulatory system. It encompasses the processes of clot formation, stabilization, and breakdown, while simultaneously confining clot formation to local tissue beds to prevent inappropriate thrombosis from damaging healthy tissues. Dysregulation of the hemostatic system can result in life threatening bleeding or thrombosis, and therefore it is not surprising that this system is highly conserved among mammals and has been extensively studied in both human and veterinary medicine.

Although the term fibrin was coined by Plato over 2000 years ago on the observation that blood formed “fibers” once it left the body,1 our more modern understanding of coagulation has advanced most rapidly in the last 150 years. While platelets and their critical role in hemostasis were originally described in the mid 1800’s,1 the “classic” coagulation model proposed in 1905 by Morawitz included only 4 factors: “thrombokinase,” prothrombin, fibrinogen, and calcium.2 Discoveries of additional clotting factors and standardization of their names to a roman numeral system by The International Committee on the Nomenclature of Blood Coagulation Factors in 1954 resulted in proposal of the traditional “waterfall” cascade model of hemostasis that is still widely taught in medical schools today.3,4 (Figure 1.1)
This model was helpful in understanding the role of the serine protease coagulation factors as zymogens and in interpretation of commonly used coagulation tests such as the prothrombin time (PT) and partial thromboplastin time (PTT). However, it is obviously overly simplistic in that the extrinsic pathway alone cannot create a stable clot as evidenced by the severe bleeding tendencies in hemophilia A (lack of fVIII) and B (lack of fIX). Conversely, patients deficient in fXII show no clinical bleeding abnormalities in vivo despite an incomplete intrinsic pathway.\(^2,^5\) Additionally, the role of platelets is considered as a separate entity in these models, with a platelet plug forming initially at the site of vascular injury (primary hemostasis) and then being stabilized by a fibrin meshwork (secondary hemostasis) despite the acknowledgement that a phospholipid membrane, and platelet involvement, is necessary for either intrinsic or extrinsic pathway to function.\(^6,^7\)

These gaps in previous models have led to the more recent development of the “cell-based” model of hemostasis, summarized by Hoffman and Monroe in 2001.\(^8\) This model continues to expand as the role of multiple cell types including platelets, endothelial cells, white blood cells, erythrocytes, as well as cell membrane fragments from these cell types (microparticles) is increasingly understood, and helps explain the localization of coagulation to specific cell membrane surfaces. The cell-based approach is generally divided into 3 phases; initiation, amplification, and propagation\(^5,^9\) (Figure 1.2), with some models preferring a 2-step system, grouping initiation and amplification as one initiation step.\(^10\)

*Initiation:*
Exposure of activated or non-encrypted Tissue Factor (TF) is widely accepted as the first step in initiation of in vivo coagulation. TF is generally localized on cells not exposed to flowing blood under normal conditions, such as fibroblasts and smooth muscle cells. It can also be facultatively expressed on the surface of cells exposed to blood such as activated platelets, macrophages, endothelial cells, and microparticles in states of inflammation or cell damage. While an alternative splice variant of soluble circulating TF has been described, its role in coagulation is still unclear.\(^1\) Once TF is exposed to blood, fVIIa is able to bind TF. fVII is the only coagulation factor that circulates in small amounts in its activated form, but is unable to activate subsequent coagulation factors until it is complexed with TF. The source that activates fVIIa is still under much debate; fVII can be activated by fIXa, fXa, fXIIa, fIIa, and plasmin, as well as by other proteases; it has also been proposed to undergo autoactivation.\(^10\) Once fVIIa is bound to TF in the presence of Ca\(^{2+}\), this complex is able to activate fX and fIX. fXa can then bind with fVa to form the prothrombinase complex. The source of fVa at this step is likely multifactorial. Small amounts of fV can be activated by fX alone in the presence of an appropriate membrane and adequate calcium concentration, can be released from activated platelets and activated in the presence of thrombin (fIIa), or be activated by non-coagulation cellular proteases such as elastase and cathepsin.\(^9,11\) The small amounts of thrombin generated by the prothrombinase complex in the initiation phase act as a “spark,” which, if large enough and sustained enough, drives the process into the amplification step. The thrombin generated in initiation can activate fXI, create more fVa to complex with fXa, cleave VIII from vWF and activate it, and can activate fIX. The fXa/fVa prothrombinase complex is initially localized to the TF-bearing cell, as
any fXa that diffuses away from this surface will be rapidly inactivated by tissue factor pathway inhibitor (TFPI) or antithrombin (AT). Once TFPI is bound to fXa, it also becomes a much more potent inhibitor of the TF-fVIIa complex, providing further local negative feedback during initiation. While TFPI can have some inhibitory effects on fIXa, this effect is far weaker. Therefore if enough fIXa and fXIa are produced in the initiation phase, they can diffuse away from the initiation surface and participate in amplification and propagation on other procoagulant membrane surfaces, classically activated platelets.\textsuperscript{9}

\textit{Amplification:}

The initial damage that exposes TF bearing cells in the initiation phase also exposes subendothelial collagen and von Willebrand factor (vWF), so concurrent platelet recruitment and activation at the site of damage is occurring during the initiation phase.\textsuperscript{8} During the amplification phase, the fIIa “spark” produced in initiation further activates platelets, activates platelet-derived fV, activates fXI, and cleaves and activates fVIII from vWF. The amplification phase is essentially setting the stage on an appropriate cell membrane surface (generally perceived as activated platelets) for co-localization and assembly of coagulation factor complexes to create large-scale production of thrombin in the propagation phase.\textsuperscript{5}

\textit{Propagation:}

In propagation, fIXa generated in the initiation phase (or by the fXIa activated by thrombin in the amplification phase) complexes with fVIIIa and Ca\textsuperscript{2+} on platelet cell surfaces to form the “tenase” complex.\textsuperscript{1} As previously discussed, the small amounts of fXa made by TF-VIIa in the initiation phase is rapidly degraded when it moves away
from the cell surface, so formation of the tenase complex on platelets during propagation allows for generation of large amounts of fXa on an appropriate pro-coagulant membrane surface. \(^9\) This much larger amount of fXa generated by the tenase complex joins with fVa and Ca\(^{2+}\) to form the prothrombinase complex, which activates fII. The so called “thrombin burst” produced by the prothrombinase complex is now made in sufficient quantity to cleave large amounts of fibrinogen and form a stable clot. \(^{13}\) The large amount of thrombin produced in this phase also has several other roles, including further platelet activation, fXIII activation leading to fibrin cross linking and increased clot stability, and activation of anticoagulant and antifibrinolytic pathways such as protein C/protein S and activation of thrombin-activateable fibrinolysis inhibitor (TAFI). \(^{14}\)

One of the most important concepts of the cell based model is the role various cell membranes plays in supporting and localizing generation of the tenase and prothrombinase complexes. While coagulation may be initiated on any TF bearing cell, the amplification and especially propagation steps require a suitable cell surface microenvironment to function optimally. \(^5\) Platelets are by far the most important and best studied cell surface for the propagation phase of coagulation, yet other cell types are also incorporated and involved in clot formation, including white blood cells, red blood cells, endothelial cells, and microparticles. \(^{15}\)

When platelets are activated, they swap procoagulant phospholipids such as phosphatidylserine (PS) in their phospholipid membranes to the external membrane leaflet. \(^9\) These phospholipids are believed to play a critical role in localization and assembly of co-factors fVIIIa and fVa with their respective factors IXa and Xa, and greatly promoting the speed of the enzymatic reactions. This is likely due to interaction
of these cofactors with the negative charge of the phospholipid “head” of PS as opposed to other neutral phospholipids.\textsuperscript{9} Factors IIa, VIIa, IXa, and Xa, carboxylated by Vitamin K, also interact with PS in a calcium-dependent manner to co-localize with their cofactors and substrates on the cell surface.\textsuperscript{1} The critical nature of this interaction is underscored by the life-threatening bleeding that occurs in the absence of Vitamin K. Platelet cell surface receptor proteins for multiple coagulation factors such as PAR-1 and GP1b also help to localize coagulation factors to PS-dense lipid rafts, which facilitate enzymatic interactions on the platelet cell surface and act as transmembrane signaling domains for further platelet activation.\textsuperscript{1,5,8,16} Platelets also protect clots from fibrinolysis as they release plasminogen activator inhibitor 1 (PAI-1), and release myosin, which may mask cleavage sites on fibrin.\textsuperscript{15} Platelets increase clot strength by retraction and strongly influence fibrin structure, resulting in radially dense and thinner fibrin fibers, which are more resistant to fibrinolysis.\textsuperscript{15,17,18}

While platelets are by far the most important and best-studied cell surface for the propagation phase of coagulation, the role of red blood cells as active participants in hemostasis is becoming more and more apparent. It has been demonstrated that anemic patients have a tendency to have an increased bleeding time (BT) and increased risk of clinical bleeding compared to non-anemic patients, and that anemic, thrombocytopenic patients can have their bleeding risk reduced by RBC transfusion alone.\textsuperscript{19,20} This concept, called “anemic coagulopathy,” implies a more active role of the RBC in clot formation than previously thought.\textsuperscript{21}

RBCs are the major contributor to whole blood viscosity, and appropriate RBC volume is necessary for RBC aggregation near the center of blood vessel during flow, aka
“axial migration.”

This effect plays an important rheological role in helping to sequester platelets closer to the endothelium during laminar intravascular blood flow. RBCs may play a similar rheological role in sequestering leukocytes to the periphery in post-capillary venules, improving endothelial contact for margination. Axial RBC aggregation also reduces frictional resistance and decreases local viscosity near the endothelium. This promotes plasma skimming and the Fahraeus-Lindqvist effect, resulting in relatively decreased RBC content in the microcirculation, decreased blood viscosity, and decreased flow resistance.

Decreased wall shear stress will also decrease nitric oxide production, and increase platelet activation, favoring a more procoagulant hemostatic balance. In disease states of very high hematocrit, such as polycythemia vera, very high blood viscosity may even restrict blood flow in small vessels and increase thrombotic risk.

RBCs play a role in coagulation at the molecular level as well. They release the platelet agonists ATP and ADP under conditions of low pH, low PaO₂, or high shear stress, as might be expected in damaged vasculature. They also augment platelet production of eicosanoids such as thromboxane, and platelet release of 5-HT, both of which cause further platelet activation and recruitment. Hemoglobin, the most prevalent protein in RBCs, scavenges nitric oxide, a platelet inhibitor, further augmenting platelet activity. When exposed to situations such as high-shear stress, complement attack, oxidative stress, or pro-apoptotic stimuli, RBCs can also express a more procoagulant lipid membrane that allows for prothrombinase assembly, similar to platelets. These properties may explain some of how RBCs and RBC microparticles stimulate thrombin generation and enhance platelet activation and aggregation. Finally, incorporation of
red blood cells into a fibrin clot changes the structure of the fibrin network, reducing fiber
diameter and increasing clot resistance to fibrinolysis.\textsuperscript{29} Taken together with the clinical
evidence, all of these factors seem to indicate that increasing red cell mass should favor a
hypercoagulable state, and decreased red cell mass a hypocoagulable state.

Given the importance of cells in our increasing understanding of hemostasis, it
becomes clear why traditional methods of monitoring hemostasis such as the PT and PTT
may be poorly reflective of \textit{in vivo} coagulation. These plasma-based assays artificially
divide the clotting cascade into intrinsic and extrinsic paths and are considered complete
once fibrin is generated, despite the fact that the majority of thrombin generation occurs
in the propagation phase after the initial formation of fibrin.\textsuperscript{5,30} While these tests can be
helpful in determining hypocoagulable states caused by factor deficiency or inhibition,
they cannot be used to detect hypercoagulable states or the effects of cellular impairment
in coagulation.\textsuperscript{31,32} Given our increasing understanding of the complex interactions
between cells and plasma coagulation proteins, there is increasing interest in more global
assessments of hemostasis that include all of these factors.\textsuperscript{33} As a result, viscoelastic
hemostasis assays such as the thrombelastograph hemostasis analyzer or TEG\textsuperscript{©}
(Haemonetics Corporation, Braintree, MA, USA), Sonoclot coagulation and platelet
function analyzer or Sonoclot\textsuperscript{©} (Sienco Inc., Arvada, CO, USA), and rotation
thromboelastometer (ROTEM\textsuperscript{©}) (Penta- pharm GmbH, Munich, Germany) have become
widespread in both the human and veterinary coagulation literature in the past 20 years;
for the purposes of this review, TEG will be discussed as it is the methodology used in
the studies described herein. While the principles behind viscoelastic technologies are
similar, the results obtained by these different methodologies are not interchangeable and cannot be directly compared.\textsuperscript{32,34}

1.2 Thromboelastography (TEG)

1.2.1 Mechanism of TEG and tracing interpretation

Thromboelastography was first introduced by Dr. Hartert in 1948, but did not become widely used in the USA until the 1980’s when it gained popularity in monitoring coagulation during liver transplantation.\textsuperscript{31,35} The methodology of the TEG is as follows:\textsuperscript{36} (Figure 1.3) The substance to be analyzed is placed in a sterile plastic cup warmed by the machine to $37^\circ$C. The substance may be unaltered as in the case of non-anticoagulated whole blood, or may be recalcified +/- additionally activated to initiate clot formation up to a total volume of 360uL of substance within the cup. Once the sample is added to the cup, the cup is immediately raised so that a stationary pin is submerged in the well of the cup and is circumferentially contacted by the sample. The cup gently rotates in a $4^\circ$-$45'$ arc back and forth in 10-second intervals. When a clot starts to form, the clot will bind to the pin suspended within the cup and generate torque on the pin, which is translated into an electrical signal and the characteristic TEG tracing is generated (Figure 1.3). The amount of torque generated on the pin depends on the viscoelasticity of clot development over time, and as such the tracing generated gives information about the kinetics and strength of clot formation in the sample.

TEG can be performed on any blood product, but is most often used to analyze whole blood, platelet-rich plasma, and platelet-poor plasma. Coagulation-factor deficient plasma and blood mixed with or exposed to various biomaterials have also been described in research settings.\textsuperscript{37} The most clinically relevant sample is whole blood, but
the sample chosen in a research setting will reflect which components of clotting are
under investigation. Whole blood can be used as a point-of-care test without
anticoagulation within 4 minutes of the sample being drawn from the patient (so called
“native” activation or N-TEG), or with recalcification of citrated blood (citrated native or
CN-TEG).32 These samples can be further activated by the addition of substances such as
kaolin (K-TEG) or tissue factor (TF-TEG) to initiate clotting via the intrinsic or extrinsic
pathways, respectively, or the addition of both in the RapidTEG assay (R-TEG).32
Depending on the clinical circumstances, other substances such as heparinase may be
added to assess the effect of administered heparin on patient coagulation as compared to
non-heparinased samples. The addition of antifibrinolytics, such as tranexamic acid, to
assess the effects of inhibition of fibrinolysis has also been described.38,39 Finally,
specific TEG tests such as platelet mapping or functional fibrinogen levels use specific
activators (ADP and arachidonic acid for platelet mapping) or inhibitors (monoclonal
glycoprotein IIb/IIIa receptor antagonists for functional fibrinogen levels, ReoPro®) to
compare those modified TEG tracings to control citrated K-TEG samples. These assays
gauge the inhibition of platelet activity by various platelet inhibitors, or the contribution
of fibrin to final clot strength.38,40 Given the broad range of TEG methodology used
clinically and in research settings, it is no surprise that one of the difficulties in
interpreting TEG data is that results performed via different methodologies are not
directly comparable.41,42 In fact, even results performed with identical methodologies but
different laboratories still show significant variability, creating difficulty in standardizing
TEG across institutions.43
The characteristic TEG tracing is composed of 6 variables: Reaction time (R), Rate of clot formation (K), alpha angle ($\alpha$ or angle), Maximum amplitude (MA), Clot Lysis at 30 minutes (CL30), and Clot Lysis at 60 minutes (CL 60).\(^{32}\) (Figure 1.3). R is the time in minutes from initiation of the TEG to the first detectable fibrin formation causing torque on the pin, and is described as the point at which the tracing deviates by 2 mm from the baseline.\(^{38}\) R times are generally correlated with coagulation factor activity.

In a recent study of canine TF and EA activated ROTEM (equivalent to TF-TEG and K-TEG), concentration of fVIII was highly correlated with TF-CT (equivalent to TEG-R), and concentration of fIX with EA-CT, consistent with activation through the extrinsic and intrinsic pathways, respectively.\(^{44}\) R times may also be prolonged with thrombocytopenia as the lack of appropriate procoagulant surface membranes may delay thrombin formation as previously discussed.\(^{45}\) The clot formation time (K) is the time (minutes) from the end of R time (2 mm deviation from baseline) until the tracing reaches 20 mm amplitude from baseline. In samples where an amplitude of 20mm is never reached, or MA is <25mm, the K values should not be used, but rather the alpha angle used as the primary representation of clot kinetics.\(^{38}\) The alpha angle ($\alpha$) is a line drawn from the end of R (the beginning of coagulation) tangential to the TEG curve. Both K and angle represent the rate of increase of the elastic shear modulus of the clot, i.e. how quickly and how strongly the clot structure is forming.\(^{45}\) Not surprisingly, these are the most complex values in the tracing as they can reflect changes in coagulation factor quantity or function, platelet number or function, hematocrit, and fibrinogen concentration.\(^{32,45}\) The maximum amplitude (MA) is recorded at the largest distance between the two arms of the tracing. MA reflects the maximum strength (maximum
shear modulus) achieved by the clot and can be influenced by multiple factors similar to angle and K. Interestingly, although the fact that increasing Hct correlates with decreasing MA is well established, it is frequently ignored in descriptions of MA in TEG reviews, which focus only on the contribution of fibrin, fXIII, and platelets. In a recent study of canine ROTEM, the factors most strongly predictive of MCF (equivalent to TEG-MA) were Hct, platelets, and fibrinogen. The TEG variables have been correlated with the phases of the cell-based model of coagulation, with the R time roughly reflecting initiation and amplification, and the angle, K, and MA reflecting the propagation phase. The TEG will also record the time taken to reach MA (TMA), which represents the time needed from sample initiation to form a stable clot. Normal in-house as well as published CN-TEG reference ranges for humans, greyhounds, and non-greyhound dogs are shown in Table 1.1, however, as TEG “normals” are highly variable between techniques and institutions, current guidelines recommend generation of normal reference ranges at each institution performing TEG.

Additional values are often derived to represent specific aspects of coagulation kinetics and stability. Global clot strength (G) is calculated as \( 5000 \times \text{MA} / (100 - \text{MA}) \). Based on Dr. Hartert’s original work, this equation uses the MA to estimate the maximum elastic shear modulus of the clot in dynes/cm\(^2\). This gives actual units of force per area to clot strength rather than the arbitrary “mm” of MA on the tracing. The coagulation index (CI) is an equation that combines the 4 main TEG variables to generate one number as a representation of overall coagulability of the sample. It is described in dogs as \( \text{CI} = 0.1227 \times \text{R} + 0.0092 \times \text{K} + 0.1655 \times \text{MA} - 0.0241 \times \text{angle} - 5.0220 \). Normal CI in humans is between -3 and +3, and values below -3 are considered hypocoagulable,
while values above +3 are considered hypercoagulable; relevant CI values in dogs are unknown.\textsuperscript{57} While the summation of all TEG variables into one number may help simplify interpretation, it also may mask valuable information the tracing provides about where defects in clotting occur along the tracing in real time.\textsuperscript{32}

One of the large advantages of viscoelastic testing is its ability to look at fibrinolysis. The values CL30 and CL60 are derived from the amplitude of the TEG tracing at 30 and 60 minutes after MA (A30 and A 60, respectively), and then compared to MA to represent the percent of maximal clot strength remaining, i.e. \( CL30 = 100 \times \frac{A30}{MA} \) and \( CL60 = 100 \times \frac{A60}{MA} \). The parameters LY30 and LY60 may be a more accurate way of looking at fibrinolysis, as they look at the area under the TEG curve at 30 and 60 minutes after MA, respectively (Figure 1.4). LY30 and LY60 are expressed as a percentage change from MA. The lysis values will change in opposition, with a low CL30 and a high LY30 being indicative of fibrinolysis. The clot lysis time (CLT) is how long it takes the TEG tracing to drop by 2 mm once MA has been achieved; it represents the time it takes the samples to undergo a set amount of fibrinolysis. While this use of TEG is well documented in human medicine,\textsuperscript{58} the ability of TEG to detect fibrinolysis in veterinary clinical studies is not well documented or defined, and in both humans and animals may require addition of set amounts of tissue plasminogen activator (tPA) to the assay to adequately standardize detection of fibrinolysis.\textsuperscript{59,60} With the addition of tPA to CN-TEG, K-TEG and TF-TEG, Spodsberg et al were able to demonstrate fibrinolysis in healthy dogs, and decreased fibrinolysis in sick dogs, leading them to hypothesize that resistance to fibrinolysis may be a mechanism of hypercoagulability in sick dogs that would not be detected by traditional TEG.\textsuperscript{60}
Based on the work of Neilsen et al., a set of parameters can be generated from the mathematical first derivative of the TEG value G at various amplitudes over time (Figure 1.4). The advantage of these values is that they are expressed in metric units of elastic resistance to more accurately describe changes in clot strength. The maximum thrombin generation (MTG) is the first derivative of the velocity of the increase in clot strength, which begins as G starts to increase and ends once clot strength stabilizes (MA). The time to maximum rate of thrombin generation (TMG) is the time needed to reach MTG. The area under the MTG curve is total thrombus generation (TTG) represents the total change in elastic resistance until the clot strength stabilizes. This has also been referred to as the TEG_{TG} in the veterinary literature. Similar values are generated for fibrinolysis, including the maximum rate of lysis (MRL), time to maximum rate of lysis (TML), and area of clot lysis (ACL).

While there are several values that can be measured or derived from the TEG tracing as described above, one of its greatest advantages is the quick visual assessment of the tracing that is representative of changes in overall clot strength over time (Figure 1.5).

1.2.2 Limitations of TEG

While the TEG is becoming an extremely popular diagnostic tool for assessment of coagulation in human and veterinary medicine for all of the reasons discussed above, like any test it has limitations. It can be influenced by pre-analytical factors, variation in repeatability of testing and testing methodology, and does not fully represent all factors involved in coagulation, such as endothelial reactivity and rheological variables.
TEG can be influenced by multiple pre-analytical factors such as venipuncture methodology, blood storage container and time, citrate concentration of the sample, temperature, and signalment of the patient. The site of venipuncture does not appear to have an effect on canine TEG tracings. The method of blood collection (syringe vs. vacutainer, delayed vs. immediate citration) can cause statistically significant differences in TEG variables. Whether or not these changes are clinically significant is debatable and varies by study. Current PROVETS guidelines state that there is insufficient evidence to recommend one standardized blood collection system, but do suggest the use of evacuated tubes and 21-Ga or larger needles. Traumatic venipuncture shortens R time in healthy dogs, but can be mitigated by the use of a discard tube as recommended by the manufacturer. The use of IV catheters of varying gauge to sample blood has not been shown to significantly affect TEG result compared to direct clean phlebotomy, but the use of appropriate discard sample sizes and/or heparinase cups should be considered if the catheter has been heparinized.

Due to the difficulty in immediate analysis of fresh whole blood, most veterinary studies are performed on blood anticoagulated with citrate and recalcified immediately prior to starting the TEG analysis. To our knowledge, there are no veterinary studies evaluating the optimum citrate concentration for TEG samples or concentration of calcium to add to the TEG cup to optimize results; current guidelines recommend 3.2% buffered sodium citrate tubes filled to a strict 1:9 ratio and activation with 0.2M CaCl₂. One study on human blood documented increasing hypercoagulability in recalcified citrate samples up to 33mmol/L, with progressive hypocoagulability noted at Ca²⁺ concentrations >39mmol/L, and recommended an optimal value of 2.1mmol/L for
recalcification of citrated blood.\textsuperscript{71} TEG results from recalcified citrated blood are not directly comparable to those obtained from native (fresh non-citrated whole blood) samples.\textsuperscript{42} Citrated blood run with no activator or with kaolin activation will demonstrate progressive hypercoagulability as it sits over time. This is due to the fact that citration does not completely prevent some thrombin generation in the blood, likely due to increasing fXIIa activity from contact activation.\textsuperscript{67,72} The sample can also be influenced by the temperature at which it incubates until run on the TEG, with warmer samples showing more hypercoagulable TEG variables than those held at room temperature.\textsuperscript{67} Repeated sampling from the same tube has also been shown to increase hypercoagulability of the sample, likely due to increased contact activation.\textsuperscript{42} Current recommendations are to hold citrated blood at room temperature for 30 minutes prior to sample acquisition.\textsuperscript{68} While most samples are stored in the glass citrate tube prior to running on the TEG, different tubing surfaces have been shown to cause differences in TEG values, and so standardization of storage methodology is recommended.\textsuperscript{74}

The repeatability and variation in viscoelastic testing continues to be problematic. While a study on biological variation in coagulation parameters in dogs showed a low degree of individuality (suggesting reference intervals would be useful in predicting abnormal samples), it is important to note that this study was performed on fresh frozen plasma from healthy research beagles and may not reflect variation seen in a more clinically applicable heterogeneous population using whole blood.\textsuperscript{75} Similarly, even though the TEG-ROTEM working group used homogenous samples of FFP, they were unable to satisfactorily standardize TEG and ROTEM results between institutions.\textsuperscript{43} In a human study on TF-TEG and K-TEG, the intra-individual variation repeatability of TEG
within the same healthy person sampled on 3 different days had a coefficient of variance <10% for all values except R. However in patients with Hemophilia undergoing therapy with rVIIa, intra- and inter-patient variability was very high, rendering TEG unsuitable for monitoring therapy in this population. TEG variability may need to be determined depending on the underlying disease state of the patient population. In a study performed on healthy horses, the intra-individual CV was >10% for all values except MA and TF-activated alpha in horses sampled once weekly for 5 weeks. In the same study, intra-assay variation was shown to be low regardless of the method of activation used, leading the authors to presume variability in pre-analytical factors was the cause of the large intra-individual variation. While multiple studies have attempted to create normal TEG reference values for dogs, the difficulty in standardizing TEG across institutions has been well established. Therefore it is recommended at this time that each institution derive its own normal values for each method of activation.

To mitigate the high degree of pre-analytical variation that is noted in citrated whole blood samples, the use of activators (most commonly kaolin and TF) has been recently recommended by veterinary guidelines. In a recent study investigating use of TEG in equine subjects, the use of kaolin and TF showed a significant improvement in reducing variation in TEG due to pre-analytical variation. Multiple studies have demonstrated that TEG or ROTEM values obtained via one method of activation are not directly comparable to another method of activation. Which method of activation is preferable is currently undetermined and may depend on the process being studied, as kaolin will activate via the intrinsic pathway and tissue factor via the extrinsic pathway.
To shorten acquisition of TEG variables as much as possible, the use of dual activation with kaolin and tissue factor, so called “Rapid TEG” or R-TEG has also been described.\textsuperscript{83}

During analysis, multiple factors can influence the TEG independently of the patient’s underlying coagulation status. Due to the hanging pin-in-cup setup of the TEG, it is prone to vibrational or motion interference, and is recommended to keep the TEG in an area where it is unlikely to encounter motion artifact.\textsuperscript{38} Inter-operator differences may be significant, especially if they affect sample handling, agitation, or time from recalcification to initiation of TEG recording. Hemolysis has been shown to shorten R time and decrease MA,\textsuperscript{85} while lipemia of a blood sample also influences TEG parameters, but only at very elevated levels.\textsuperscript{86}

Viscoelastic coagulation variables have been shown to differ based on signalment.\textsuperscript{80} While gender and age have been shown to influence TEG results in human studies, one canine study did not shown an impact of gender on kaolin-activated TEG, within the limitation that approximately 30\% of the dogs in the study were neutered.\textsuperscript{79} To our knowledge, the effect of age alone on TEG variable in canines has not been investigated. Significant species differences in viscoelastic coagulation as measured by ROTEM were shown to exist, an important factor to keep in mind in translational medicine between laboratory animals and human medicine.\textsuperscript{87} Published human and canine and canine internal reference CN-TEG values from The Ohio State University blood bank are shown in Table 1.1

As previously discussed, the wide variation in TEG methodologies and inherent variation in the test itself makes comparisons of data extremely difficult. Efforts to standardize TEG in both human and veterinary medicine are ongoing, including a recent
publication with an extensive overview of the current status of viscoelastic testing in veterinary medicine. However absolute standardization, while necessary for overall comparability especially in experimental setting, will not be feasible for all clinical populations. For example, current veterinary guidelines recommend standardization of TEG analysis at 37°C. There is evidence that TEG results are influenced by temperature, so TEG consistently performed at 37°C will not detect changes in coagulation that may be altered by patient body temperature, such as hypothermic coagulopathy.

The most important limitation of the TEG is that, like all other methods of coagulation testing, the assay is performed outside of the blood vessel, and outside of the patient. The TEG can assess whole blood under low-shear rotational conditions. It cannot assess the rheological effect of blood flowing in a vessel, which plays a critical role in tissue perfusion, microvascular dynamics, and cell-endothelial interactions. It does not contain any endothelium from the patient, and therefore all aspects of coagulation relating to endothelial dysfunction, activation, or interaction between the endothelium or subendothelial matrix and cellular or protein coagulation factors are not assessed. Finally, the TEG assesses the sample of blood taken from the patient at that moment in time, and may not reflect a coagulation pattern that is rapidly changing without serial evaluation. The very advantage of TEG as a global point-of-care test that can be performed serially in a patient with rapidly changing coagulation status is also a difficulty in that the tracing may not indicate the exact nature of the hemostatic problem in the patient to guide therapy, requiring additional testing to pinpoint the cause of the coagulopathy. Despite these limitations, TEG has gained popularity in human and
veterinary medicine as both a clinical and research tool.

1.2.3 Clinical utility of TEG in human and veterinary medicine

In the USA, TEG began to gain notice in the 1980’s for monitoring of coagulation during liver transplantation. The desire for a more global assessment of hemostasis in liver disease and transplantation is unsurprising given that these conditions result in a complex interplay between decreased factor synthesis, increased fibrinolysis, platelet dysfunction, as well as a well-documented risk of thrombosis during or after the procedure.\textsuperscript{58,92} TEG-guided transfusion during orthotopic liver transplantation was shown as early as 1985 to reduce the need for blood product administration.\textsuperscript{92} TEG has been shown more recently in a prospective clinical trial to reduce FFP transfusion compared to PT/INR guided monitoring, but did not reduce 3-year mortality, possibly due to small sample size.\textsuperscript{93} While TEG is commonly discussed as a method to reduce blood product transfusion during transplantation, the exact utility and algorithm remain under debate.\textsuperscript{94} There is evidence that TEG does reduce the amount of blood product used in patients with severe trauma or at risk for massive transfusion, but no mortality benefit has yet been reported.\textsuperscript{95,96} TEG has also been used in human medicine to diagnose hypercoagulability and hyperfibrinolysis associated with acute and chronic liver disease outside of a transplantation scenario.\textsuperscript{58} While liver transplant is not routinely performed in veterinary medicine, studies documenting mixed coagulation changes in liver disease\textsuperscript{97} and hypercoagulability in extra hepatic biliary obstruction have been performed.\textsuperscript{98}

The use of TEG to reduce bleeding and transfusion volume is best documented in cardiac bypass. In a randomized, blinded, prospective trial, it has been demonstrated that a TEG guided transfusion algorithm reduced the need for post-operative FFP and platelet
transfusion in the TEG group as compared to a group with transfusion guided by platelet count, PT/ACT, and fibrinogen levels alone.\textsuperscript{99} TEG was shown to reduce re-exploration in bleeding post-cardiac bypass patients and had a higher specificity than traditional tests to predict the need for transfusion.\textsuperscript{100,101} While there is still debate as to the standardization of TEG studies and high variability in study designs for TEG-guided transfusion, the Audit and Guidelines Committee of the European Association for Cardio-Thoracic Surgery (EACTS) gives a grade B recommendation to the use of TEG in guiding post-operative transfusion, but states that further studies are required before it can be called the standard of care.\textsuperscript{102} A recent Cochrane review covering mostly cardiac bypass cases (1 liver transplant study was also included) found a reduction in bleeding when TEG was used to guide transfusion strategy, but no reduction in morbidity or mortality.\textsuperscript{103} To our knowledge, there are currently no studies evaluating TEG to guide transfusion decision-making in veterinary transfusion medicine.

TEG has gained recent popularity in trauma patients in the diagnosis of peri-trauma related hyper and hypocoagulable states, and in guiding transfusion related to acute traumatic coagulopathy (ATC).\textsuperscript{104} TEG has been shown to be predictive of the need for transfusion in human blunt trauma patients and is superior to traditional coagulation testing in predicting transfusion requirement in penetrating trauma.\textsuperscript{105,106} TEG studies of human trauma have found multiple coagulation abnormalities, including hypercoagulability, hypocoagulability, and hyperfibrinolysis, further increasing our understanding of the complexity of the coagulation abnormalities that can occur with trauma.\textsuperscript{104,107,108} In animal models of bleeding caused by hypothermia or hemodilution, TEG was a better indicator of coagulopathy than traditional tests such a PT.\textsuperscript{109,110}
Algorithms for R-TEG guided transfusion therapy in humans suffering from traumatic coagulopathy or severe bleeding have been reported to decrease mortality and transfusion requirements at individual institutions, but multicenter prospective trials are still lacking.\textsuperscript{104,111} One of the benefits of using viscoelastic coagulation monitoring in severe hemorrhage may actually be due to the faster turnaround time of these tests in diagnosing coagulopathy, leading to earlier intervention in coagulopathic patients as compared to more traditional coagulation assays.\textsuperscript{83} Recently, 2 veterinary papers used TEG to attempt to diagnose ATC in veterinary patients. Based on the G value obtained with K-TEG within 6 hours of trauma, one study found 33\% of dogs to be hypercoagulable.\textsuperscript{112} In contrast, another study found dogs with low angle, MA, and G as generated by K-TEG were more likely to need blood product and less likely to survive, but that dogs became hypercoagulable over time as assessed by MA and G.\textsuperscript{113} Wiinberg et al explored the use of a low G value on TF-TEG to predict bleeding compared to other measurements of coagulation (aPTT, PT, D-dimer, fibrinogen, platelet count) in dogs suspected of being hypocoagulable.\textsuperscript{114} They found that a low TEG G value had a higher PPV and NPV than interpretation of the coagulation panel to diagnose clinical bleeding in dogs. This study did not explore whether TEG variable could predict which dogs would be likely to require transfusion in a prospective fashion.

In veterinary medicine, it is most common to use TEG to try to predict hypercoagulable states or predict outcome in diseases suspected to cause hypercoagulability. A study exploring TEG in parvoviral enteritis found hypercoagulability as evidenced by an increased MA in citrated native TEG.\textsuperscript{115} As is common in many of the earlier papers of TEG in veterinary medicine, this paper did not
discuss the Hct of the 9 dogs, which was likely low given that most dogs with parvo are young and have gastrointestinal bleeding as well as hemodilution from fluid therapy. In dogs with cancer, citrated native TEG (CN-TEG) has been used to demonstrate hypercoagulability via increased TEG$_{TG}$ in dogs with carcinoma, and TF-TEG to demonstrate mostly hypercoagulability in dogs with mixed cancers as evidenced by increased G value (with only dogs with metastatic disease having hypocoagulability).\textsuperscript{63,116} TF-TEG has also been used to demonstrate 66% of dogs with mixed cancers had hypercoagulable TEGs as evidenced by increased G value, with again the only hypocoagulable patients having metastasis or disseminated lymphoma.\textsuperscript{117} Hypercoagulability using the variables K, angle, and MA in CN-TEG and K-TEG has been found in dogs with protein-losing nephropathy (PLN) compared to healthy dogs,\textsuperscript{118} but this was not different than hypercoagulability in dogs with non-PLN renal failure in another study.\textsuperscript{119} In protein losing enteropathy, CN-TEG demonstrated hypercoagulability by R, K, angle, and MA in all patients compared to healthy controls.\textsuperscript{120} In dogs with immune-mediated thrombocytopenia, K-TEG was initially hypocoagulable as determined by MA, but became hypercoagulable in all dogs once platelet counts exceeded $>40 \times 10^9$/L.\textsuperscript{121}

Canine immune-mediated hemolytic anemia, well known for its thrombotic risk in dogs, has also been investigated with TEG. Using CN-TEG, Sinnott et al found the majority of dogs (33/39) with IMHA were hypercoagulable as indicated by CI; the 6 dogs who were classified as normocoagulable all died.\textsuperscript{122} Fenty et al found similar results, with IMHA dogs having hypercoagulable tracings prior to any therapy as determined by K, angle, MA, and G on K-TEG as compared to control dogs.\textsuperscript{123} In an assessment of CN-
TEG changes in dogs with IMHA over course of treatment, all dogs were hypercoagulable on admission per K, angle, and MA values. Over 5 days, R, K, and angle became more hypercoagulable, while MA decreased slightly over time. MA at admission was negatively correlated with Hct (r=-0.451, spearman’s rank correlation), but correlation of MA with change in Hct over time was not performed. The authors correlated a lower MA at admission with increased risk of death at discharge and 30 days. Several studies have used CN-TEG, K-TEG, and TF-TEG to assess hypercoagulability in canine hyperadrenocorticism; all found significant increase in MA as well as other hypercoagulable variables on presentation in HAC dogs compared to healthy dogs.

Two studies found that TEG variables may improve but do not normalize with therapeutic control of HAC, and one study found no difference in TEG variables between untreated, treated, and “sick” dogs without HAC. Wide variation in TEG findings in veterinary studies of disease categories is common and has been seen in multiple studies of generally ill dogs such as those in an ICU population, septic peritonitis, SIRS, endotoxin, and DIC. One interesting trend is for dogs with presumably pro-inflammatory hypercoagulable diseases such as IMHA and septic peritonitis to have worse outcomes if their TEG tracings are normo- or hypocogulable. This is presumed to indicate progression of disease into the hypocoagulable phase of DIC, which is associated with a worse prognosis. In fact, in a study focusing solely on dogs with suspected DIC, those with hypocoagulable G values had worse outcomes than those with hypercoagulable G values.

It is important to note that of the above studies, only hypocoagulable TEG results in classically “hypercoagulable” conditions such as IMHA, septic peritonitis, and DIC have
been associated with outcome data in dogs. Hypercoagulable tracings in dogs have not yet been proven to be associated with thrombotic risk in any condition. In fact, in dogs with known thrombi, only 50% were found to be hypercoagulable as defined by an elevated G value on K-TEG. Indeed, the ability for TEG to predict postoperative thromboembolic events appears extremely variable in the much more extensive human literature, and so clinical decision making based on TEG results should be undertaken cautiously given the lack of evidence in human or veterinary medicine for improved outcomes based on TEG guided anticoagulation.

TEG has been used in both human and veterinary medicine to assess the effects of various drugs on the coagulation system. It has been used in dogs to assess anticoagulants such as unfractionated heparin, dalteparin, clopidogrel, and cytochalasin D and abciximab. Similar to human studies, while veterinary studies have shown the ability of TEG tracings to detect the effects of various anticoagulants on the hemostatic system, guidelines for the use of TEG to monitor the effects or direct dosages of these medications in clinical patients is lacking.

1.2.4 Influence of Hematocrit (Hct) on TEG tracings

While experimental and quality control assays of TEG are often performed on plasma due to the ability to store and ship identical samples, clinical TEG assessment is most often performed on whole blood to get the most global in vitro representation of the coagulation system. The impact of fibrinogen and platelets on the TEG tracing are always discussed in interpretation of these tracings, but it is of note how infrequently other cellular factors, especially red blood cells, are mentioned in the discussion of TEG tracing interpretation. This is especially puzzling given the significant body
of evidence that TEG is strongly influenced by hematocrit (Hct). As early as 1989 it was demonstrated that canine blood reconstituted with normal platelet counts and fibrinogen levels to Hcts of 10%, 30%, and 50% showed significantly increased angle and MA with decreasing Hcts.\textsuperscript{142} In a paper documenting TF-ROTEM (equivalent to TF-TEG) performed in 71 healthy non-anemic dogs and in 31 dogs with EA-TEM (equivalent to K-TEG) with Hct ranging between 41-61%, decreasing Hct was correlated with all ROTEM parameters becoming more hypercoagulable.\textsuperscript{44} Similarly, Iselin et al showed that in reconstituted human blood with static platelet counts, Hcts decreasing from 40% through 10% corresponded with progressively shorter K, increased angle, increased MA, and increased G.\textsuperscript{46} TEG performed on naturally anemic humans following chemotherapy for hematological malignancies (in remission) showed a significantly increased MA and G for patients with [Hb] < 10 (Hct < 30%).\textsuperscript{143} In the same study, transfusion-dependent chronically anemic humans were studied before and after receiving 3U of pRBC. These patients had an average baseline [Hb] of 8.3 (Hct 25%) prior to transfusion, which increased on average to approximately Hct 34% after transfusion with either fresh or stored pRBC. The MA and G values decreased in both groups at 1 and 24 hours after transfusion, correlating with the increased Hct levels. Hct has been shown to have a similar effect on multiple viscoelastic methodologies including ROTEM\textsuperscript{©}, Sonoclot\textsuperscript{©}, and ReoRox\textsuperscript{©}, implying this influence is consistent across all methodologies of viscoelastic clot assessment.\textsuperscript{142,144} In chronically iron-deficient anemic human patients, all ROTEM values were hypercoagulable compared to non-anemic gender and age matched controls, despite no difference in the endogenous thrombin potential between groups.\textsuperscript{48} Endogenous thrombin potential is a test of how much thrombin can be
generated in a blood sample, and would be expect to be elevated in a truly hypercoagulable patient. The lack of elevation of this test lends further support to the concept that the effect of Hct on viscoelastic methodologies of coagulation is an \textit{in vitro} phenomenon rather than reflective of true \textit{in vivo} changes in coagulation.

Obviously dilution of whole blood with anything other than PRP results in reduction in multiple factors including platelets and fibrinogen in addition to Hct, therefore it is not surprising that in blood significantly diluted with saline or other non-plasma products, MA decreases, despite a decrease in Hct.\textsuperscript{145} But when Hct is diluted with autologous PRP rather than saline, a hypercoagulable tracing is observed.\textsuperscript{146} In fact, PRP without any RBCs has a greater G value than equivalent whole blood in both CN-TEG and TF-TEG.\textsuperscript{147} Progressive anemia in platelet-deficient whole blood has a greater increase in MA than in blood with platelets, implying Hct has strong influences on MA and G in TEG independent of platelets, and this effect may be even more pronounced in low platelet states.\textsuperscript{47} Most recently, a study was performed on 13 laboratory dogs where temporary mild anemia was created by removal of 1U of pRBC with return of plasma and 250mL LRS to the donors to restore blood volume. On day 3 after phlebotomy, all TF-ROTEM parameters with the exception of CT (the ROTEM equivalent of R) became hypercoagulable; this difference resolved at day 21 when RBC values had returned to baseline.\textsuperscript{50}

The opposite trend is also seen; that increasing Hct is associated with “hypocoagulable” TEG tracings. In an experimental mouse model that over expressed erythropoietin to a Hct of 85\%, angle and MA were significantly decreased compared to wild-type mice of the same age.\textsuperscript{148} Similarly, in horses made polycythemic \textit{in vivo} via
administration of phenylephrine to cause splenic contraction, ROTEM values all showed significant hypocoagulability associated with increased Hct within 5 minutes of phenylephrine administration. However, all values returned to baseline at 2h post administration once Hct had also returned to normal. No other parameters including PT, PTT, platelets, fibrinogen, or thrombin-antithrombin levels were different at any time point, implying the differences in ROTEM values were likely influenced by Hct alone.\textsuperscript{149}

Why Hct has this effect on viscoelastic methodologies of coagulation is unknown. Previous explanations for \textit{in vitro} hypercoagulable TEG tracings associated with anemia have included an increase in the relative amount of plasma proteins to red blood cells, a decreased citrate: plasma protein ratio in anemic samples, increased contact activation of anemic blood with the TEG cup, or decreased red cell mass allowing a tighter fibrin meshwork.\textsuperscript{47,149-151} While decreasing the Hct in a fixed volume of blood will increase the total amount of plasma in the sample, it will not change the concentration of protein in the plasma, which is the main determinant of enzyme kinetics.\textsuperscript{152} This makes a “dilutional” explanation for anemic hypercoagulable TEG tracings less likely, as no true dilution occurs. A relative decrease in the absolute amount of plasma present in the cup due to the presence of RBCs has been recently proposed as an alternate theory to dilution,\textsuperscript{44,50} and correlation of ROTEM parameters with coagulation factor levels was improved after mathematical correction for plasma volume.\textsuperscript{44} However, experiments designed to increase absolute amounts of coagulation proteins within a constrained volume will by definition increase concentration and enzyme kinetics, confounding experimental proof of this concept. Decreased citrate relative to plasma
volume is also an unlikely explanation, given a recent paper using canine samples that showed hypercoagulable TEG tracings at mildly low Hct, even after adjusting citrate concentration.\textsuperscript{50} Similarly, in many \textit{in vitro} studies, blood is drawn from non-anemic donors and anticoagulated prior to component separation, therefore changes in citrate concentration should not affect the findings.\textsuperscript{46,47,146} Increased contact activation of less viscous blood with the pin/cup surface is also unlikely given that similar variation in TEG parameters with Hct have previously been observed despite the use of activators.\textsuperscript{44,47,50} Given that none of these potential explanations seem to adequately account for these changes, it suggests that other factors might be at play. As Hct is the main determinant of whole blood viscosity,\textsuperscript{22} and TEG is a viscoelastic method of monitoring coagulation, it seems plausible that changes in whole blood viscosity may cause a mechanical bias of the TEG tracing. Alternatively, changes in red cell mass affecting fibrin network strength and structure might be another possible explanations for this phenomenon.\textsuperscript{151}

1.3 Whole blood viscosity measurement

Viscosity is a reflection of resistance to flow, and is an intrinsic property in the movement of any fluid. It is defined as the ratio of the shear stress to the shear rate during flow of the fluid. The shear stress is the force applied to one layer of the fluid, causing it to flow over the other layers at a given rate (shear rate). Velocity of flow is determined by the internal friction of the fluid layers moving over one another as demonstrated in (Figure 1.6). For homogeneous “Newtonian” fluids like water, the viscosity of the fluid is an intrinsic property that will always be the same at a given temperature, and will display a constant viscosity independent of flow rate. For non-
Newtonian fluids, the relationship between shear stress and shear rate is non-linear, and the viscosity of the fluid will change depending on the rate of flow.

Blood is a non-Newtonian fluid, mainly because the cellular elements suspended within the plasma will aggregate at lower flow rates and deform at higher flow rates. This means that at high shear rates, blood will be less viscous than at low shear rates. Whole blood viscosity is primarily determined by the red cell mass, deformability of red cells, and red cell aggregation, but is also influenced by plasma proteins, white blood cells, and platelets. As previously discussed, whole blood viscosity, especially as pertaining to red blood cell aggregation and laminar flow of blood in the vasculature plays an important role in endothelial reactivity and margination of other cell types towards the periphery of flow.

The viscosity of blood and other fluids is measured using a viscometer. Viscometers generally fall into 3 types: tube or capillary viscometer, falling-body viscometers, and rotational viscometer. Capillary viscometers involve recording the time it takes for the substance to flow a given distance through a tube of known diameter. Falling-body viscometers drop a sphere or move a pin through the substance; the time needed for the object to fall or move a certain distance through the fluid is recorded. While these types of devices work well for Newtonian fluids, in non-Newtonian fluids such as blood it is necessary to determine the shear stress and shear rate independently. Rotational viscometers place a small amount of the substance between 2 surfaces (i.e. between the cup and pin in the TEG). The amount of force (torque) needed to move one surface relative to the other, or the speed of rotation at a given torque, is used to determine viscosity. While there are many ways in which blood viscosity can be
measured, due to the small amount of blood needed (0.5 mL) and the ability to control the shear rate, cone and plate viscometry or similar rotation methods are preferred for the measurement of whole blood. Cone and plate viscometry involves placing whole blood between a very shallow rotating cone and a fixed plate at a fixed temperature. The cone rotates and the more viscous the fluid, the more torque will be needed to rotate the cone at a given speed (Figure 1.7). Using the measured torque, and a given a known shear rate, fluid viscosity can be calculate in units of millipascal-second (mPa-s) or centipoise (cP). While blood viscosity will vary among species and shear rate, whole blood viscosity for normal dogs with Hct ranging from 41-53% was determined previously on our cone and plate viscometer (with a shear rate of 150 s⁻¹) to range from 3.74-5.2, with an average value around 4.5 cP.

1.4 Study rationale

The critical need to identify and appropriately treat coagulation disorders in our patients has led to great interest in and expanded use of viscoelastic methodologies of coagulation testing. As such, TEG been used in numerous applications for both human and veterinary clinical medicine as well as in research settings. Given the significant investment of time, money, and potential clinical implications associated with use of these methodologies, it behooves us to fully understand their strengths and limitations as we go forward in their use. While the impact of Hct on TEG is well established, it is often overlooked in clinical studies and the role of all cellular components on the TEG is ignored in plasma based research models. In order to compensate for the effect that various states of anemia may have on clinical TEG samples, we need to understand why red cells have such an effect on this viscoelastic assessment of coagulation. We
hypothesize that as the main contributor to whole blood viscosity, changes in Hct influence the shear modulus of the clot on the pin, causing an artifactual mechanical bias that appears as “hypercoagulability” *in vitro*. This is concerning as clinical studies have demonstrated that *in vivo*, anemia should cause hypocoagulability; therefore this TEG finding may cause misinterpretation of a patient’s true coagulation status. In order to test this hypothesis, we manipulated the viscosity of canine blood samples while holding Hct fixed in various states of anemia. We used 2 viscosity modifying agents, alginate and carboxymethylcellulose to account for possible artifacts caused by the agents themselves. Finally, to investigate the effect of changing Hct on non-manipulated canine blood samples, we investigated the change in TEG tracings in naturally anemic dogs immediately before and after packed red blood cell transfusion.
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Figure 1.1: The classic “waterfall” coagulation pathway. Adapted from Pallister CJ and Watson MS (2010). *Haematology*. Scion Publishing. pp. 336–347
Figure 1.2: cell based model of coagulation. (A) Initiation. TF-VIIa complex generated fXa locally at the site of tissue damage, leading to a small thrombin (fIIa) “spark” being generated (B) Amplification. The thrombin “spark” sets the stage by activating fXia, fVa, fVIIIa, and platelets. (C) Propagation. The colocalization of the “tenase” and “prothrombinase” complexes on pro-coagulant cell surfaces results in the generation of large amounts of thrombin, which cleaves fibrinogen to fibrin to form a stable clot. See text for more details. ECM = extra-cellular matrix, vWF = von Willebrand factor, Ca = calcium

(continues)
(Figure 1.2 continued)

(C)
Figure 1.3: (A) TEG mechanism showing the pin seated in a rotating cup, with the substance to be tested (blood) between the two. (B) The classic TEG tracing generated by the movement of the pin as the blood clots. See text for more details on TEG variables.
Figure 1.4: (A) TEG lysis variables. The CL30 = [A30/MA] x 100; for the upper tracing, CL30 is zero. LY30 is [striped area/total area in rectangle since MA] x 100. (B) First derivative TEG values. See text for further detail. MTG = maximum thrombus generation, TMG = time to MTG is the time needed to reach MTG. TEG_{TG}/TTG = total thrombus generation, MRL = maximum rate of lysis, TML = time to MRL, ACL = area of clot lysis.
Figure 1.5: Classically described “eyeball” diagnoses based on appearance of the TEG tracing (from TEG user manual, 2007).
Figure 1.6: Viscosity. During flow, force (F) is applied to the surface of the fluid over an area (A). This is the shear stress. The shear stress causes theoretical layers of the fluid to flow at different velocities (v); the velocity of \( v_1 \) will be less the \( v_0 \) due to the internal friction of the fluid. The difference in the conceptual velocity layers (\( v_0 - v_1 \)) is the shear rate. Viscosity is the ratio of shear stress to shear rate. Adapted from 156

Figure 1.7: Schematic of a cone and plate viscometer. The fluid to be analyzed is placed between a shallow cone and plate (arrow). The cone is rotated at a known speed (shear rate). By measuring the torque needed to maintain the shear rate against the drag of the fluid, the viscosity of the fluid can be calculated.
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Chapter 2: ALGINATE

2.1 Introduction

Thromboelastography is a whole blood hemostatic assay that has recently undergone a renaissance in human and veterinary medicine as the role of cellular components in coagulation have become better understood.\textsuperscript{1,2} The Thrombelastograph\textsuperscript{TM} (TEG, Haemonetics) measures changes in the viscoelastic strength of clot formation throughout the phases of clotting and fibrinolysis.\textsuperscript{3,4} The main advantage of TEG is its ability to assess global clotting of whole blood, including the states of hypercoagulability and fibrinolysis.

Whole blood viscosity is mainly determined by red cell mass under physiologic conditions, although plasma protein, especially fibrin, may contribute to a lesser extent.\textsuperscript{5,6} Several previous \textit{ex vivo} and \textit{in vitro} human and animal studies have shown that under conditions of high hematocrit (Hct), viscoelastic methodologies of whole blood coagulation show tracings consistent with hypocoagulability.\textsuperscript{7-9} Conversely, under anemic conditions, tracings are indicative of hypercoagulability.\textsuperscript{10-14} These effects are opposite to the clinical observation that anemia results in prolonged bleeding times independent of platelet count or hemostatic proteins, a concept known as “anemic coagulopathy,” which can be improved by correcting anemia alone.\textsuperscript{15-18} Similarly, in patients with erythrocytosis, a tendency towards clotting or stasis of blood is seen.\textsuperscript{5,19} This discrepancy between clinical observations and TEG data is concerning. The “hypercoagulable” TEG
tracings noted in anemic disease states may be due a true prothrombotic state caused by the underlying disease, but could also be attributed to effects of anemia on the TEG itself.

Manipulation of whole blood viscosity independent of Hct would allow for better understanding of the contribution of each to the TEG tracing. This can be facilitated by the use of a substance such as alginate (ALG), which serves to alter viscosity without affecting Hct. Alginates are unbranched polysaccharides found in bacteria such as Pseudomonas aeruginosa and algae; commercial products are all derived from brown algae, and are used in many applications from food thickeners to wound dressings. Alginates are composed of 2 uronic acid monosaccharides; D-mannuronic (M) and L-guluronic (G) acid, linked by β(1->4) and α(1->4) glycosidic bonds (Figure 2.1). Alginates isolated from different algae will have different M/G compositions, which can affect the properties of the alginate. Higher G ratios are associated with increased gel formation in the presence of divalent cations as adjacent G-G residues form a binding site for the cation. Effects of ALG on coagulation have not been well explored, with one study finding ALG with mostly M groups causing a prolonged thrombin times and PTT, and ALG with mostly G groups shortening PT. Thrombin generation as measured by TAT complexes were increased in samples incubated with high-G ALG groups but not for other ALGs, and platelet aggregation was stimulated by all materials, but more so by G-ALG. The calcium-donating effects of ALG, its gel forming ability, and its biocompatibility are all reasons why ALG has been used extensively in wound dressings. Alginates have also been used in drug delivery systems, and as scaffolds.
or as carriers for implantation of cell transplants or tissue engineering. ALG solutions are compatible with blood and have been used intravenously in previous studies to increase blood viscosity in studies of microcirculatory flow.

We hypothesize that changes in TEG tracings associated with anemia are due to decreased blood viscosity resulting in a hypercoagulable appearance to the TEG tracing, rather than a representation of a true in vivo phenomenon of “hypercoagulability” associated with anemia. We also hypothesize that normalization of blood viscosity in anemic blood with ALG will reduce or eliminate the hypercoagulable tracing despite a persistently low hematocrit.

2.2 Materials and Methods

Preparation of samples:

All fresh whole blood samples were obtained from healthy non-Greyhound blood donor dogs who were pre-screen as part of the OSU Veterinary Teaching Hospital blood donation pool. All dogs had normal packed cell volume/total protein (PCV/TP) levels (PCV 45-54%, TP 5.7-7.2 g/dL) on the day of phlebotomy. Blood collection was approved by the OSU Institutional Animal Care and Use Committee. A flowchart of the sample processing method is outlined in Figure 2.2. In brief, samples were collected from a jugular vein using a 19G butterfly needle attached to a Vacutainer set. The first 0.5-1.0 mL of blood from the draw was discarded into a red top tube to clear the line of air and remove activated tissue factor (Garcia 2012). Subsequently, 4-6 citrated tubes were filled to manufacturer’s recommended volumes with 4.5 mL whole blood to 0.5 mL 3.2% citrate (1:9 citrate
to blood ratio) to yield a final concentration of 0.32% citrate in a final volume of 5 mL per tube. The time of phlebotomy was noted.

Samples were then centrifuged at 400 $\times g$ at room temperature for 3 minutes. The platelet-rich plasma (PRP) was removed and evaluated with a point-of-care hematology analyzer to obtain PRP platelet count. The remaining sample was centrifuged at 1370 $\times g$ for 10 minutes, and the platelet-poor plasma (PPP) separated from the packed red blood cells (pRBCs). The PPP and pRBC were analyzed with the same hematology analyzer to obtain the platelet concentration and Hct. Each sample was then reconstituted to create one of three Hct values (45%, 20%, or 10%). Platelet counts were normalized between 150,000-300,000 by adding variable amounts of PRP based on the platelet concentration of the PRP. The order in which each Hct value was formulated from each phlebotomy event was systematically randomized by rolling a die for each dog to avoid sampling order bias; all samples for each dog were drawn the same day with a new phlebotomy for each Hct condition.

Sodium alginate was dissolved in 0.9% NaCl to make stock solutions of 2% and 0.075%, which were handled according to manufacturer’s recommendations. Each reconstituted whole blood sample for each Hct condition was divided in half, and equal volumes of either saline (SAL) or alginate (ALG) solution were added to each to make a final volume of 2.0 mL for each sample (Table 2.1, Figure 2.2). The amount of ALG added to each Hct condition was determined in preliminary studies as the amount needed to bring the viscosity of the sample to match approximate normal canine blood viscosity of 4 centipoise (cP). The final Hct and platelet
count for each SAL diluted sample was determined using the same hematology analyzer; Hct and platelet count of the paired ALG sample was presumed to be identical.

**Thromboelastography analysis**

The paired SAL and ALG samples were gently mixed by inverting 5-10 times then immediately run simultaneously on a 2-channel thromboelastography analyzer. All samples were kept at room temperature until run on the TEG. A volume of 340 µL from each sample was mixed with 20 µL of 0.2 M CaCl$_2$ in the pre-warmed TEG cup at 37°C to a final volume of 360 µL, and analysis was started immediately. No activator was used as our laboratory reference ranges are for citrated native blood without activator. The TEG values R, angle, K, MA, and G were recorded. G was calculated by the software using the following equation: $G = \frac{(5000MA/(100-MA))/1000}$. The channel assigned to either saline or alginate sample was systematically randomized as previously described.

To assess the effect of possible ALG gel formation in the TEG, 340uL of the 2% ALG stock solution was combined with 20uL of 0.2M CaCl$_2$ and run on the TEG.

To determine the effect of calcium concentration on samples manipulated with ALG or saline, a stock solution of calcium chloride 600mg/mL (approximately 6.0M) was made from ACS Grade >98% anhydrous CaCl$_2$ (MW 110.99). This stock was subsequently diluted to yield solutions of 1M, 0.6M, 0.4M and 0.2M (control) so as to have increasing amounts of calcium without altering volume in the
TEG cup (20uL of CaCl$_2$ was always added to the cup). To assess the effect of ALG binding calcium in the TEG cup leading to hypocoagulable samples as compared to control, 10% Hct + SAL was compared to 10% Hct + ALG reactivated with 20uL of 6M, 1.1M, 0.6M, 0.4M, and 0.2M (control) of CaCl$_2$.

We defined a hypercoagulable TEG tracing in this study as having a shortened R and K time, and an elevated MA, angle, and G as compared to canine reference ranges for our institution. As the appropriate definition of what constitutes a “hypercoagulable” TEG tracing is currently under debate in veterinary medicine,$^{26}$ we report all TEG values and assess them independently. Similarly, defined a hypocoagulable tracing as the opposite for all variables, as previously described.$^{4}$ Normal canine TEG reference values for our institution are listed in Table 2.1 in parenthesis under each variable and are represented by dotted outlines in Figure 2.4.

Viscosity measurement

The viscosity of each of the paired samples was measured on a cone and plate viscometer.$^{1}$ The cup was pre-warmed to 37$^\circ$ C via circulating water bath.$^{1}$ A blood sample (0.5 mL) was placed in the center of the cup and immediately placed on the viscometer to start cone rotation. The viscosity was measured after 10 seconds of equilibration at 20 rpm (150 $^{-1}$ shear rate). A 10-second time period from initiation of rotation was chosen for recording of viscosity, as viscosity of whole blood will continue to drop as time goes on due to sedimentation of red blood cells. Repeated readings (2-3) were performed for each sample and the average viscosity for each sample recorded in cP.
Statistics

Summary statistics (mean and standard deviation) are provided for each variable (Table 1). Due to the small number of dogs (n=7), non-parametric statistics were used for comparisons. Wilcoxon/Kruskal Wallis rank sum tests were used to compare platelet count, viscosity, hematocrit, and TEG variables across all SAL and all ALG conditions; post-hoc pairwise comparisons were performed with the Steel-Dwass method. Wilcoxon signed rank tests were used for pairwise comparisons between SAL and ALG groups of the same Hct condition.

P-values of <0.05 were considered statistically significant. Data were analyzed using a commercial statistical package.

2.3 Results

Seven dogs were used; 3 spayed females and 4 castrated males. Breeds included 2 Boxers, 2 Labrador Retrievers, and 1 each German Shepherd Dog, Rottweiler, and mixed breed. All dogs were >25 kg of body weight. Mean age was 5 years (range 3-8 years). Mean Hct, platelet count, time from phlebotomy to TEG analysis, and TEG variables are outlined in Table 2.1. All samples were run on the TEG a median of 46 minutes (range 37-61 min) after initial blood draw. There was no significant difference in platelet concentration between groups. Viscosity was higher in 20%Hct+ALG (4.4 ±0.2 cP) compared to 10%Hct+ALG (4.1±0.1 cP) and 45%Hct+ALG (4.0±0.2 cP) (p=0.04); 10%Hct+ALG and 45%Hct+ALG were not different. Viscosity was not significantly different between 45%Hct+ALG and 45%Hct+SAL (3.88±0.2 cP). Viscosity was lower (1.5±0.1 cP) in 10%Hct+SAL
compared to 10\% Hct+ALG (4.1±0.1 cP) (p<0.016). Similarly, it was lower in
20\% Hct+SAL (2.1±0.1 cP) compared to 20\% Hct+ALG (4.4±0.2 cP) (p<0.016).
Viscosity was different between all SAL groups (p<0.006) as outlined in Table 2.1
and Figure 2.4.

The differences between TEG parameters and their significance are
illustrated in Figure 2.4, and a sample TEG tracing from all conditions in one dog is
shown in Figure 2.5. Overall, the 10\% Hct+ALG and 20\% Hct+ALG conditions
showed hypocoagulable tracings as compared to the other conditions; there were no
significant differences between 10\% Hct+ALG and 20\% Hct+ALG for all recorded
TEG variables. 10\% Hct+ALG and 20\% Hct+ALG had a significantly increased R
and K, and decreased angle, MA, and G as compared to their respective SAL
samples (p=0.016), and as compared to 45\% Hct+ALG (p<0.01). 20\% Hct+SAL and
10\% Hct+SAL both had hypercoagulable TEG tracings as defined by a shorter K,
and higher angle, MA, and G when compared to 45\% Hct+SAL (p<0.014). While
the MA and G values were statistically different (p=0.016) between the
45\% Hct+ALG and 45\% Hct+SAL groups, given the significant overlap of the data
ranges (Figure 2.4), we do not consider this difference to be biologically significant,
especially given that G is calculated using MA.

ALG 2\% alone + 0.2M CaCl₂ generated flatline tracings in preliminary data;
however repetition of this experiment did occasionally result in small tracings as shown
in Figure 2.3. TEG tracings in either SAL or ALG conditions runs with [CaCl₂] > 0.6M
resulted in only flat line tracings for control SAL samples (not shown). Representative
changes in TEG values at [CaCl₂] at 0.2M, 0.4M and 0.6M are shown in Figure 2.6. SAL
samples become more hypercoagulable at 0.4M, but at 0.6M progress to more hypocoagulable than 0.2M baseline. Similarly, ALG samples at 0.4M become hypercoagulable compared to 0.2M, but return to baseline at 0.6M. The large difference in tracings between SAL and ALG are preserved despite increasing [CaCl₂].

2.4 Conclusions

The 10% and 20%Hct+SAL conditions in this study had significantly higher angle, MA, and G values, while 10% and 20%Hct+ALG conditions had significantly lower angle, MA, and G values. Our data also showed that the 10%Hct+SAL and 20%Hct+SAL conditions had hypercoagulable TEG tracings compared to the 45%Hct+SAL condition, consistent with previous studies in people and dogs on the effect of anemia on TEG tracings.¹⁰⁻¹⁴,²⁷ As the SAL and ALG conditions of the same Hct were made from one stock solution and therefore had essentially identical concentrations of red blood cells, fibrin, clotting factors, and platelets, the only difference between the paired ALG and SAL samples was the addition of ALG to alter viscosity.

Explanations for *in vitro* hypercoagulable TEG tracings associated with anemia have included an increase in the relative amount of plasma proteins to red blood cells, a decreased citrate: plasma protein ratio in anemic samples, increased contact activation of anemic blood with the TEG cup, or decreased red cell mass allowing a tighter fibrin meshwork.⁹,¹¹,²⁸ As previously discussed, many of these explanations fall short, leaving changes in whole blood viscosity, or changes in red cell mass affecting fibrin network strength and structure as possible explanations for this phenomenon. In this study, we found significant differences when RBC mass
was held static but viscosity was altered with ALG. There are two possible explanations for the TEG changes observed between ALG and SAL samples of the same Hct in this study. The first is that changes in blood viscosity influence the viscoelastic TEG methodology, resulting in a mechanical bias of the TEG tracing. The second possibility is that the ALG itself exerts a direct effect on clot kinetics and strength, and is not as biologically inert as has been previously proposed.²⁹ While autologous red cells or plasma proteins could have been used to alter whole blood viscosity, we would not have been able to separate the effect of these native components on coagulation from the effect of viscosity.

As it is impossible to separate the chemical properties of alginate from the experimental changes in blood viscosity, we cannot rule out a direct effect of alginate on clot formation as an alternate explanation for the results of this study. Other intravenous colloidal agents such as hydroxy-ethyl starches (HES) have been investigated in TEG studies and have been shown to alter TEG tracings.³⁰⁻³⁴ HES have been proposed to affect TEG by a number of mechanisms, including reduced platelet adhesion and aggregation, inhibition of thrombin/fibrin and fibrin/fXIII interactions, and enhanced fibrinolysis.³¹,³³,³⁴

While HES and alginates are both polysaccharide chains, they have significant structural differences. HES are composed of branched glucose chains (amylopectin), with variable hydroxyethylation of the C2 and C6 positions,³⁵ and alginates are unbranched polysaccharides composed of variable amounts of mannuronic (M) and guluronic (G) acids (Figure 2.1).²¹ Whether alginates have a
clinical effect on coagulation that is similar to that of HES is currently unknown, and could be an alternate explanation for our results.

Alginates are known to form hydrogels when mixed with divalent cations such as calcium.\textsuperscript{21} We used sodium alginate to avoid affecting the calcium concentration in the TEG cup, but some degree of calcium binding by the alginate is possible. This could have made less calcium available for interaction with clotting factors and contributed to overall hypocoagulability. However, increasing the concentration of CaCl\textsubscript{2} added to the TEG cup up to 0.6M caused only a minor increase in the MA of the ALG tracing and did not resolve the significant difference in TEG tracings seen between the 10\% Hct+SAL and 10\% Hct+ALG samples. (Figure 2.6). Therefore, calcium sequestration by ALG causing decreased clotting factor activity cannot be blamed for the majority of the difference between anemic SAL and ALG samples in this study. In fact, increasing [CaCl\textsubscript{2}] > 0.4M began to decrease clot speed and strength in SAL control samples, likely due to high salt concentration effecting protein-protein interactions and solubility. This is not surprising given that high salt concentration is known to lower protein solubility; this effect of “salting out” is exploited in ion-exchange chromatography to purify proteins.\textsuperscript{36}

Alginates have been shown to increase red blood cell aggregation.\textsuperscript{37} Subjective increases in sedimentation rate for ALG samples (as compared to their SAL counterparts) were noted in this study, but RBC aggregation was not directly measured. While further investigation into the effects of RBC aggregation on TEG tracings is warranted, previous \textit{in vivo} studies of RBC aggregation have
demonstrated an association with increased risk of thrombosis,\textsuperscript{5,38} while previous \textit{in vitro} studies have yielded mixed results.\textsuperscript{39,40} Whether \textit{in vivo} findings of increased clot formation due to RBC aggregation should also result in \textit{in vitro} hypercoagulable TEG tracings is unknown.

The results of this experiment support the theory that changes in viscosity may drive the “hypercoagulable” TEG tracings seen with anemia. To our knowledge, this is the first experiment to demonstrate that manipulating blood viscosity with ALG independent of red cell mass can reverse the expected hypercoagulable changes to TEG tracings in anemic blood. Moreover, instead of the expected normalization of the TEG tracings with normalized viscosity, the alginate treated samples became hypocoagulable. These findings are more consistent with the \textit{in vivo} clinical finding that anemia is associated with prolonged bleeding times.\textsuperscript{15-18} The unexpected hypocoagulability seen in the anemic ALG samples could be due to unmasking of underlying true hypocoagulability of anemic blood once the effect of hypoviscosity was removed, or could be due to direct effects of the alginate itself on clot formation.

In order to assess whether the changes seen in this experiment were due to effects of viscosity on TEG tracings or due to intrinsic properties of ALG affecting coagulation, the experiment was repeated with a different viscosity-modifying agent, sodium carboxymethylcellulose (CMC), as reflected in the next chapter.
2.5 References


30. Casutt, M., Kristoffy, A., Schuepfer, G., Spahn, D. R. & Konrad, C. Effects on coagulation of balanced (130/0.42) and non-balanced (130/0.4) hydroxyethyl starch or gelatin compared with balanced Ringer's solution: an in vitro study using two different viscoelastic coagulation tests ROTEM and SONOCLOT. British Journal of Anaesthesia 105, 273–281 (2010).


Figure 2.1: Chemical structure of ALG
Figure 2.2: Flow chart of method of sample preparation
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Table 2.1: ALG vs. SAL at 10, 20, and 45% HCT. * = Significantly different from same Hct condition of opposite group (SAL or ALG), P < 0.05. † = Significantly different from baseline (the 45% Hct condition of the same (SAL or ALG) group), p < 0.05.
Figure 2.3: ALG + Ca run on TEG without blood; CMC + Ca run on TEG without blood
Figure 2.4: Bar graphs for ALG vs. SAL at 10, 20 and 45% Hct. Lines are medians and outer boxes represent quartiles. Whiskers represent range of data. * = Significantly different from same Hct condition of opposite group (SAL or ALG), $P<0.05$. †= Significantly different from baseline (the 45% Hct condition of the same (SAL or ALG) group), $p < 0.05$. The area outlined by dotted lines represents normal reference ranges for TEG variables and canine whole blood viscosity.
Figure 2.5: Example tracing of all ALG and SAL Hct conditions in 1 dog

Figure 2.6: Representative changes in TEG tracing at [CaCl$_2$] at 0.2M, 0.4M and 0.6M in 10%Hct + SAL and 10%Hct + ALG
CHAPTER 3: CARBOXYMETHYLCELLULOSE

3.1 Introduction

Thromboelastography is a hemostatic assay that has the advantage of being able to assess global clotting of whole blood, including the states of hypercoagulability and fibrinolysis. Interest in the TEG has grown in both human and veterinary medicine as the role of cells in clotting have become more understood.\textsuperscript{1,2} The TEG measures the changes in the viscoelastic strength of clot formation throughout the phases of clotting and fibrinolysis and generates a characteristic tracing representing the kinetics of clot formation and breakdown.\textsuperscript{3,4}

Several previous \textit{ex vivo} and \textit{in vitro} human and animal studies have shown that under conditions of low hematocrit (Hct), viscoelastic methodologies of whole blood coagulation show tracings consistent with hypercoagulability.\textsuperscript{5-10} The opposite is seen under conditions of elevated Hct, where tracings are consistent with hypocoagulability.\textsuperscript{11-13}

However, multiple studies have documented the clinical observation that anemia results in prolonged bleeding times, independent of platelet count or hemostatic proteins.\textsuperscript{14-17} This “anemic coagulopathy,” can be improved by correcting anemia alone. Similarly, in patients with erythrocytosis, a tendency towards thrombosis is seen.\textsuperscript{18,19} This discrepancy between TEG tracings and clinical observations is of concern. Whole blood viscosity is largely determined by Hct, although plasma protein, especially fibrin, contributes to a lesser extent.\textsuperscript{19,20}
“hypercoagulable” TEG tracings noted in previous studies of anemic patients may have been influenced by the viscosity effect of Hct on the TEG itself, as it is a viscoelastic measurement.

Manipulation of whole blood viscosity at a static Hct would allow for better understanding of the independent effect of viscosity on TEG tracings. Previous work with the viscosity-modifying agent alginate showed that TEG tracings with low Hct, normal viscosity were significantly hypocoagulable when compared to low Hct, low viscosity controls. However, as the effect of viscosity and that of the chemical properties of alginate cannot be separated, it was impossible to know if those findings were due to the change in viscosity or an effect of alginate itself.

To further explore the effects of altering whole blood viscosity independent of Hct, we chose to alter viscosity using a different material; sodium carboxymethylcellulose. Cellulose is an extremely common polysaccharide found in many plant species, but its applications are limited as it is not water-soluble. Carboxymethylcellulose is a man-made anionic polymer that is created by the addition of carboxymethyl groups on to a cellulose chain (Figure 3.1). It is widely used in oral food and drug preparations as a binder, and has been reported to have no biological activity. It has been used intraperitoneally in horses to reduce adhesion formation after intestinal surgery and has been used in nanoparticle synthesis for antineoplastic compound. In one study of horses administered CMC intraperitoneally for adhesion prevention, precipitation of the presumed CMC was noted in blood smears up to 9 days after administration, but no systemic events related to IV CMC were noted in this retrospective review.
In this *in vitro* study, we use CMC to alter whole blood viscosity and assess TEG parameters in conditions of anemia (Hct 20% and Hct 10%) as compared to saline diluted controls. We hypothesize that samples with low Hct, viscosity normalized with CMC will be less hypercoagulable than low Hct, low viscosity control samples, similar to our previous findings with ALG. Additionally, we hypothesize that this difference is not due to calcium binding by CMC, and will not be eliminated by increasing the \([\text{Ca}^{2+}]\) of the sample.

3.2 Materials and Methods.

Preparation of samples:

All fresh whole blood samples were obtained from healthy non-Greyhound blood donor dogs at The Ohio State University Veterinary Medical Center; all dogs had normal packed cell volume/total protein (PCV/TP) levels (PCV 45-54%, TP 5.7-7.2 g/dL) on the day of phlebotomy. Blood collection was approved by the OSU Institutional Animal Care and Use Committee. Samples were collected and processed using the same protocol outlined in the ALG experiment (Figure 2.2), with the exception that CMC was used in place of ALG, and samples were centrifuged at 400 X g in a similar centrifuge of a different brand due to availability.\(^M\) Sodium carboxymethylcellulose\(^N\) was dissolved in 0.9% NaCl to make a 2% stock solution, which was handled according to manufacturer’s recommendations. The reconstituted whole blood sample of either 10% or 20% Hct was then divided in half, and equal volumes of either saline (SAL) or carboxymethylcellulose (CMC) solution were added to make a final volume of 2.0 mL for each sample (Table 3.1). The amount of CMC added to each Hct condition was determined in preliminary studies. Briefly,
varying amounts of CMC based on trial and error preliminary data (not shown) were added to the various HCT conditions until the viscosity approximately matched a normal canine blood viscosity of 4 centipoise (cP) as previously determined in our laboratory.\textsuperscript{29} The final Hct and platelet count for each SAL diluted sample was determined using the same hematology analyzer;\textsuperscript{E} Hct and platelet count of the paired CMC sample was presumed to be identical to their paired SAL sample.

Thromboelastography analysis

The paired SAL and CMC samples were gently mixed by inverting 5-10 times then immediately run simultaneously on a 2-channel thromboelastography analyzer.\textsuperscript{G} All samples were kept at room temperature until TEG analysis. For initial CMC experiments, 340 µL of each sample was mixed with 20 µL of 0.2 M CaCl\textsubscript{2} in the pre-warmed TEG cup at 37° C to a final volume of 360 µL, and analysis was started immediately. No activator was used to maintain consistency with previous ALG results, and our laboratory reference ranges are for citrated native blood without activator. The TEG values R, angle, K, MA, and G were recorded. G was calculated by the software using the following equation: $G = \frac{5000MA}{(100-MA)} \times 1000$. The channel assigned to either SAL or CMC sample was systematically randomized as previously described.

We defined a hypercoagulable TEG tracing in this study as having a shortened R and K time, and an elevated MA, angle, and G as compared to canine reference ranges for our institution. As the appropriate definition of what constitutes a “hypercoagulable” TEG tracing is currently under debate in veterinary medicine,\textsuperscript{30} we report all TEG values and assess them independently. Similarly, defined a
hypocoagulable tracing as the opposite for all variables, as previously described. Normal canine TEG reference values for our institution are listed in Table 3.1 in parenthesis under each variable and are represented by dotted outlines in Figure 3.2 (b-f).

Calcium adjustment samples:

To determine the effect of calcium concentration on samples manipulated with CMC or saline, a stock solution of calcium chloride 0.4M was made from ACS Grade >98% anhydrous CaCl$_2$. Blood samples from 9 of the same dogs used in the earlier CMC experiments were drawn and reconstituted to a Hct of 10% as described above. These samples were divided and equal volumes of CMC or SAL were added to create a final volume of 2mL. They were then run simultaneously on the TEG; 340 µL of each sample was mixed with 20 µL of 0.4 M CaCl$_2$ in the pre-warmed TEG cup at 37°C to a final volume of 360 µL, and analysis was started immediately. No activator was used for consistency with previous data. Results from these TEG tracings were compared with those from the previous 10%Hct data run with 0.2M CaCl$_2$ to evaluate if increasing available [Ca$^{2+}$] that may be bound to CMC would negate the differences seen in the first experiment.

Viscosity measurement

The viscosity of each of the paired samples was measured on a cone and plate viscometer in an identical manner as described in the ALG experiment (shear rate 150 s$^{-1}$).

Statistics
Summary statistics (mean and standard deviation) are provided for each variable (Table 3.1). Normality was tested using the Shapiro-Wilk test. While most TEG parameters were normally distributed, some (most often R and K) were not; due to this as well as the small number of dogs (n=11, n=9 for calcium experiments), non-parametric statistics were used for all comparisons. Wilcoxon signed rank tests were used for pairwise comparisons between SAL and CMC groups of the same Hct condition and to compare 10%Hct conditions recalcified with either 0.2M or 0.4M CaCl₂. Kruskal Wallis rank sum tests were used to compare 10%Hct+SAL, 10%Hct+CMC and 45%Hct+SAL all at 0.2M [CaCl₂]; post-hoc pairwise comparisons were performed with the Steel-Dwass method. P-values of <0.05 were considered statistically significant. Data were analyzed using a commercial statistical package.  

3.3 Results.

Eleven dogs were included in this study; 7 castrated males and 4 spayed females. Breeds included 4 American Pitt Bull terriers, 2 boxers, 2 mixed breeds, and 1 each Golden Retriever, American Bulldog, and Labrador Retriever. As all dogs were selected from our blood donor pool, all were >25kg of body weight. Median age was 3.5 years (Range 3-7 years). Two dogs were no longer available for blood collection at the time of the calcium data collection, and so only 9 of the original 11 dogs were used for that arm of the study.

Mean (± standard deviation) for Hct, platelet count, time from phlebotomy to TEG run, TEG variables, and amount of CMC added to samples are outlined in Table 3.1. All TEG samples were run on average 43 minutes from time of blood
draw (range 39-51 min). There was no significant difference in platelet concentration between groups. Viscosity was significantly higher in all samples containing CMC than their corresponding SAL samples (p=0.001), as designed. When comparing 10%HCT+SAL to 10%HCT+CMC, R (p=0.004) and K (p=0.005) were significantly shorter, and angle (p=0.007), MA (p=0.002), and G (p=0.002) were significantly greater. Similarly, when comparing 20%HCT+SAL to 20%HCT+CMC, R (p=0.007) and K (p=0.006) were significantly shorter, and angle (p=0.02), MA (p=0.004), and G (p=0.001) were also greater for SAL than CMC. Box and whisker plots outlining these differences are illustrated in Figure 3.2 (a-f), and an example tracing from 1 dog illustrated in Figure 3.3.

In 10%Hct+SAL vs. 10%Hct+CMC, when the [CaCl$_2$] was increased to 0.4M some differences were noted when compared to the original 0.2M 10%Hct tracings. K (p=0.02), MA (p=0.01), G (p=0.03) and viscosity (p=0.004) were still different between 10%Hct+SAL and 10%Hct+CMC, but R (p=0.1) and angle (p=0.3) were no longer statistically different (Table 3.1, Figure 3.4, a-f). While K, MA, and G remain statistically different between SAL and CMC samples at the higher 0.4M [CaCl$_2$], the average difference between these values decreased for all 3 variables (Table 3.2). Platelets remained not significantly different between groups (p= 0.12). TEG tracings performed on 340uL of the stock 2%CMC solution mixed with 20uL 0.2M CaCl$_2$ repeatably showed only flat line tracings as demonstrated in Figure 2.3.

Comparison between 10%Hct+SAL (low viscosity, low Hct), 10%Hct+CMC (normal viscosity, low Hct) and 45%Hct+SAL (normal viscosity, normal Hct) were
performed at 0.2M \([\text{CaCl}_2]\) as shown in Figure 3.5. As designed, viscosity was significantly different between 10\%Hct+SAL and both 10\%Hct+CMC and 45\%Hct+SAL, but was not different between CMC conditions and 45\%Hct +SAL. There were no differences between any groups for R. All other TEG variables were significantly “hypercoagulable” for the 10\%Hct+SAL compared to 45\%Hct+SAL, consistent with the data in the ALG experiments. 10\% Hct+SAL was also “hypercoagulable” for MA and G compared to 10\%Hct+CMC, but not for any other variables. While there was a definite trend for all TEG variables except R to be “hypercoagulable” in the 10\%Hct+CMC as compared to 45\%Hct+SAL, this did not reach statistical significance. An example composite tracing of these 3 conditions in 1 dog is shown in Figure 3.6

3.4 Discussion

Compared to the previous experiment with ALG, the differences in the TEG tracings between CMC and SAL samples at both 10\% Hct and 20\% Hct were not nearly as large in this study. This suggests that a direct effect of ALG on coagulation, rather than the change in viscosity, may have created a large amount of the difference seen in the previous study. This is likely due to a previously unknown effect of ALG on coagulation, possible similar to the effect of HES as discussed in the previous chapter. However, a statistical difference between samples whose viscosity was altered with CMC compared to SAL controls was still found in this experiment. This may indicate that viscosity still plays a role in altering the TEG tracing, albeit to a lesser extent than initially suggested in the ALG experiment. Again, because the chemical properties of
CMC cannot be separated from its viscosity altering properties, we cannot rule out that interactions of CMC with the coagulation system were responsible for this difference.

While the coagulation effects of CMC in blood have not to our knowledge been investigated, an *in vitro* study of the coagulation effects of fully soluble hemostatic fiber (FHF) used in topical wound dressings (of which the main component is CMC) has been performed.²⁷ This study demonstrated increased coagulation in citrated blood mixed with FHF as indicated by shortened aPTT and thrombin time, increased platelet attachment rate, and increased blood viscosity. This study concluded that the mechanisms of hemostasis caused by FHF was due to increased contact activation and formation of a mechanical plug allowing for platelet aggregation.²⁷ However the hemostatic effects of pure carboxymethylcellulose have not been investigated, and IV preparations of nanoparticles coated with CMC in drug delivery systems showed no significant hematological or biochemical abnormalities in mice 5 days post-injection.²⁸ Therefore any effect of CMC on coagulation would be expected to cause increased hypercoagulability compared to SAL, opposite to what was seen in this study.

Because the difference between CMC and SAL control was much smaller than in our previous ALG experiment, we explored whether calcium binding by CMC was causing the decrease in hypercoagulability in samples manipulated with CMC compared to SAL controls. As can be seen in Fig 2.6, maximal coagulation is achieved in SAL control samples at 0.4M [CaCl₂]; at increasing calcium concentrations, coagulation is inhibited until SAL samples show flat-line TEG tracings with [CaCl₂] at and above 1M. At 0.4M [CaCl₂], R and angle were no longer statistically different, and while K, MA, and G were still statistically significantly different, the average difference between SAL
and CMC decreased for all of these variables (Table 3.2). This implies that binding of Ca$^{2+}$ by CMC may account for a portion of the difference between CMC and SAL. However, maximizing the Ca$^{2+}$ available for binding does not completely resolve the difference, indicating that viscosity and/or the CMC itself are contributing to the decrease in hypercoagulable changes between CMC and SAL conditions.

Despite having the same viscosity and Hct, the findings for this study were very different than our previous findings with ALG. While not the initial goal of the ALG study, the discovery of possible direct hemostatic effects of ALG is still a worthwhile finding. Previous studies of the impact of ALG on coagulation focus primarily on its pro-coagulant properties as a hemostatic dressing.$^{31}$ Whether ALG truly has anticoagulant properties similar to other colloids when used intravenously in vivo is unknown; the study by Segal et al showed variable effects of ALG on coagulation in vitro depending on the G/M ratio, with ALG containing more G groups having a hypercoagulable effect on coagulation testing. Intravenous ALG compounds may also have renal toxicity depending on their composition, which may limit intravenous uses as well.$^{32}$ Intravenous ALG compounds have been used previously in laboratory animal models to study the effect of viscosity on the microcirculation, and so it may be useful to know the impact that ALG may have on organ toxicity and the coagulation system.$^{29,33}$

We attempted to distinguish the impact of viscosity from that of red cell mass by comparing the 10%Hct+SAL (low viscosity, low Hct), 10%Hct+CMC (normal viscosity, low Hct) and 45%Hct+SAL (normal viscosity, normal Hct) at a normal 0.2M [CaCl$_2$] as shown in figures 3.5 and 3.6. Unsurprisingly as shown in our original ALG study, 10%Hct+SAL was different than 45%Hct+SAL for all TEG
values except R. As demonstrated earlier in this experiment, 10%Hct+CMC was also different from 10%Hct+SAL for the values MA and G. The loss of significance for the variables K and angle between 10%Hct+CMC and 10%Hct+SAL that were found earlier in this experiment is likely due to loss of statistical power when comparing multiple variables, as opposed to the paired comparisons between only CMC and SAL conditions of the same Hct. Similarly, 10%Hct+CMC was not statistically different that 45%Hct+SAL for any TEG variable, however there was a clear trend for 45%Hct+SAL to have a longer K and a smaller angle, MA, and G than 10%Hct+CMC, indicating that despite having the same viscosity, the 45%Hct+SAL tracings appear more hypocoagulable than the 10%Hct+CMC tracings. This is exemplified in a composite from 1 dog in Figure 3.6. The lack of statistical difference between the 10%Hct+CMC and 45%Hct+SAL is again likely due to the small sample size and the fact that these are unpaired samples, which decreases our statistical power as compared to paired samples within the same study. The clear trend for normal Hct samples of the same viscosity to be less hypercoagulable than anemic samples implies that whole blood viscosity does not account for all of the difference seen between samples of varying Hct.

Given that changes in viscosity cannot fully explain the changes in TEG tracing seen with anemia, it raises the question as to the role of red cell mass and the physical presence of red blood cells within the clot. Previous work has shown that incorporation of RBC into the fibrin network alters the fiber diameter and density of the fibrin clot. However, the notion that red blood cell incorporation into a fibrin “weakens” the meshwork would seem to be in contrast to in vivo studies which have
found that increasing Hct actually decreases the risk of bleeding.\(^{16,35-37}\) None of the rheological effects of flowing red cells in a vessel, such as erythrocyte aggregation and platelet margination increasing platelet-endothelium interactions, can be assessed by a non-flowing system such as TEG.\(^{38,39}\) In fact, in low-shear situations, red blood cells tend to form rouleaux or aggregates, which may change the homogeneity of their incorporation into a fibrin structure.\(^{39}\) During \textit{in vitro} studies investigating clots containing RBCs, the clots must be rotated during formation to prevent settling of the RBCs and uneven distribution within the clot.\(^{40}\) Even within individual thrombi removed surgically from large arteries, the RBC content of the clot can vary significantly within various areas of an individual thrombus.\(^{41}\) Similarly, RBC content of venous thrombi (so called “red clots”) is known to be significantly higher than in arterial thrombi (so called “white clots”),\(^{42}\) so the content and distribution of red cells in clots \textit{in vivo} can vary significantly in clots formed in different vessels and even within an individual clot. While electron microscopy of small sample portions of clots formed in TEG has been accomplished,\(^{43}\) to our knowledge no study investigating the distribution of red cells throughout a clot formed by TEG has been performed.

The incorporation of RBCs into a fibrin meshwork has been shown to affect the viscoelastic properties and fibrinolytic tendencies of a fibrin meshwork. The viscoelasticity of fibrin is highly related to fibril thickness and number of branch points.\(^{44}\) Increasing RBC content of clots makes them resistant to fibrinolysis.\(^{41}\) This is likely due to the production of thinner fibers in RBC rich areas, which are more resistant to fibrinolysis.\(^{41,45}\) Increasing RBC content also increased fibrin pore
size,\textsuperscript{34} which may play a role in allowing diffusion or exchange of material in a flowing system that is not appreciable in a non-flowing system such as TEG. Therefore, the roles that RBCs play in vivo to favor clot stability may not be detectable in a low-shear no-flow system such as TEG, but changes in fibrin structure caused by RBC incorporation into clots may influence clot viscoelasticity in vitro in ways that may be interpreted as clot “weakness” as assessed by TEG.

Using a strain controlled dynamic time sweep test, Gersh et al demonstrated that increasing the amount of RBCs in a clot resulted in peak viscous and elastic modulus at 10\% Hct; further addition of RBCs increased the viscous to elastic modulus ratio, significantly above Hcts of 20\%.\textsuperscript{40} This was interpreted as the viscoelastic nature of the RBCs themselves becoming a significant force in the clot and overwhelming the integrity of the fibrin structure. Unfortunately, the viscoelastic data generated by TEG cannot be directly compared other viscoelastic methodologies of assessing clot or fibrin dynamics. Therefore, correlations between various methodologies of clot assessment are currently impossible.\textsuperscript{46}

Red blood cells have been demonstrated to express procoagulant membranes and can act as membrane surfaces for thrombin generation.\textsuperscript{47} Increased thrombin generation would be expected to cause hypercoagulable TEG tracings, however in a recent review,\textsuperscript{48} research indicated RBC membranes as the main surface for generation of meizothrombin, an intermediate cleavage product of prothrombin that can have anticoagulant properties through interactions with thrombomodulin, resulting in generation of protein C. This is unlikely to be the mechanism of “hypocoagulable” TEG tracings with increasing Hct as the TEG does not contain
endothelium. However, it further illustrates that the full extent of red blood cell participation in thrombosis has not been fully realized, and that the formation of a clot in vivo will have far more complex cell-cell and cell-protein interactions than in an in vitro system such as the TEG. While in vitro tests may indicate increasing red blood cell content “weakens” a fibrin meshwork, in vivo studies clearly indicate that RBCs may have a procoagulant effect, possibly due to their effects on rheology, increased thrombin-generation, platelet adhesion and stimulation, and antifibrinolysis.\textsuperscript{39,41}

From this data, we conclude that alterations in whole blood viscosity may play a small role in influencing TEG tracings independent of red cell content. However, as normalization of blood viscosity could not return TEG tracings to baseline as compared to non-anemic samples, there is likely a role attributable to red blood cell mass itself, likely due to the effect of incorporation of red blood cells on fibrin meshworks formed in vitro. This study reinforces the notion that effects of Hct on TEG are likely an in vitro phenomenon, which can complicate interpretation of TEG in clinical conditions of variable Hct. It is important to remember that clot formation in the TEG cup is under a specific set of in vitro conditions that is not representative of all factors contributing to clot formation and strength in vivo. TEG tracings may be influenced by factors that affect blood viscosity (plasma proteins, intravenous medications or fluids) and should be interpreted accordingly. Similarly, caution should be taken in interpreting “hypocoagulability” caused by increasing RBC mass in TEG tracings as these findings are likely in vitro artifacts of the testing methodology.
3.5 References


38. Uijttewaal, W. S., Nijhof, E. J., Bronkhorst, P. J., Hartog, Den, E. & Heethaar, R.


Table 3.1: CMC vs. SAL at 10 and 20% Hct. † = significantly different than same symbol at p<0.05. * = significantly different than same symbol at p<0.05
Figure 3.2: Bar graphs CMC vs. SAL at 10 and 20% Hct. * or † = significantly different than same symbol at p<0.05.
Figure 3.3: example tracing of 10% SAL vs. CMC in one dog
Figure 3.4: 10%Hct+SAL vs. 10%Hct+CMC at 0.2M CaCl$_2$ and 0.4. * or † = significantly different than same symbol at p<0.05.
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<th><a href="mailto:CMC@0.2M">CMC@0.2M</a></th>
<th>Average difference 0.2M</th>
<th><a href="mailto:SAL@0.4M">SAL@0.4M</a></th>
<th><a href="mailto:CMC@0.4M">CMC@0.4M</a></th>
<th>Average difference 0.4M</th>
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<td>245(±25)</td>
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<td>275(±32)</td>
<td>275(±32)</td>
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<td>R (min) (1.0-6.1)</td>
<td>3.2(±1)$^\dagger$</td>
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<td>66.2(±2.8)$^\dagger$</td>
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<td>64.3(±3.5)*</td>
<td>3.4(±3.5)</td>
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<td>G (d/sec) (3104-10020)</td>
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<td>8089.9 (±800.3)$^\dagger$</td>
<td>1806.9 (±1457.7)</td>
<td>10638.8 (±1824.2)*</td>
<td>9111.8 (±1401.7)*</td>
<td>1527 (±1567.1)</td>
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<td>3.9(±0.1)$^\dagger$</td>
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<td>4(±0.3)*</td>
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<td>2.5</td>
<td>N/A</td>
<td>0</td>
<td>2.5</td>
<td>N/A</td>
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</table>

Table 3.2: 10%Hct SAL vs. CMC at 0.2M and 0.4M CaCl$_2$. $^*$ or $^\dagger$ = significantly different than same symbol at p<0.05.
Figure 3.5: Comparison of 10%Hct + SAL (low Hct, low viscosity), 10% Hct + CMC (Low Hct, normal viscosity) and 45% Hct + SAL (normal Hct, normal viscosity). * or † = significantly different than same symbol at p<0.05.
Figure 3.6: TEG tracing comparison of 10% Hct + SAL (low Hct, low viscosity), 10% Hct + CMC (Low Hct, normal viscosity) and 45% Hct + SAL (normal Hct, normal viscosity) in 1 dog
CHAPTER 4: INFLUENCE OF RED BLOOD CELL TRANSFUSION ON TEG TRACINGS IN NATURALLY ANEMIC DOGS

4.1 Introduction

In the previous studies, we explored the effect of manipulation of Hct and whole blood viscosity in an \textit{in vitro} system. These studies confirmed that the TEG has a bias towards hypercoagulability in canine whole blood samples manipulated to be anemic \textit{in vitro}. It was also determined that manipulation of whole blood viscosity toward normal reverses some of these changes, independent of HCT. However, not all of the TEG changes are explained by the effect of viscosity, suggesting there may be some contribution attributed to the direct effect of red cell mass. Furthermore, the impact that the degree of anemia has on TEG tracings in naturally anemic dogs has not, to our knowledge, been investigated. Previous studies have demonstrated that canine whole blood samples manipulated to be anemic \textit{in vitro} and \textit{in vivo} demonstrate this bias,\textsuperscript{1,2} and decreasing Hct was strongly correlated with “hypercoagulable” ROTEM values in dogs with normal Hct.\textsuperscript{3} However, the impact of changing Hct on TEG in dogs that are naturally anemic has not been explored, and this is the population in which the effect of anemia on the TEG is most clinically relevant. Therefore we hypothesized that Hct would be negatively correlated with TEG tracings indicative of “hypercoagulability” and that following transfusion, TEG values would become less hypercoagulable despite minimal change in underlying disease status over a short period of time.

4.2 Materials and Methods
Dogs presenting to The Ohio State University Veterinary Medical Center with anemia requiring red blood cell transfusion were eligible for enrollment in this study. Dogs were excluded from enrollment if they had received any blood product within the last 30 days, if transfusion was started prior to obtaining the pre-transfusion blood sample, if other major interventions such as surgery, massive transfusion, or large volume fluid resuscitation was needed at any point during transfusion, if owner consent was not obtained, or if dogs were <4kg or the attending clinician felt enrollment was against the clinical interest of the patient for any reason. Owner consent was obtained for all dogs enrolled in this study. This study was approved by the OSU Institutional Animal Care and Use Committee, the OSU Veterinary Medical Clinical Trials Office, and the Veterinary Medical Center Clinical Research Advisory Committee.

Twenty-eight dogs were enrolled in this study; 6 dogs were subsequently excluded. Four were excluded due to lack of a post-transfusion sample as the dog was discharged or was taken to surgery before the sample could be obtained; 2 dogs had pre-transfusion samples that could not be assessed, either due to delay in TEG analysis >30 minutes or TEG error. Twenty-two dogs had appropriate pre and post transfusion data to be analyzed and were included in the following data. Signalment, weight, underlying disease process, volume of transfused blood, and pre and post transfusion CBC data were collected. Patient data was further subdivided into 3 broad categories of anemia: bleeding, destruction, and non-regenerative. Pre-transfusion samples were taken within 1 hour prior to starting red blood cell transfusion, and post-transfusion samples were obtained at the time of post-transfusion PCV, within 1-3 hours of finishing the transfusion so as to minimize phlebotomy events. As these were clinical patients, site
and technique of sampling could not be standardized between patients and was not always consistent between pre and post transfusion samples. If blood was drawn from an IV catheter or central line, care was taken to flush the catheter with non-heparinized saline first and draw an appropriate waste sample (>3cc) prior to taking the study sample. Otherwise samples were drawn by direct venopuncture using butterfly needles and tubing attached to a syringe, or 22-18g needles attached directly to a syringe. All venipuncture was performed by skilled ICU technicians or the primary investigator (ACB). Care was taken to add 1.8 mL of blood to a citrated tube containing 0.2mL sodium citrate for a final 1:9 citrate: blood ratio. The time of phlebotomy was recorded on the tube.

All venipuncture was performed by skilled ICU technicians or the primary investigator (ACB). Care was taken to add 1.8 mL of blood to a citrated tube containing 0.2mL sodium citrate for a final 1:9 citrate: blood ratio. The time of phlebotomy was recorded on the tube.

All samples were analyzed on a hematology analyzer prior to TEG analysis to obtain Hct, platelet count, and white blood cell count before and after red blood cell transfusion.

Thromboelastography analysis

All samples were run on channel 1 of the TEG. Samples were kept at room temperature for 30 minutes until TEG analysis. 340 µL of each sample was mixed with 20 µL of 0.2 M CaCl₂ in the pre-warmed TEG cup at 37°C to a final volume of 360 µL, and analysis was started immediately. No activator was used to maintain consistency with previous results, and our laboratory reference ranges are for citrated native blood without activator. The TEG values R, angle, K, MA, and G were recorded. G was calculated by the software using the following equation: G = (5000MA/(100-MA))/1000.

We defined a hypercoagulable TEG tracing in this study as having a shortened R and K time, and an elevated MA, angle, and G as compared to canine
reference ranges for our institution. As the appropriate definition of what constitutes a “hypercoagulable” TEG tracing is currently under debate in veterinary medicine,\textsuperscript{12} we report all TEG values and assess them independently. We defined hypocoagulable variables as the opposite for all variables, as previously described.\textsuperscript{4} Normal canine TEG reference values for our institution are listed in Table 4.2 in parenthesis under each variable and are represented by dotted outlines in Figure 4.1.

Viscosity measurement

The viscosity of the remainder of each sample was measured after TEG analysis on a cone and plate viscometer.\textsuperscript{1} The cup was pre-warmed to 37° C via circulating water bath.\textsuperscript{3} A blood sample (0.5 mL) was placed in the center of the cup and immediately placed on the viscometer to start cone rotation. The viscosity was measured after 10 seconds of equilibration at 20 rpm (150 \textsuperscript{-1} shear rate). A 10-second time period from initiation of rotation was chosen for recording of viscosity, as viscosity of whole blood will continue to drop as time goes on due to sedimentation of red blood cells. Repeated readings (2-3) were performed for each sample and the average viscosity for each sample recorded in cP.

Statistics

Summary statistics (mean and standard deviation) are provided for each variable (Table 4.2). Normality was tested using the Shapiro-Wilk test. As many TEG parameters failed normality testing, non-parametric statistics were used for all comparisons. Wilcoxon signed rank tests were used for pairwise comparisons between pre- and post- transfusion sample data within the same patient. Kruskal Wallis rank sum tests were used to compare the anemia categories bleeding,
destruction, and non-regenerative as outlined below and in Table 4.1; post-hoc pairwise comparisons were performed with the Steel-Dwass method. P-values of <0.05 were considered statistically significant. Spearman’s rho value was calculated for non-parametric correlation between change in Hct or Plt and change in TEG values. Data were analyzed using a commercial statistical package.

4.3 Results

Twenty-two dogs completed the study; 10 were spayed females, 10 castrated males, 1 intact female, and 1 intact male. Five dogs were of mixed breed, 3 Labrador retrievers, and the remaining were 1 each: Golden Retriever, American Bulldog, Giant Schnauzer, Greyhound, Wirehaired Fox Terrier, Pekinese, Cocker Spaniel, Staffordshire Terrier, Pomeranian, Pit Bull, Pembroke Welsh Corgi, Doberman, Miniature Schnauzer, and Basset Hound. Ten dogs presented for bleeding from various causes, 6 for Immune-Mediated Hemolytic Anemia (IMHA), and 6 for non-regenerative anemia (Table 4.1). The median Hct pre-transfusion was 11% (range 6-26%) and median Hct post-transfusion was 19% (range 12-31%). On average, each dog received 11 (±2) mL/kg of packed red blood cells, and the average Hct increased by 7% (±3) after transfusion. Time between starting pre and post TEG samples was a median of 5 hours and 37 minutes (range 3:15-8:10). Pre vs. Post transfusion TEG variables are outlined in Figure 4.1 and Table 4.2.

As expected, the Hct increased significantly between pre and post transfusion time points (p = 0.0001). Viscosity also increased significantly as expected with increasing Hct (p=0.0007). Platelets and WBC were both significantly decreased in post-transfusion samples (p<0.008). There was no significant difference between pre and post transfusion samples for the TEG variables R, K, and angle. MA (p=0.03) and G
(p=0.007) decreased significantly in post-transfusion samples. A decreasing change in MA (increased delta MA, pre minus post) was significantly (p=0.03, r=0.5) correlated with increasing change in Hct; linear regression analysis is shown in Figure 4.2.

Decrease in Plt count was also significantly correlated with decreasing MA (p=0.002, r=0.6) and decreasing G (p=0.005, r= 0.6). Decreasing Plt count was significantly correlated with increasing Hct (p= 0.002, r= 0.6).

The pre-transfusion values and post-transfusion values were further assessed between each anemia category (Table 4.3 and Figure 4.3). Before transfusion, dogs with IMHA were significantly more anemic than bleeding dogs (p=0.04). Dogs with IMHA also had a significantly higher WBC count before transfusion than dogs with non-regenerative anemias (p=0.02). Dogs with IMHA had a significantly shorter K (p=0.002), and greater angle (p=0.008), MA (p=0.03), and G (p=0.03) than bleeding dogs. TEG values for dogs with non-regenerative anemia tended to fall between those two groups but were not statistically different from either, likely due to small sample sizes and the large variation within groups. R time and blood viscosity were not significantly different between any groups.

After transfusion, the differences noted between pre-transfusion groups persisted, with IMHA dogs having a significantly lower Hct than bleeding dogs (p=0.03), and a significantly higher WBC count than dogs with non-regenerative anemias (p=0.01). Dogs with IMHA still had a significantly shorter K (p=0.003), and greater angle (p=0.01), MA (p=0.03), and G (p=0.03) than bleeding dogs. R time and blood viscosity were not significantly different between any groups after transfusion.

4.4 Discussion
Data from this study showed that there is a relationship between increasing Hct and decreasing MA values in naturally anemic dogs, as has been documented in other species previously.\textsuperscript{5,6} in experimentally induced mild anemia in dogs,\textsuperscript{2} and in non-anemic dogs.\textsuperscript{3} However, unlike these previous studies, we did not find a correlation between increasing Hct and evidence of reduced hypercoagulability in any of the other TEG values. This is most likely due to the larger variation present in a cohort of dogs naturally anemic from multiple causes, where other factors of their underlying disease state may cause greater variability in these TEG values. Both platelet count and WBC count decreased following transfusion of pRBC. This is attributed to a dilutional effect of the RBC transfusion on the other cell types, and was also seen in a similar study in humans with platelets measured before, 1 hour, and 24 hours after transfusion,\textsuperscript{7} and in a horse model of increased red cell mass.\textsuperscript{5} As expected, whole blood viscosity was also significantly higher after transfusion.

When separating the transfused dogs by general disease category, there was a trend for dogs with IMHA to be the most anemic and most hypercoagulable, and for bleeding dogs to be the least anemic and least hypercoagulable. Dogs with non-regenerative anemia tended to fall in the middle, and likely due to high variability and small sample size, this group was not statistically different than IMHA or bleeding dogs. Dogs with IMHA are known to be clinically hypercoagulable and at risk for thrombosis, (Weiss 2006) and tend to become anemic over a longer period of time than acutely bleeding dogs, so it is not surprising that they are transfused at lower Hcts. Further, given the inflammatory nature of IMHA, these dogs would be expected to have hypercoagulable tracings and higher WBC counts than non-regenerative dogs of the same
Hct. It also stands to reason that bleeding dogs should have less hypercoagulable tracings compared to IMHA dogs as they are also loosing platelets and clotting factors. These dogs will also often need to be transfused at higher Hct levels as they have less time to compensate for their anemia and also have concurrent vascular volume loss.

Interestingly, despite the fact that they were clinically bleeding to the point that they required transfusion, the TEG tracings of dogs in this category were widely variable, including both hypercoagulable and hypocoagulable tracings, as indicated by the large variance seen in Figure 4.3. While a previous study indicated that a low G value in TF-TEG was both sensitive and specific for identifying bleeding dogs, 5/10 dogs in our bleeding group had G values that were above the upper end of our reference range. The 5 dogs that were hypercoagulable as identified by their G value had GI bleeding (3), pre-op bleeding from a left adrenal mass, and post-op bleeding after removal of a liver lobe. The 5 hypocoagulable dogs had hemangiosarcoma (3), facial trauma (1), and post-op splenectomy bleeding (1). While studies of cardiac bypass have shown TEG to be helpful in predicting surgical causes of bleeding vs. coagulopathic causes of bleeding, further prospective data will be needed to see if this holds true in veterinary patients.

Based on the slope of the best fit line, approximately every 1% increase in Hct my cause a decrease in MA of 0.5mm (±0.2). This is similar to the contribution of Hct (-0.598) to the multivariate linear modeling performed for the maximum clot firmness (MCF) of ellagic acid activated thromboelastometry (equivalent to MA for kaolin-activated TEG) by Smith et al in non-anemic canine blood samples (Figure 4.2). This model was also able to predict TE-ROTEM MCF (equivalent to TF-TEG MA) in experimentally induced mild anemia in dogs with strong correlation with Hct. In our
study, the maximum decrease in MA (7mm) occurred in a dog whose Hct increased by 12%. While the pre vs. post transfusion difference in MA was statistically significant, the clinical significance is difficult to assess. There is no data in veterinary medicine and sparse data in human medicine as to the appropriate triggers to drive TEG-guided transfusion or anticoagulation therapy, and no data at this time to indicate that the use of TEG-guided therapy has a mortality benefit over more traditional assays. Therefore it is impossible to say what the impact of Hct on TEG values used for clinical decision-making should be. However, given the relatively small changes in TEG parameters despite manipulation of Hct with pRBC transfusion, we feel that changes in Hct within the ranges studied here are unlikely to change TEG tracings enough to alter clinical decision making.

Limitations of this study include lack of standardization of blood sampling technique, a clinically heterogeneous sample of anemic patients, and a small sample size for each cause of bleeding. It is our policy that dogs not receive other medications while they are being transfused, but some patients in this study had already received medications such as steroids and one dog was on chronic clopidrogel, which may alter TEG tracings. None of these medications were administered between pre and post sample collection, and so theoretically should not have had major effects on coagulation during that short period of time. We limited TEG collection to immediately before and after transfusion in patients needing non-massive transfusion to try to assess only the impact of RBC mass on TEG parameters, but time effects of these medications or rapid change in disease status cannot be completely excluded as possible effects on TEG tracings. Because of the large inherent variation in a population of dogs receiving red
blood cell transfusion for a variety of causes, we also used paired statistical methods within the same patient over time to assess only the pre vs. post change within an individual.

Another limitation is the lack of measurement of fibrin/fibrinogen levels as recently recommended by PROVETS guidelines. While we believe the short time frame makes significant changes in these levels unlikely, without direct measurement we cannot rule out changes in fibrin/fibrinogen levels impacting pre and post pRBC transfusion TEG tracings. Platelets also decreased significantly in the post-transfusion group, which cannot be excluded as a contributor to the decreased MA seen in that group. However, in an in vivo model, it is impossible to transfuse only pRBCs and not cause a dilutional decrease in platelets. Platelet products for transfusion are not widely available in veterinary medicine, nor would it be ethical to transfuse them unnecessarily in this clinical population. As such the changes seen with solely pRBC transfusion in this study are most similar to widespread transfusion practice in veterinary medicine. The overall drop in platelets was about 18% from baseline, and a similar drop in WBC to about 12% from baseline was noted. Given that transfusion of 11mL/kg of pRBC should be expected to stay mostly within the intravascular space in this short time frame, an approximate 12-14% dilution of total canine blood volume (assumed 80-90mL/kg) by pRBC would be expected. This leads us to conclude that these decreases are most likely a result of dilution, but additional procoagulant effects of RBCs on platelet activation/consumption and WBC activation and margination as previously discussed cannot be ruled out.
The effect of a change in platelet count of approximately 35,000/L on TEG tracings is difficult to assess. In a study looking at TEG performed on native whole blood in women with pre-eclampsia, MA did not start to decrease in correlation with platelet count until the platelet count was <100,000/L. Similarly, an in vitro study by Oshita et al predicted that significant changes in the CN-TEG variables MA and K were only seen at platelet counts <66,000/L. However, in that same study a strong correlation ($r^2 = 0.74$) was seen between the Log$_{10}$[Plt] and MA even at higher platelet concentrations. Similarly, a high correlation between Log$_{10}$[Plt] and the CN-TEG variables MA ($r=0.987$) and K ($r=-0.86$) were seen in one study where it was commented that the logarithmic nature of this relationship means that fairly large changes in platelet number or function would be needed to cause a significant change in MA. In a study by Smith et al, the overall contribution of Plt to ellegin-activated thromboelastometry MCF was an approximate increase of MCF by 0.04 for every 1000/L increase in platelets. Other studies have also indicated that overall platelet mass in addition to platelet number, as well as platelet-fibrin interactions contribute to the generation of TEG variables. Therefore specific additional assays such as the addition of fibrin and platelet inhibitors are needed to assess the contribution of each parameter to the TEG tracing. While the presumed dilutional decrease in platelets count in response to red blood cell transfusion cannot be eliminated as a possible cause of the TEG changes seen in this study, it does not explain the increased MA seen in response to decreasing Hct in vitro when platelet counts are held constant in both our previous in vitro work and that of others.

In our previous studies, we have shown that viscosity does have a small impact on TEG tracings. As increasing the red blood cell content of the patient will also increase
whole blood viscosity, it is likely that a small component of the impact seen in this study is due to the change in whole blood viscosity. However this change in TEG tracings caused by viscosity is relatively small as we have previously demonstrated. Additionally, this study was focused on assessment of the impact of changing red blood cell content in TEG tracings of naturally anemic dogs; it is inevitable that when changing red cell content, viscosity will also change. Therefore, for the goal of assessing clinical impact on TEG tracings, it makes most sense to consider the effects of red cells and viscosity together.

This study focused on the effects of Hct on TEG tracings in naturally anemic dogs as this is the population in which we are most likely to run and interpret a TEG in a state of changing Hct. However, we can only comment on the effect Hct has on TEG within the range of anemia present in this clinical population. It is possible that as RBC content of the TEG cup increases, there may be a greater impact on MA or on other TEG values besides MA and G; however, the change in MA caused by Hct in this study was similar to that found by McMichael et al for dogs with experimentally induced milder anemias. Further work in dogs with a broader range of Hct and broader disease states will be needed to fully assess the impact Hct has on TEG. Similarly, this study selected dogs that were already determined to need RBC transfusion over a moderate (2-4 hour) period of time. It therefore excludes patients receiving intraoperative or more urgent transfusions, and has a selection bias for dogs in clinical need of RBC transfusion as determined by the attending clinician.

In conclusion, increasing Hct in naturally anemic dogs does have a statistically significant impact on the TEG variables MA and G, causing these variables to become
less hypercoagulable as Hct increases. As we currently have no understanding of what constitutes a clinically significant change in TEG values, it is difficult to comment on whether the relationship between Hct and MA would have an impact on clinical decision-making based on TEG analysis. However, it seems fair to say that the impact of Hct on TEG values in naturally anemic dogs below Hcts of 30% is relatively small, and large changes in TEG tracings should be considered to reflect other underlying coagulation abnormalities in the patient and not be attributed to an artifact of changing Hct.
4.5 References


<table>
<thead>
<tr>
<th>Bleeding (10)</th>
<th>Destruction (6)</th>
<th>Non-regenerative (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Post-op liver lobectomy for hepatocellular carcinoma</td>
<td>(6) IMHA</td>
<td>(3) PRCA</td>
</tr>
<tr>
<td>(1) Post-op splenectomy for splenic hematoma</td>
<td></td>
<td>(1) B-cell lymphoma</td>
</tr>
<tr>
<td>(1) Pre-op bleeding, left adrenal tumor</td>
<td></td>
<td>(1) Waldenstrom's macroglobulinemia</td>
</tr>
<tr>
<td>(1) Diffuse HSA with pulmonary bleeding</td>
<td></td>
<td>(1) Non-regen anemia—open (CKD vs. PRCA vs. Phenobarbital toxicity)</td>
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<tr>
<td>(1) Lymphoma and HSA, urinary bleeding</td>
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<td></td>
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<tr>
<td>(1) HSA bleeding into R thigh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) HBC, severe facial trauma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) GI bleeding (not due to ITP)</td>
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Table 4.1: Causes of bleeding in dogs needing pRBC transfusion
<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-transfusion</th>
<th>Post-transfusion</th>
<th>Average difference</th>
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</thead>
<tbody>
<tr>
<td>Hct (%) (45-54)</td>
<td>11.8 (±4.5)*</td>
<td>18.6 (±4.5)*</td>
<td>6.8 (±3.1)</td>
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<tr>
<td>Plt (10^9/L) (108-433)</td>
<td>188.9 (±163.6)*</td>
<td>154.1 (±117)*</td>
<td>-34.7 (±82.2)</td>
</tr>
<tr>
<td>WBC (10^9/L) (4.1-15.2)</td>
<td>24.5 (±16.8)*</td>
<td>21.8 (±15.2)*</td>
<td>-2.7 (±4.5)</td>
</tr>
<tr>
<td>Viscosity (cP) (3.74-4.94)</td>
<td>2.23 (±0.6)*</td>
<td>2.7 (±0.6)*</td>
<td>0.5 (±0.5)</td>
</tr>
<tr>
<td>R (min) (1.0-6.1)</td>
<td>3.5(±1.6)</td>
<td>3.5(±1.5)</td>
<td>0.02(±1.3)</td>
</tr>
<tr>
<td>K (min) (0.9-3.6)</td>
<td>2.1(±3.8)</td>
<td>1.9(±2.2)</td>
<td>-0.2(±1.7)</td>
</tr>
<tr>
<td>Angle (°) (51.8-73.4)</td>
<td>71.6(±15)</td>
<td>69.7(±13.3)</td>
<td>-1.9(±4.5)</td>
</tr>
<tr>
<td>MA (mm) (43.9-67.9)</td>
<td>71.2(±17.4)*</td>
<td>69.3(±16.1)*</td>
<td>-1.9(±3.5)</td>
</tr>
<tr>
<td>G (d/sec) (3104-10020)</td>
<td>18585.4(±13470.6)*</td>
<td>15740.2(±10345.6)*</td>
<td>2845.23(±4150.6)</td>
</tr>
</tbody>
</table>

Table 4.2: TEG pre vs. post transfusion variables for all dogs
Figure 4.1: Bar graphs pre vs. post all causes. † = statistically different at p<0.05. Normal reference ranges indicated within dashed boxes. Box and whiskers represent median with interquartile range and whiskers are 1.5x interquartile distance with outliers represented as dots.
(Figure 4.1 continued)
Figure 4.2: Linear regression of ΔHct vs. -ΔMA for all dogs. Representative equations showing Hct contribution as measured by thromboelastometry, adapted from Smith et al 2012.
Figure 4.3: Bar graphs divided by underlying cause of anemia. * and † = statistically different from same symbol at p<0.05. Normal reference ranges indicated within dashed boxes. Box and whiskers represent median with interquartile range and whiskers are 1.5x interquartile distance with outliers represented as dots.
(Figure 4.3 continued)
<table>
<thead>
<tr>
<th></th>
<th>IMHA (n=6)</th>
<th>Non-regen (n=6)</th>
<th>Bleeding (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>Hct (%) <strong>(45-54)</strong></td>
<td>9 (+2.2)*</td>
<td>15.3 (+2.9)+</td>
<td>10.7 (+2.5)</td>
</tr>
<tr>
<td>Plt (10^9/L) <strong>(108-433)</strong></td>
<td>222 (+152)</td>
<td>191 (+147)</td>
<td>278 (+231)</td>
</tr>
<tr>
<td>WBC(10^9/L) <strong>(4.1-15.2)</strong></td>
<td>40.5(+22.1)*</td>
<td>36.6(+20.4)+</td>
<td>12.1(+6.1)*</td>
</tr>
<tr>
<td>Viscosity(cP) <strong>(3.74-4.94)</strong></td>
<td>2.4 (+0.4)</td>
<td>2.8 (+0.4)</td>
<td>2.5 (+0.9)</td>
</tr>
<tr>
<td>R (min) <strong>(1.0-6.1)</strong></td>
<td>3.2 (+0.7)</td>
<td>3.4 (+1)</td>
<td>3.0 (+1.3)</td>
</tr>
<tr>
<td>K (min) <strong>(0.9-3.6)</strong></td>
<td>0.8*</td>
<td>0.85 (+0.1)+</td>
<td>1.2 (+0.7)</td>
</tr>
<tr>
<td>Angle (°) <strong>(51.8-73.4)</strong></td>
<td>81.7 (+2.8)*</td>
<td>78 (+6.9)+</td>
<td>75.5 (+10.3)</td>
</tr>
<tr>
<td>MA (mm) <strong>(43.9-67.9)</strong></td>
<td>82.7 (+8.3)*</td>
<td>81.3 (+7.7)+</td>
<td>75.5 (+11.7)</td>
</tr>
<tr>
<td>G (d/sec) <strong>(3104-10020)</strong></td>
<td>29,866 (+15458)*</td>
<td>25548 (+11630)+</td>
<td>19,207.37 (+10587.8)</td>
</tr>
</tbody>
</table>

Table 4.3: Pre vs post transfusion variables by disease state. * = significantly different (p<0.05) between pre-transfusion variables.  
+ = significantly different between post-transfusion variables
CHAPTER 5: CONCLUSION

Viscoelastic methodologies of monitoring coagulation continue to grow in popularity both as research tools and as clinical tests. It is therefore essential to understand the limitations of these tests as well as factors that may bias their results and possibly impact research conclusions or clinical decision-making. The effect that low Hct causes a more “hypercoagulable” TEG tracing is well established in multiple species, despite the commonly noted effect that increasing Hct in vivo commonly reduces bleeding risk. This discrepancy is often ignored in the use of TEG in patients where anemic conditions are common, such as renal failure, IMHA, and hypoadrenocorticism.

The series of investigations performed were designed to explore whether changes in red cell mass or changes in whole blood viscosity are responsible for the alterations in TEG (a viscoelastic methodology) that have been documented with changes in Hct (specifically anemia). One experiment determined that normalizing viscosity with ALG resulted in TEG tracings that were significantly less hypercoagulable compared to control samples with matched Hct. However, it could not be determined whether ALG itself may also have direct impact on in vitro coagulation, and thereby TEG tracings. Therefore we repeated the experiment using a difference viscosity-modifying agent, CMC. While there was still a statistically significant reduction in the “hypercoagulability” of the TEG tracing in CMC samples compared to controls, this difference was far less than what was seen in the ALG experiments. This implies that the large differences seen between ALG
and SAL controls of the same Hct in the first study are likely due to an effect of ALG rather than viscosity, as similar changes in CMC samples of the same viscosity were not seen. While studying the effects of ALG on coagulation was not our initial purpose, this is still an important finding. To our knowledge, possible anticoagulant effects of ALG on TEG tracings have not been previously reported, and could impact the use of this material in future *in vivo* and *in vitro* studies of viscosity.

The CMC study showed a less dramatic difference between SAL and CMC samples than the ALG study, but there was still a statistically significant decrease in the hypercoagulability of CMC TEG tracings compared to controls. This difference was reduced but not eliminated despite doubling the Ca$^{2+}$ content of the TEG cup, indicating that Ca$^{2+}$ binding by CMC may play a small role in creating this difference. However, the difference between CMC and SAL tracings persisted even in maximum [Ca$^{2+}$], leading to the conclusion that viscosity does have a small impact on TEG tracings. Yet the change in viscosity does not explain the entire effect caused by red cell mass, as samples with the same viscosity but higher Hct still show a trend towards decreasing hypercoagulability. Therefore we conclude that the impact of red cell mass on TEG tracings is likely a combination of viscosity and the impact that incorporation of RBCs has on the formation of the fibrin network in the TEG cup. While *in vitro* tests may indicate increasing red blood cell content “weakens” this fibrin meshwork, *in vivo* studies clearly indicate that RBCs have a procoagulant effect, likely due to a combination of the rheological, thrombin-generating, platelet adhesion and stimulation, and antifibrinolytic effects of red blood cells. This reinforces the notion that effects of Hct on TEG are likely an *in*
vitro phenomenon, which complicates interpretation of TEG in clinical conditions of variable Hct.

To explore the degree of influence that this artifact would have on TEG tracings of dogs with clinically relevant anemia under conditions of changing Hct, we investigated the impact Hct had on TEG tracings before and after pRBC transfusion. Increasing Hct in naturally anemic dogs does have a statistically significant correlation with the TEG variables MA and G, trending with these variables to become less “hypercoagulable” as Hct increases. Within the range of Hct values in this study (6-30%), this impact was relatively small, with approximately every 1% increase in Hct causing a decrease in MA of around 0.5mm (±0.2). While it is difficult to say whether this amount of change would impact clinical decision making, large changes in TEG tracings likely reflect the underlying disease process rather than the effect of Hct.

Further research is needed to fully explain the effect of RBCs on TEG tracings. Assessment of RBC distribution within clots formed in TEG cups by scanning electron microscopy or separate fluorescent labeling of RBC and fibrin strands would allow for visualization of RBC distribution within the fibrin network. This would also reveal whether the fibrin network and RBC incorporation within that network was uniformly distributed within the TEG cup. Using an inert “bead” of similar size to RBCs and studying the impact of incorporation of those “beads” into fibrin meshworks formed in TEG cups could also help clarify whether this influence of RBCs is solely based on creating a wider fibrin meshwork, or if there are intrinsic viscoelastic or biochemical properties of the RBC membrane or cellular
contents that play a role in influencing TEG clot formation. As the impact of RBCs on fibrinolysis is a possible in vivo effect of RBCs on clot stability that is not easily seen with citrated native TEG, further work using tPA spiked TEG samples might better explore this effect. Similar experiments such as those in this thesis using other activators such as K-TEG or TF-TEG may also help reduce variation seen in TEG variables, especially R times. Concurrent measurements of other aspect of coagulation, such as fibrinogen levels, factor activity levels, or thrombin generation potential assays would also allow for a broader investigation of the coagulation system to assess how these factors correlate with TEG tracings. From the clinical aspect of the impact of anemia on canine TEG variables, studying larger numbers of specific subsets of dogs (i.e. IMHA or traumatic bleeding) or in dogs with a wider range of Hct is needed. This will further understanding the correlation between Hct and TEG in specific disease states and further define the overall relationship between Hct and canine TEG tracings at all Hct levels. Ideally this could eventually lead to a correction algorithm to remove the artifact caused by Hct level from TEG interpretation. Finally, prospective work on the utility of TEG for predicting clinical outcomes of bleeding or thrombosis is desperately needed to elucidate the clinical applicability of this test in both human and veterinary medicine.

It is important to remember that clot formation in the TEG cup is under a specific set of in vitro conditions that does not represent all factors contributing to clot formation and strength in vivo. TEG tracings may be influenced by factors that affect blood viscosity (plasma proteins, intravenous medications or fluids) and should be interpreted accordingly. Similarly, caution should be taken in over interpreting the changes caused
by increasing RBC mass in TEG tracings as these findings are likely in vitro artifacts of the testing methodology. Viscoelastic methodologies of monitoring global hemostasis can be useful tests, but as with all testing methodologies, it is important to take their strengths and limitations into account.
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Schöchl, H. et al. Transfusion in trauma: thromboelastometry-guided coagulation factor concentrate-based therapy versus standard fresh frozen


Appendix A: Endnotes

A
BD Vacutainer Safety-Lok Blood collection set, Franklin Lakes, NJ

B
Monoject red stopper blood collection tube, 2mL. Covidien, Mansfield, Massachusetts

C
BD Vacutainer glass plasma tube, 3.2% Sodium citrate

D
Jouan CR3i centrifuge, ThermoFisher Scientific, Waltham MA

E
Idexx Procyte Dx Hematology Analyzer, Idexx Laboratories, Westbrook, Maine

F
PRONOVA™ UP LVM Batch number FP-401-06 with a G/M ratio of ≤1, 100g container, NovaMatrix Ultrapure Polymer Systems, Drammen, Norway

G
TEG R 5000, Haemonetics Corp., Niles, IL

H
ACS Grade >98% anhydrous CaCl₂ (MW 110.99) (Sigma, lot 449709)

I
DV II + Pro Viscometer with CPE-40 cone (0.8 degrees, 2.8 cm radius) and CPE-44Y plate, Brookfield, Middleboro, MA

J
Isotemp circulator, Model 900. Fischer Scientific Pittsburg, PA

K
JMP pro, SAS for Windows, SAS Institute Inc., Cary, NC

L
Monoject, Covidien, Mansfield MA

M
Beckman-Coulter Allegra 6, Brea, CA

N
Sodium Carboxymethylcellulose, Lot 89612, Hercules Incorporated, Wilmington, DE

O
Graphpad Prism, La Jolla, CA