Thromboelastographic assessment of the contribution of platelets and clotting proteases to the hypercoagulable state of dogs with immune-mediated hemolytic anemia.

Negar Hamzianpour, BSc (Hons), BVSc, MRCVS and Daniel L. Chan DVM, DACVECC, DECVECC, DACVN, MRCVS

From the Department of Clinical Science and Services, The Royal Veterinary College, University of London, Hawkshead Lane, AL9 7TA, North Mymms, Hatfield, UK

Address correspondence and reprint requests to: Daniel L. Chan, The Royal Veterinary College, University of London, Hawkshead Lane, AL9 7TA, North Mymms, Hatfield, UK . E-mail: dchan@rvc.ac.uk

Conflicts of Interest: Dr. Chan is the Editor of the Journal but did not participate in the peer review process other than as an author. The authors declare no other conflict of interest.

Presented in part as an abstract at the British Small Animal Veterinary Congress, April 2013, Birmingham, UK

Running title: Platelet contribution to hypercoagulability in IMHA

Abstract

Background*:* Hypercoagulability is a well-known feature of canine immune-mediated hemolytic anemia (IMHA) and is believed to increase the risk of thrombosis. This study was undertaken to differentiate the relative contribution of platelets and clotting proteases to this hypercoagulability using thromboelastography (TEG).

Study design*:* Retrospective observational study.

Methods*:* Thromboelastograms from 27 dogs with IMHA were retrospectively evaluated. Standard TEG parameters (R, K, α, MA), the G value and the novel parameter delta (Δ) were determined. Hypercoagulability was attributed to the platelet component of hemostasis when there was an increased G value with a normal Δ value.

Key findings*:* 19 of 27 dogs (70.4%) had ≥ 2 TEG variables suggestive of hypercoagulability, 18 (66.7%) had a hypercoagulable G value, and 11 (40.7%) had a hypercoagulable Δ value. Ten of 27 (37%) samples met the criteria for platelet hypercoagulability.

Significance*:* Our report documents the derivation and application of the Δ value to differentiate enzymatic from platelet hypercoagulability. Further studies are required to validate the use of these TEG variables in this manner. The hypercoagulable tendency in dogs with IMHA is complex and multifactorial and in some dogs this hypercoagulability may be attributed primarily to platelet hyper-reactivity. Our findings may support the use of anti-platelet drugs in some dogs with IMHA.

Keywords: canine, TEG, thrombosis, anemia, coagulation

Abbreviations

α – alpha angle

IMHA – immune mediated hemolytic anemia

K- clot formation time

MA- maximum amplitude

R – reaction time

SP – split point

TEG – thromboelastography

TMRTG - time to maximum rate of thrombus generation

Introduction

The hypercoagulable state in canine immune-mediated hemolytic anemia (IMHA) is an important cause of morbidity and mortality, resulting in an increased risk of thromboembolic disease.[1] In general, hypercoagulability can be the result of increased platelet reactivity, increased clotting factor activity, or a combination of both.[2] Despite the use of drugs to decrease clot formation, mortality in canine IMHA remains high (50-70%).[3-5] This is possibly due to a lack of knowledge of the most appropriate pharmacologic intervention for treating the hypercoagulable state in individual dogs.

Thromboelastography (TEG) is a viscoelastic test that is becoming more widely used in clinical veterinary practice. Unlike conventional plasma-based coagulation tests, TEG gives a global assessment of a patient's coagulation status, and documents clot formation from initial platelet-fibrin interactions through fibrinolysis.[6,7,18]

There are a number of standard measured values derived from the TEG tracings (thromboelastograms) that may be used to define the coagulation state of a patient [hypo- or hypercoagulable (Figure 1)].[7-9] The reaction time (R) represents the time of latency from the point when the blood was placed in the TEG analyzer to the start of fibrin formation. The R value is most representative of the initial phase of clot formation by enzymatic clotting factors. The clot formation time (K), is the time after the R time necessary for the 2 arms of the thromboelastogram to diverge by 20 mm, and alpha (α) is the angle created by the tangent to the thromboelastogram. K and α both denote the speed of fibrin formation and cross-linking, and indicate cleavage of available fibrinogen into fibrin by thrombin generated in earlier reactions. Maximum amplitude (MA) is the maximal distance between the 2 divergent arms of the

thromboelastogram; it is a direct function of the maximum dynamic properties of fibrin and platelet bonding and represents the ultimate strength of the clot.[7]

A recent human report evaluated the use of non-conventional TEG parameters (G and Δ) to characterize the etiology of a hypercoagulable state as platelet or enzymatic in origin.[10]. The TEG Δ parameter is calculated from the difference between the R time and the split point (SP, min) of the TEG tracing (R-SP, Figure 1). The SP is the first point at which the arms of the thromboelastogram diverge. The Δ value in people has a strong linear correlation with the time to maximum rate of thrombus generation (TMRTG, measured by the TEG and displayed as part of the thrombus velocity curve, Figure 1).[10] TMRTG and other velocity curve parameters may be surrogate markers of thrombin generation.[11] Thus, Δ may estimate thrombin generation and the enzymatic (clotting factor) contribution to clot formation. The G value is a calculated measure of total clot strength (G= 5000 x MA/100 - MA) and represents both the enzymatic and platelet contribution to clot formation.[8] By evaluating Δ and G, characterization of hypercoagulability as enzymatic or platelet in origin may be possible.[10]

We hypothesized that the hypercoagulable state present in dogs with IMHA could be further characterized with respect to a platelet or enzymatic etiology using TEG. This study can provide the basis for further research into the etiologic characterization of hypercoagulability in dogs using TEG.

Materials and Methods

Retrospective analysis of TEG records from dogs with IMHA at the Queen Mother Hospital for Animals, the Royal Veterinary College, between July 2008 and August 2012 was performed. The TEG database was searched for patients with a diagnosis of IMHA. Inclusion criteria were further defined to only include data from kaolin-activated TEG analyses. Several dogs had multiple TEG analyses performed during their hospitalization; only the first TEG analyzed from each case was included. For inclusion in this study, a diagnosis of IMHA included anemia (PCV<30%), evidence of hemolysis (ie, hyperbilirubinemia, bilirubinuria, hemoglobinemia, hemoglobinuria, or spherocytosis), and 1 of the following: persistent auto-agglutination, positive direct anti-globulin test (Coombs' test), regenerative anemia with spherocytosis, or non-regenerative anemia with bone marrow cytology suggestive of IMHA.

Additional information obtained from the clinical records included the sex, neutering status, age, weight and breed of each dog. Data was also gathered regarding PCV, medical treatments, and transfusions administered prior to blood collection for TEG analysis.

Blood samples were collected by careful jugular venipuncture according to our institution's standard operating procedures, using minimal stasis, a 21-Ga needle, and a syringe. Blood samples were immediately transferred into 1.3 mL, non-vacuum, polypropylene citrated tubes[a] and inverted carefully to ensure mixing to a final concentration of 1 part 3.2% buffered trisodium citrate to 9 parts whole blood. After a 30-minute incubation at room temperature, citrated whole blood samples were activated with kaolin by aliquoting 1mL of citrated whole blood into manufacturer-supplied kaolin vials[b] (containing kaolin, phospholipids and buffered stabilizers) and carefully inverted once. Immediately, 340μL of the sample was placed into a

heparinase coated cup[b] with 20 μL of calcium chloride[b] to initiate TEG analysis. The TEG analyses were performed on a single TEG machine,[c] by 1 of 4 operators, according to the manufacturer's instructions. Five TEG parameters were recorded from each thromboelastogram: R, K, α, SP, and MA. The G and Δ values were calculated by the investigators.

Hypercoagulability in the current study was defined as $\Delta < 0.45$ min, G > 10000 dynes/cm^2, or when 2 or more of the standard TEG parameters (R, K, α, MA) were indicative of hypercoagulability (ie, shortened R or K, and increased α or MA).[12] Reference intervals for all the standard parameters (R, K, α, MA) were based on our institutional reference interval (Table 2), which was derived from 27 healthy dogs that had kaolin-activated TEG analysis performed in duplicate, with the data analyzed using Robust Box Cox methods.[13,14] The upper limit of the G value calculated from the reference interval was 9400 dynes/cm^2. Based on these findings we defined hypercoagulability as G > 10000 dynes/cm^2. Similarly, Δ (R-SP) was calculated for our normal healthy controls and the normal interval was 0.45-2.8 min. Therefore we defined hypercoagulability as $\Delta < 0.45$ min. Hypercoagulability attributed to platelet hyperreactivity (ie, without a enzymatic contribution) was defined when G > 10000 dynes/cm^2 and $\Delta \geq 0.45$ min.

Data were inspected visually for assessment of data distribution. Frequency analysis of the number of cases considered hypercoagulable by each method of hypercoagulability was carried out. Non-normally distributed data were reported as the median value and the range.

Results

The search of our institution's TEG database identified 158 samples from dogs screened for IMHA. This included kaolin activated and citrated native (unactivated) samples; for this study only kaolin activated samples (n=34) were included in our analysis. Thrombelastograms

were further excluded from study because of failure to satisfy the inclusion criteria for a confirmed diagnosis of IMHA (n=2) and where repeat samples from the same dog were performed for serial TEG monitoring (n=5); in these cases, only the initial TEG trace was included in our analysis. The final analysis included TEG tracings from 27 dogs with IMHA.

Hematologic assessment documented a regenerative anemia in 21/27 (77.8%) of the dogs with the remainder showing a non-regenerative anemia. The median PCV for the population at the time of TEG analysis was 16% (range 11-27%). On the basis of the clinical history, further investigations and diagnostic tests, a final diagnosis of primary (idiopathic) IMHA was given to 22 (81.5%) of the dogs. Three (11.1%) of the dogs were found to have IMHA secondary to another cause including systemic lupus erythematosus or ehrlichia and 2 (7.4%) were not fully investigated to rule out other causes due to financial constraints or early euthanasia.

The median age of the population was 6.9 years (range 1.6 - 11.3 years). The predominant breed was Cocker Spaniel (n=8) with the rest being 1 of each of 19 other breeds. Seventeen of 27 (63%) dogs were females (15 spayed, 2 intact) and 10 (37%) were males (8 castrated 2 intact). At the time of blood sampling, 20 dogs (74%) had already received or were in the process of receiving a packed red blood cell transfusion. Additionally, 22 dogs had received some form of medical treatment prior to TEG sampling, including prednisolone/dexamethasone (n=18), azathioprine (n=9), aspirin (n=2), cyclosporine (n=1) or mycophenolate (n=1).

Nineteen dogs (70.4%) had ≥ 2 standard TEG variables (R, K, α, MA) indicative of a hypercoagulable state, Eighteen (66.7%) had a hypercoagulable G value and 11 (40.7%) had a hypercoagulable Δ value. From the total number of samples, 10/27 dogs (37%) had a hypercoagulable G value with a normal Δ value. Of the 19 dogs defined as hypercoagulable via

standard TEG parameters, 9 (47.3 %) had a normal Δ value.. A summary of TEG values obtained and the frequency of dogs classified as hypercoagulable with respect to the different methods of classification and reference intervals is shown in Tables 1 and 2.

Discussion

In this study we attempted to use TEG variables to ascertain the relative contribution of platelet and enzymatic proteases to the hypercoagulable state in dogs with IMHA. This approach was predicated on a retrospective report describing similar results in 10 critically ill human patients.[10] A major limitation of this study is the lack of validation of the parameter Δ to differentiate platelet from enzymatic hypercoagulability in dogs. This report describes the existence and derivation of this novel variable so it may be investigated in future studies.

Nineteen of the 27 dogs studied (70%) were determined to be hypercoagulable on the basis of evaluation of commonly used TEG variables (R, K, α, MA), which is consistent with previous TEG studies in dogs with IMHA.[15,19] The nature of the hypercoagulability in approximately 50% of these dogs (37% of the total number of dogs studied) was ascribed to increased platelet function.

This report demonstrates the contribution of platelet reactivity to hypercoagulability, as defined in people, in canine patients with IMHA. If confirmed, our results could be used to justify the use of anti-platelet therapies such as clopidogrel and aspirin in specific patients to reduce the risk for the development of a thrombotic event. Further studies are needed to establish if these methods can be used to identify specific hypercoagulable states and prevent thromboembolic complications in clinical cases through individually tailored thromboprophylaxis.

Future studies that document the presence of thrombi (with advanced imaging, specialized hemostatic assays, or post-mortem examinations) in animals with concurrent TEG analysis will be beneficial to determine the *in vivo* significance of hypercoagulable TEG tracings. Preliminary studies in this vein are available in abstract form.[16,17] In addition, a comparison of the incidence of thrombus formation in patients with platelet or enzymatic hypercoagulability would be beneficial by allowing us to evaluate the effect of targeted treatment for these factors with drugs such as heparins or anti-platelet drugs.

Consensus guidelines have recently been established for TEG analysis in veterinary patients.[18] Our study follows these guidelines for sample collection, handling, assay activation, test protocol, and establishment of institutional reference intervals. However, lack of standardization of the methodology for TEG analysis among veterinary centers[8,9,19] limits comparison of TEG results between institutions and hampers wider application of findings to populations of interest. These issues as well as the small number of patients used in many clinical studies present a challenge for the interpretation of TEG in veterinary patients. We recognize that these confounding factors are also important limitations of our report.

Another limitation of the current report is the lack of contemporaneous data for fibrinogen, platelet count, prothrombin time, and activated partial thromboplastin time. Fibrinogen and platelet count contribute to the characteristics of the TEG tracing; in particular, fibrinogen has a large impact on the kinetics of clot formation. Newly published consensus guidelines recommend that fibrinogen and platelet count be reported in addition to TEG results.[18] At the time of sampling, prior to the release of the consensus guidelines, these criteria were not a standard screening test for IMHA at our institution, however, future prospective evaluations of Δ should report these variables. In addition, other modalities of assessing platelet function, such as

platelet aggregometry or flow cytometry, may lend additional data to support the use of the Δ value when evaluated alongside TEG.

We attempted to minimize pre-analytical factors that are known to influence TEG analysis such as traumatic venipuncture, storage time, and temperature.[20] However, we recognize that other pre-analytic factors such as concurrent medical therapy,[21] blood transfusions, hematocrit,[22] platelet count, and fibrinogen concentration may have influenced our results. A number of patients also received blood transfusions prior to TEG sampling. As our institution uses leukoreduced packed red blood cells as the standard blood product in dogs, the impact of procoagulant microparticles typically found in non-leukoreduced blood transfusions[23] on TEG tracings is not believed to have been a factor in our study. Future prospective studies based on this report should control for these pre-analytical factors.

Our report describes initial findings that with further validation could be clinically applicable for optimizing use of thromboprophylaxis in dogs with IMHA. Our report documents the existence, derivation and potential application of the Δ variable, and suggests that TEG could be a valuable tool in the clinical assessment of hypercoagulable canine IMHA patients, in that it may allow for differentiation of enzymatic from platelet hypercoagulability. Our findings suggest that the hypercoagulable state in 50% of hypercoagulable IMHA dogs can be attributed to platelet hyper-reactivity and, in theory, justify the use of anti-platelet drugs such as clopidogrel and aspirin in such cases.

Footnotes

[a] Pediatric tube, International Scientific Supplies, Bradford, UK

[b] Haemonetics Corporation, Niles, IL, USA

[c] TEG 5000 Hemostasis Analyzer, Haemoscope Corporation, Niles, IL, USA

References

1. Kidd L, Mackman N. Prothrombotic mechanisms and anticoagulant therapy in dogs with immune-mediated hemolytic anemia. J Vet Emerg Crit Care 2013; 23(1): 3-13.

2. Kittrell D, Berkwitt L. Hypercoagulability in Dogs: Pathophysiology. Compendium: Continuing Education for Veterinarians 2012; 40: E1-E4.

3. Swann JW, Skelly BJ. Systematic review of evidence relating to the treatment of immune-mediated hemolytic anemia in dogs. J Vet Intern Med 2013; 27(1):1-9.

4. Reimer ME, Troy GC, Warnick LD. Immune-mediated hemolytic anemia: 70 cases. J Am Anim Hosp Assoc 1999; 35:384-391.

5. Grundy SA, Barton C. Influence of drug treatment on survival of dogs with immune-mediated hemolytic anemia: 88 cases. J Am Vet Med Assoc 2001; 218: 543-546.

6. Mallett SV, Cox DJA. Thromboelastography. Br J Anaesth 1992; 69(3): 307-13.

7. Donahue SM, Otto CM. Thromboelastography: a tool for measuring hypercoagulability, hypocoagulability, and fibrinolysis. J Vet Emerg Crit Care 2005; 15(1): 9-16.

8. Wiinberg B, Jensen AL, Johansson PI, et al. Thromboelastographic evaluation of hemostatic function in dogs with disseminated intravascular coagulation. J Vet Intern Med 2008; 22(2): 357-65.

9. Bauer N, Moritz A. Characterisation of changes in the haemostasis system in dogs with thrombosis. J Small Anim Pract 2013; 54(3): 129-36.

10. Gonzalez E, Kashuk JL, Moore EE, Silliman CC. Differentiation of enzymatic from platelet hypercoagulability using the novel thrombelastography parameter delta (Δ). J Surg Res 2010; 163(1): 96-101.

11. Rivard GE, Brummel-Ziedins KE, Mann KG, et al. Evaluation of the profile of thrombin generation during the process of whole blood clotting assessed by thromboelastography. J Thromb Haemost 2005; 3(9):2039-43.

12. Wagg CR, Boysen SR, Bedard C. Thrombelastography in dogs admitted to an intensive care unit. Vet Clin Path 2009; 38(4): 453-61.

13. Friedrichs KR, Harr KE, Freeman KP, et al. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. Vet Clin Path 2012; 41(4): 441-453

14. Geffre A, Concordet D, Braun J, et al. Reference value advisor: a new freeware set of macroinstructions to calculate reference intervals with Microsoft Excel. Vet Clin Path 2011; 40(1): 107-112

15. Sinnott VB, Otto CM. Use of thromboelastography in dogs with immune-mediated hemolytic anemia: 39 cases (2000–2008). J Vet Emerg Crit Care 2009; 19(5): 484-8.

16. Thawley V, Drobatz K, King L, Sanchez M. Association between results of thromboelastography (TEG) and post-mortem evidence of thromboembolism in critically ill dogs. (Abstract) J Vet Emerg Crit Care 2012; 22(S2): S15.

17. Jutkowitz A, Kinns J, Habing A, et al. CT angiography for detection of pulmonary thromboembolism and portal vein thrombosis in dogs with immune-mediated hemolytic anemia (IMHA). J Vet Emerg Crit Care 2013; 23(S1): S15.

18. Goggs R, Brainard B, deLaforcade AM, et al. Partnership on rotational viscoelastic test standardization (PROVETS): Evidence based guidelines on rotational viscoelastic assays in veterinary medicine. J Vet Emerg Crit Care 2014; 24(1):1-22

19. Goggs R, Wiinberg B, Kjelgaard-Hansen M, Chan DL. Serial assessment of the coagulation status of dogs with immune-mediated haemolytic anaemia using thromboelastography. Vet J 2012; 191(3): 347-53.

20. Kol A, Borjesson DL. Application of thrombelastography/thromboelastometry to veterinary medicine. Vet Clin Path 2010; 39 (4): 405-16.

21. Rose LJ, Dunn ME, Allegret V, et al. Effect of prednisolone administration on coagulation variables in healthy Beagle dogs. Vet Clin Path 2011; 40 (4): 426 – 434.

22. Smith SA, McMicheal MA, Gilor S, et al. Correlation of haematocrit, platelet concentration, and plasma coagulation factors with results of thromboelastometry in canine whole blood samples. Am J Vet Res 2012; 73: 789 – 798.

23. Herring JM, Smith SA, McMichael MA, et al. Microparticles in stored canine red blood cell concentrates. Vet Clin Path 2013;42(2):163-9.

Table 1: Frequency of hypercoagulability in 27 dogs with immune mediated hemolytic anemia by the different methods of TEG assessment of hypercoagulability

Method of assessment of hypercoagulability	**Nº of patients hypercoagulable**	**Percentage of patients hypercoagulable (%)**
≥ 2 of the standard TEG variables (R, K, α, MA) hypercoagulable	19	70.4
G value >10000 dynes/cm^2	18	66.7
Δ < 0.45 min	11	40.7
Platelet hypercoagulability (G value >10000 dynes/cm^2 & Δ ≥ 0.45 min)	10	37.0
Platelet hypercoagulability from patients deemed hypercoagulable by G value (n=18)	10	55.6
Platelet hypercoagulability from patients deemed hypercoagulable by ≥ 2 of the standard TEG variables (n=19)	9	47.3

Table 2: Results of thromboelastography in 27 dogs with immune-mediated hemolytic anemia

TEG parameter	**Median**	**Range**	**Institutional reference ranges***	**Reference value for hypercoagulability ***	**Nº of patients hypercoagulable**	**% of patients hypercoagulable**
R (min)	5.1	2.3 - 9.2	3-9.5	< 3	4	14.8
K (min)	1.1	0.8 - 3.1	1.6 – 5.1	< 1.6	18	66.7
α (s^o)	73.5	51.3 - 81.3	35.9 – 68.1	> 68.1	18	66.7
MA (mm)	72	44.4 - 89.4	45.5 - 65.1	> 65.1	18	66.7
G ($dynes/cm^2$)	12832.3	4000.1 - 42163.9	4200 – 9400	> 10000	18	66.7
Δ (min)	0.6	0.1 - 1.9	0.45 – 2.8	<0.45	11	40.7

*Institutional laboratory reference interval derived from 27 healthy dogs using the Robust Box-Cox method[13,14]

Figure Legends

Figure 1: Sample of a thrombus velocity curve (in grey) depicted over a standard thromboelastography (TEG) tracing, showing correlation of the Δ value with thrombus generation parameters (reprinted and adapted with permission from Elsevier).[10] R = reaction time; K = clot formation time; α^{o} = alpha angle; MA = maximum amplitude; TTG = total thrombus generation; MRTG = maximum rate of thrombus generation; TMRTG = time to maximum rate of thrombus generation; R = reaction time; SP = split point; Δ = delta.