THE EFFECT OF SAMPLE COLLECTION METHOD ON THROMBOELASTOGRAPHY IN DOGS

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ABSTRACT

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Thromboelastography allows for global assessment of hemostasis though the evaluation of the viscoelastic properties of whole blood. Many studies have been performed in both human and veterinary medicine to evaluate the hemostatic status of patients; however the effect of sample collection on thromboelastography (TEG) remains unknown. The goal of this thesis was to review the preanalytical and patient variables that affect TEG and to determine the effect of sample collection method on TEG. Furthermore, the role of platelet activation during sample collection was evaluated using flow cytometry, and markers of platelet activation are reviewed.

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TEG: Thromboelastography

ROTEM: Rotational Thromboelastometry

PSLG-1: P-selectin glycoprotein ligand

IMHA: Immune-mediated hemolytic anemia

R: R (reaction) time

K: K time

α: Alpha angle

MA: Maximum amplitude

CV: Coefficient of variation

PBS: Phosphate buffered saline

CD 61: Cluster of differentiation 61 (glycoprotein IIIa)

CD 62: Cluster of differentiation 62 (P-selectin)

PMA: Phorbol myristate acetate

FACS: fluorescence activated cell sorter

CHAPTER 1:

PREANALYTICAL VARIABLES, PATIENT VARIABLES, AND ANALYTICAL VARIABLES THAT AFFECT THROMBOELASTOGRAPHY

Introduction to Thromboelastography

Thromboelastography (TEG) is a hemostatic assay that allows for global assessment of hemostasis. TEG was originally developed in Germany in 1948,[1] but was not widely used until it became computerized. Currently, there are several instruments that assess hemostasis through the evaluation of the viscoelastic properties of blood. These instruments include the Thromboelastograph (TEG, Haemoscope Corporation, Niles, IL), the ROTEM (Pentapharm GmbH, Munich, Germany), and the Sonoclot analyzer (Sienco Inc., Arvada, CO). TEG and ROTEM are most commonly used in human and veterinary medicine and will be the focus of this review.

In human medicine, TEG is primarily used in liver transplant and cardiac surgery patients in order to guide transfusion therapy.[2-4] Use of TEG has been associated with a decrease in the use of transfusion products.[4] More recently, TEG has been used to detect hypercoagulable states in people including following trauma.[5-8] In veterinary medicine, TEG has been used most commonly to detect hypercoagulable states seen during diseases such as neoplasia,[9] parvovirus infection,[10] immune-mediated hemolytic anemia,[11,12] and protein-losing enteropathy.[13] It has also been correlated with clinical bleeding in dogs.[14]

Unlike traditional coagulation assays, TEG uses whole blood to analyze coagulation. This allows for assessment of the interaction of platelets, red and white blood cells, coagulation factors, anticoagulant factors, and the fibrinolytic system. While this is an advantage of TEG as it may more accurately reflect in vivo hemostasis, it may also make TEG more susceptible to preanalytical variation. This preanalytical variation may mask hemostatic abnormalities in patients, or may falsely lead a clinician to believe that a patient has a hemostatic abnormality when there is not really one present.

Preanalytical Variables

Sampling Technique

Many preanalytical variables may cause significant changes in TEG tracings, beginning with sample collection. There has been a recent effort to standardize TEG for human patients, with an initial goal of establishing a standardized collection method.[15]

In human medicine, the effect of sample site was evaluated in cardiac surgery patients.[16,17] Initially, it was demonstrated that samples simultaneously collected from venous and arterial catheters differed significantly.[17] The authors concluded that the differences were large enough that decisions regarding transfusion of both platelets and fresh frozen plasma may have been altered depending on the site of sample collection.[17] This study was followed by a second study to evaluate the reason for the difference between arterial and venous samples. The

authors collected 3 blood samples from patients undergoing cardiac surgery, one from an arterial line and 2 from different venous ports.[16] The authors demonstrated that shear stress created from the diameter of the catheter was likely responsible for the differences seen between arterial and venous samples.[16] The attributed this difference to possible platelet activation during sample collection.[16]

In veterinary medicine, the effect of sample collection technique on TEG has been evaluated.[18] One study evaluated the effect of sampling from various sizes and locations of intravenous catheters on kaolin-activated TEG.[18] Two relatively small (18 and 20 gauge) catheters were placed in either cephalic vein, and two larger catheters (13 and 14 gauge) were placed using different techniques in either jugular vein.[18] The authors found no difference amongst the collection techniques with regards to kaolin-activated TEG.[18] Based on results from human studies,[16,17] one may speculate that the catheters with a smaller diameter would result in more coagulable TEG results due to shear stress. However, the samples collected through the two catheters with the smallest diameter were collected directly into tubes containing citrate, whereas the samples collected from the two catheters with the largest diameter (and therefore possibly lower shear stress) were first collected into a plain syringe followed by transfer to a tube containing citrate.[18] A slight delay to contact with citrate may result in a more coagulabe TEG tracing. The delay present in the sample technique involving the larger catheters may have made these samples more coagulable, therefore making the results similar to those samples exposed to a potentially higher shear stress. An alternative explanation is that the use of

an activator resulted in less preanalytical variation from different sample collection techniques, as has been previously suggested.[19] Activators speed the initiation of coagulation, shortening the R and K times and therefore may mask changes in the initiation phase of coagulation caused by preanalytical variables.

Discard Tube

Another debatable aspect of sample collection for TEG analysis is the value of a discard tube prior to collecting the sample for analysis. Previously, it was thought that a discard tube was needed for traditional hemostatic tests due to potential tissue factor activation during venipuncture.[20] However, studies have since provided evidence that a discard tube is not necessary and it is no longer recommended for traditional hemostatic testing in human medicine.[21,22] The need for a discard tube for TEG has been evaluated in veterinary medicine. The authors found that a discard tube was not necessary if clean venipuncture was achieved.[a] However, if venipuncture was challenging, a discard tube could negate the effects of suboptimal venipuncture. As venipuncture may be more challenging in veterinary medicine than in human medicine, a discard tube may be more useful in veterinary patients.

Hemolysis

The effect of induction of post-sampling hemolysis on TEG and ROTEM has been evaluated in veterinary medicine.[23,24] Mechanical induction of hemolysis in equine blood resulted in significantly less coagulable tracings compared to samples that did not have hemolysis.[24] This was true for non-activated, ellagic acid-activated, and tissue factor-activated ROTEM.[24] Similarly, in a study assessing both mechanical and freeze-thaw induction of hemolysis in canine blood, hemolyzed blood resulted in TEG tracings that were significantly less coagulable when compared to the non-hemolyzed sample.[23] The authors used kaolin activation for this study.[23] In both of these studies, hemolysis was induced artificially and after sample collection.[23,24] The induction of hemolysis may have altered the membranes of cells or activated platelets, thus accounting for the changes seen in TEG and ROTEM. Further studies are needed to assess the effect of hemolysis during sample collection. Use of activators does not appear to eliminate the preanalytical variability of hemolysis in horses or dogs.

Interoperator Variability

Interoperator variability has been examined for equine TEG.[25] There was less interoperator variability when tissue factor was used as an activator compared to when non-activated TEG was used.[25] Sample collection was standardized using a butterfly catheter with collection of blood into a syringe followed by transfer to several vacuum tubes. Interoperator

variability was not assessed using the same horses with different people collecting blood which would have been a better assessment of interoperator variability. Additionally, samples were transferred to different numbers of citrate tubes, possibly allowing for variable time between collection and contact with citrate which may have contributed to interoperator variability. Because of reduced interoperator variability with use of tissue factor activation, the authors recommended tissue factor-activated TEG be used in horses to allow more reliable comparisons among operators.[25]

Sample Type

Native whole blood (no anticoagulant) is the most common sample collected for TEG analysis of human patients.[26,27] Recommendations in human medicine state that native samples should be analyzed within 6 minutes of collection.[28] However, in facilities where the TEG instrument is centralized or separated from patients, anticoagulation with citrate makes TEG a more widely useful diagnostic tool because it allows delayed analysis and reserves the sample during transport to the analyzer.

In human medicine, there are conflicting results of several studies comparing TEG results from native blood and from blood anticoagulated with citrate. A study in 8 healthy adults found no difference between TEG performed on native blood within 4 minutes of collection compared to recalcified citrated blood following a 30 minute rest period.[26] A significant difference was found, however, when the citrated sample rested 120 minutes prior to testing; the stored sample

was slightly more coagulable.[26] In human patients with severe liver disease, there were no differences in TEG results between native blood samples and recalcified citrated blood samples held at room temperature for 1-2 hours.[29] This supports use of citrate anticoagulation to allow time for samples to be transported to a central laboratory. In contrast, a study in children[27] and a separate study in adults[30] found that recalcified citrated whole blood was significantly more coagulable following a 30 minute rest period or a one hour rest period, respectively, compared to native whole blood. A different study assessed the effects of citrated sample storage over time. Using celite activation, there were significant differences between native and recalcified citrated blood. Initially, the recalcified citrated blood appeared less coagulable than then native blood, but it progressively increased in coagulability over time.[31] The authors attributed this change to incomplete inhibition of thrombin generation in the citrated samples.[31]

The effect of citrate anticoagulation on kaolin-activated TEG has been assessed in dogs.[32] TEG performed on native whole blood within 6 minutes of sample acquisition was not significantly different from TEG of citrate-anticoagulated whole blood that was held at room temperature for 1 hour prior to activation with kaolin and recalcification.[32] Citrate-anticoagulated whole blood may be an acceptable substitute for native whole blood in dogs, while in contrast, most human studies report significant differences between these two sample types. Use of kaolin as an activator or differences between canine and human hemostasis may account for this discrepancy.

Sample Transport

Samples often have to be transported to a laboratory for analysis. Pneumatic tube transport is sometimes used in hospitals for efficient transport of samples. One study in human medicine evaluated the effect of pneumatic tube transport on TEG and other hemostatic tests.[33] The authors found that transport via pneumatic tube resulted in a significantly shorter R compared to samples that were manually transported to the laboratory; therefore, manual transport of blood samples for TEG was recommended.[33]

Time until Sample Analysis

Native whole blood used for TEG is typically analyzed within 4-6 minutes of collection in human patients. In order to make TEG a more widely available test, blood is often anticoagulated with citrate. When blood is exposed to citrate for anticoagulation, TEG results vary over time. In humans, citrated blood becomes more coagulable with time after 120 minutes of storage.[26] However when an activator such as celite or kaolin is used, blood appears to be stable for a longer period of time, ranging from 60 minutes[34] to 8 hours.[31] Some studies also report that blood can be analyzed by kaolin-activated TEG or ROTEM anytime from 0 through 30 minutes after sampling.[35,36]

Canine blood samples seem to yield less stable TEG results than human samples over time. When tissue factor was used as an activator of canine samples, blood stored for 120

minutes was more coagulable than blood stored for 30 minutes.[37] However, when tissue factor or kaolin was used as an activator for ROTEM, samples were stable from 0 through 30 minutes.[19] If no activator was used, blood was more coagulable over time.[19] In horses, blood becomes more coagulable over time when TEG is performed without an activator.[38] Using ROTEM with activators for analysis, citrated equine blood is relatively stable for up to 20 hours.[24] When comparing sample results, the samples should be tested at the same time post-sampling to eliminate time-related variations. The use of activators may eliminate some of the time-dependent variability in ROTEM.[19]

Temperature

Most commonly, citrated blood samples are held at room temperature prior to recalcification for TEG analysis. Clinically, many samples must be transported to a laboratory for analysis and transport at room temperature is more feasible. Additionally, if samples are to be held at body temperature (37 °C in people), sample tubes should be prewarmed to prevent temporary cooling of blood during collection. This creates challenges because samples are often collected in various settings and a heating block for keeping tubes warm is not feasible in all areas. Because of concern about the hemostatic effects of cooling and rewarming, the effect of storage temperature has been evaluated in humans and dogs. In a study of human samples, there were no significant TEG differences in native blood and citrated blood stored at room temperature for 30 minutes or at 4 °C for up to 150 minutes.[26] Similarly, no ROTEM

differences were found for citrated canine blood held at 37 °C or room temperature.[19] However, blood samples held a 4 °C compared to those held at room temperature were significantly different in equine blood stored for 20 hours.[24] Holding blood at room temperature or 37 °C may be most appropriate due to the potential for platelet damage at colder temperatures.[39]

Patient Variables

Gender

Men and women are known to have different tendencies for thrombosis, with women tending to be more prothrombotic.[40,41] TEG and ROTEM are also significantly different between men and women, with women being more coagulable,[30,35,42,43] even when pregnancy and use of oral contraceptives are excluded.[30] The prothrombotic tendencies of women are detectable via TEG following trauma, with women being significantly more hypercoagulable than men immediately after the traumatic event.[7]

In veterinary medicine, the effect of gender on coagulation as assessed by TEG has only been evaluated in one canine and one equine study.[25,32] There were no gender differences in TEG results in either study. Many of the animals were intact in both studies, making a lack of sex hormones unlikely to be the cause for the lack of difference between the groups. Larger

studies including both intact and neutered animals are needed to determine if gender influences TEG as it does in humans.

Pregnancy

Pregnancy causes a state of hypercoagulability in people[44-46] and this can be demonstrated with TEG.[42,47,48] There are no veterinary studies evaluating the effect of pregnancy on TEG.

Age

Hypercoagulability is associated with increasing age and increasing age has been associated with increasing coagulability as assessed by TEG.[30,35,49] However, a study comparing kaolin-activated TEG in children to that in adults did not show age-related differences.[50] The lack of a difference between adults and children may be due to the use of kaolin as an activator, or age-associated coagulation changes may only be detectable in older adult populations using TEG. There are not published veterinary studies assessing the association of age and TEG results; however, caution should be used when using adult reference intervals to interpret TEG in neonates.

Analytical Variables

Activators

Many different activators have been used for TEG in both human and veterinary medicine. Activators such as celite and kaolin act by initiating the intrinsic pathway, while others, such as tissue factor, work by initiating the extrinsic pathway. Alternatively, native blood can be used without an activator or blood anticoagulated with citrate can be recalcified and analyzed without one of these activators. Activators commonly lead to accelerated coagulation and differences in TEG results compared to native blood or recalcified citrated blood.[34,51-53] Some authors have suggested that activation of a sample may minimize susceptibility to preanalylitcal variation and should therefore be used.[19] However, other authors have suggested that strong activators such as tissue factor may mask hemostatic abnormalities in some patients.[52,53] There is no consensus in human or veterinary medicine regarding the use of activators, but reference intervals should be established for each activator used by the institution.

Conclusions

Many preanalytical variables affect TEG results and should be considered when interpreting TEG results. An effort is being made in human medicine to make recommendations for standardization of TEG to allow for more global comparison of results both in the clinical and research arenas.[15] Until a standard approach to TEG is established, results from TEG and

comparison to reference intervals must be made with careful consideration of the possible influences of preanalytical variables.

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CHAPTER 2:

EFFECT OF SAMPLE COLLECTION METHOD ON THROMBOELASTOGRAPHY IN DOGS

Introduction

Thromboelastography (TEG) allows for global assessment of hemostasis through evaluation of the viscoelastic properties of whole blood throughout the entire process of coagulation from initiation to final clot formation and fibrinolysis. TEG has been used to evaluate coagulation in dogs with neoplasia,[1] canine parvovirus infection,[2] consumptive coagulopathy,[3] immune-mediated hemolytic anemia,[4] and hemorrhage.[5] TEG is also being used clinically to diagnose coagulation abnormalities, especially hypercoagulable states, and to direct therapy, such as anticoagulant therapy.[6] However, there are no published guidelines for such use, and there is little published regarding the effect of sample collection method on TEG in human or veterinary medicine.[7-9]

Blood samples for TEG are being collected by various methods without knowledge of the impact of collection method. Samples for TEG in published research have been collected using a 21 g butterfly catheter attached to a vacuum tube containing sodium citrate,[1,3,5,10,11] via 21 g needle into a syringe before transfer to a vacuum tube containing sodium citrate,[12] using a needle directly attached to a vacuum tube,[2,11] and from a central venous catheter.[2,3] Multiple

collection methods have been used in some studies,[2,3,11] while methods of sample collection are sometimes not reported.[4] If significant effects of sample collection variables are not recognized, TEG results may be interpreted inappropriately and patient management may be adversely affected.

Current veterinary recommendations for collection of blood for traditional plasma-based hemostasis testing include collecting blood directly into a syringe containing citrate or into a citrated vacuum tube.[13] However, there are no reports of TEG results generated from blood collected directly into a syringe containing citrate.

It has been recommended that blood for routine coagulation testing of human patients be collected with clean venipuncture from a large vein directly into a citrated vacuum tube.[14] Moreover, vacuum tubes with less vacuum force are preferred because strong vacuum forces during blood collection have been associated with platelet activation.[15] A group created to standardize human TEG in a clinically applicable manner has acknowledged that standardization should begin with sample collection, but a standard sample collection protocol has not been established.[16]

The primary goal of this study was to determine if TEG results for canine blood samples collected directly into a syringe containing citrate differ from those collected with vacuum and/or delay to citrate. We hypothesized that vacuum collection and delay to contact with citrate significantly affect thromboelastography results. To assess the diagnostic significance of any

collection-related effects, we also sought to determine the expected non-activated TEG findings in apparently healthy dogs whose blood was similarly collected into citrate-containing syringes. The few published reference values for canine TEG were either established for activated TEG[10,17] or by using an unspecified collection technique.[4,18]

Materials and Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee. For each comparison of sample collection methods, blood was collected from twelve healthy dogs that were privately owned or part of a research breeding colony. For establishment of expected TEG values, blood was collected from 40 clinically healthy dogs. Of these 40 dogs, 24 were also used for studies 1 and 3 described below. Dogs were clinically healthy based on physical examination and history, but laboratory testing was not done to exclude clinically-occult disease. Dogs were excluded if they had received medications other than heartworm and flea preventive within the preceding two weeks.

Sample Collection

Variations in sample collection method were compared in three different studies. For each study, paired 2.7 mL blood samples were collected in random order (Microsoft Excel, Redmond, WA) from the right and left jugular veins of 12 dogs. All samples were collected

with a 20 g one-inch needle into a 3 mL plastic syringe (3 mL BD Luer-Lok syringe with 20 g × 1 in BD PrecisionGlide needle, Franklin Lakes, NJ). All syringe samples were gently transferred following removal of the needle to a plain plastic tube just prior to sample evaluation to facilitate pipetting (Sarstedt, Newton, NC). Samples were excluded if venipuncture was not successful on the first attempt or if any needle redirection was necessary.

Study 1 (effect of delay to anticoagulation): One blood sample was drawn into a syringe containing 0.3 mL of 3.2% sodium citrate. The paired sample was drawn into an empty syringe and then transferred without vacuum within 5 seconds to a tube containing 0.3 mL of 3.2% sodium citrate (BD Vacutainer Plus Citrate Tubes, Franklin Lakes, NJ). For the transfer, the needle was removed from the syringe and the top was removed from the vacuum tube to allow for as little agitation to the sample as possible during transfer.

Study 2 (effect of vacuum): One blood sample was drawn into a syringe containing 0.3 mL of 3.2% sodium citrate and transferred without vacuum to a plain plastic tube (BD Vacutainer No Additive (Z) Plus Tubes, Franklin Lakes, NJ). The paired sample was drawn into a syringe containing 0.3 mL of 3.2% sodium citrate and then transferred by vacuum to a plain plastic vacuum tube (BD Vacutainer No Additive (Z) Plus Tubes, Franklin Lakes, NJ).

Study 3 (combined effect of delay to anticoagulation and vacuum): One blood sample was drawn into a syringe containing 0.3 mL of 3.2% sodium citrate. The paired sample was drawn into an empty syringe and then transferred by vacuum within 5 seconds to a tube containing 0.3 mL of 3.2% sodium citrate (BD Vacutainer Plus Citrate Tubes, Franklin Lakes, NJ).

Establishment of expected TEG results: Blood was drawn into a syringe containing 0.3 mL of 3.2% sodium citrate. The 24 dogs from studies 1 and 3 that had one sample collected by this method were included. Blood was collected from an additional 16 apparently healthy dogs.

Sample Analysis

Samples were rested for 40 min at room temperature (20-23 °C) before being analyzed in duplicate, simultaneously, with two Haemoscope Thromboelastograph Analyzers (Thromboelastograph Hemostasis Analyzer 5000, Haemonetics, Niles, IL) at 39 °C according to the manufacturer's instructions and including assessment of control samples. A temperature of 39 °C was used to mimic the canine physiologic state. Briefly, 340 µL of citrated whole blood was pipetted into a reagent cup provided by the manufacturer, and 20 µL of 0.2 M calcium chloride was added to initiate coagulation. The cup was then gently raised to the pin and measurements were initiated. R and K times, alpha angle (α), and relative maximum amplitude (MA) were recorded.

Statistical Analysis

Statistical analysis was performed using commercially available software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego CA USA, www.graphpad.com). For paired samples in each study phase, R, K, α, and MA were compared with the Wilcoxon matched pairs test. Intra-assay variation was determined for paired samples

from each study by calculating the coefficient of variation (CV) using the standard deviation divided by the arithmetic mean. Intersubject variability was calculated for each study by comparing the variance of each collection method. $P < 0.05$ was considered statistically significant.

For determination of expected values, commercially available software (Analyse-it Method Evaluation edition for Microsoft Excel, Leeds, United Kingdom) was used to identify the central 95% of values. A nonparametric approach was used because of the small number of individuals and the non-normal distribution of data for some of the parameters.

Results

Expected values: Expected values for non-activated TEG (39 °C) using samples collected into citrate-containing syringes were generated from 40 clinically healthy dogs as the central 95% of values (Table 1).

Study 1 (effect of delay to anticoagulation): The median R for blood drawn into a syringe followed by transfer to a tube containing citrate was significantly shorter than that for paired blood samples drawn from the contralateral jugular vein into a syringe containing citrate, (median of 8.0 min versus 14.7 min), $P = 0.0005$. K was also significantly shorter (median of 2.2 min versus 6.6 min), $P = 0.0005$, α was significantly greater (median of 61.1° versus 29.5 °), $P = 0.0005$, and MA was significantly greater (median of 55.4 mm versus 46.8 mm), $P = 0.0025$, when anticoagulation was slightly delayed (Figure 1). The differences between paired samples, reported as median (low-high), were 7.2 min (5.2-10.4) for R, 4.3 min (2.6-6.5) for K, 28.5 °

(18.3-38.6) for α, and 7.4 mm (3.5-17.2) for MA. Median values for K and α in samples with delay to anticoagulation were outside the expected intervals established for samples drawn directly into a syringe containing anticoagulant (Table 1).

Study 2 (effect of vacuum): Median α and MA were significantly greater in blood exposed to vacuum: 34.7 ° versus 29.2 ° (P = 0.0034) and 49.6 mm versus 44.1 mm (P = 0.0134), respectively. The median differences between paired samples were relatively small: 1.9 ° (-0.8-16.0) for α, and 3.1 mm (-3.2-10.6) for MA. R and K times trended toward being shorter in the blood exposed to vacuum, but neither reached statistical significance (median R of 12.7 min versus 15.3 min, P = 0.0522, and median K of 5.9 min versus 6.8 min, P = 0.1099) (Figure 2).

Study 3 (combined effect of delay to anticoagulation and vacuum): All 4 parameters were significantly different between the two collection methods. The median R was significantly shorter in the blood exposed to a delay to anticoagulation and vacuum compared to the paired samples drawn into syringes containing citrate (4.7 min versus 16.3 min), P =0.0025. The median K showed similar results with a K of 2.0 min in the delay to anticoagulation plus vacuum sample as compared to 8.2 min in the blood drawn directly into citrate (P=0.0005). The α and MA were both significantly greater in the delay to anticoagulation plus vacuum method (median α of 63.1 ° vs 26.1 °, P= 0.0005, and median MA of 52.2 mm vs 39.6 mm, P= 0.0005) (Figure 3). The differences between paired samples, reported as median (low-high), were 11.7 min (3.0-19.2) for R, 6.6 min (1.4-15.8) for K, 38.0 ° (13.8-48.9) for α, and 13.0 mm (4.4-18.0) for MA. When there was a delay to anticoagulation plus vacuum, the median values for R, K, and α were

outside the expected intervals established for samples drawn directly into a syringe containing anticoagulant (Table 1).

Intra-assay variability: CVs for each collection method were generally good (Table 2). Except for K, all CVs were less than 11%.

Intersubject variability: There was significantly less intersubject variability in R ($P=0.0003$), K ($P<0.0005$), and α ($P=0.01$) when blood exposed to delay in anticoagulation plus vacuum was compared to blood drawn directly into a syringe containing citrate. When blood was drawn with a delay to anticoagulation alone compared to blood drawn directly into citrate, there was significantly less intersubject variation in K ($P=0.02$). There were no differences in intersubject variability when blood was exposed to vacuum compared to blood not exposed to vacuum.

Discussion

Sample collection method significantly affects TEG results in clinically healthy dogs. Samples collected by vacuum or with a delay to citrate anticoagulation appeared more coagulable (shorter R and K times and greater α and MA values) than paired samples collected directly into citrate without vacuum from each dog's contralateral jugular vein.

Blood with delayed exposure to citrate anticoagulation, with or without vacuum, was more coagulable by TEG than blood drawn into a syringe containing citrate. Collection directly into tubes or syringes containing citrate has been recommended for coagulation testing in veterinary medicine.[13] However, many reports reflect the common clinical practice of collecting

blood by syringe with subsequent transfer to vacuum tubes. This is done for several reasons. In many dogs and cats, vacuum collection is limited to the jugular vein because smaller vessels may collapse from the force of the vacuum; however, blood collection from the jugular vein may not be possible due to coagulopathy, anticoagulant therapy, or preservation of the jugular veins for central venous catheter placement. Additionally, samples are often collected from catheters in order to avoid repeated venipunctures, but this makes collection directly into a vacuum tube impossible. Collection into a citrated syringe is uncommon clinically due to lack of commercially available pre-citrated syringes. This may be problematic given that vacuum and delay to anticoagulation yielded samples with greater coagulability than the samples collected with immediate contact between blood and citrate. We did not investigate the reason for this difference, but we speculate that transfer-induced trauma and delayed control of coagulation and platelet activation may initiate hemostatic pathways that promote clot formation and increased overall clot strength.

In study 2, blood collected and then exposed to vacuum was more coagulable than blood collected and not exposed to vacuum. Vacuum collection is expected to promote shear stress, and shear stress is known to cause platelet activation and result in platelet aggregation.[19,20] TEG results generated for human cardiopulmonary bypass patients have been shown to be significantly affected by collection through different intravenous catheters, and the differences in TEG tracings were considered likely due to differences in shear stress and subsequent platelet aggregation.[8] In our study, paired blood samples were exposed to similar shear stress during the collection process, but during transfer to the plastic tube, the sample transferred with vacuum had greater shear stress than the sample transferred without vacuum. Therefore, greater shear stress

leading to platelet activation and aggregation may have contributed to the more coagulable TEG tracings in the group of samples exposed to vacuum.

When sampling included both vacuum and delay to anticoagulation, a common practice in veterinary critical care units, all four TEG parameters were significantly more procoagulant and had substantially less intersubject variability than with the other collection methods. This collection method also yielded some of the lowest CV values, which may have contributed to a reduced intersubject variability but which do not explain differences in median values. The greater coagulability of these samples suggests the possibility that in vitro promotion of hemostasis by this common sample collection method may accelerate coagulation, thereby decreasing variability and masking true in vivo hemostatic abnormalities in sick patients, though this is speculative. Alternatively, although the authors have no specific support for this interpretation, collection directly into citrate may increase variations in hemostasis and yield results that are not reflective of the true hemostatic state in vivo. For example, increased variability might arise from varied forces and shear stress associated with syringe collection. However, all samples in this study were collected by one individual who made a concerted effort to apply a similar amount of gentle suction each time. Whatever the reason for the differences in results with these collection methods, there is clearly a need to interpret results using reference intervals based on the same collection method.

Similarly, the values generated by non-activated TEG for the 40 clinically healthy dogs in this study are notably more variable than those generated for healthy dogs with samples activated by tissue factor or kaolin.[10,17] Although it is conceivable that some dogs with subclinical disease were included in our population, thus broadening the intervals, it is also possible that

activators may override influences of vacuum and delayed anticoagulation, thus narrowing intervals. If activators can reduce such preanalytical variation, as has been shown and suggested to be advantageous[21] one can hypothesize that they may mask certain patient hemostatic abnormalities as well. This has been reported in dogs with Scott Syndrome and horses with acute gastrointestinal disease.[22,23]

The effects of sample collection on TEG parameters were great enough to be of diagnostic importance. Median K and α values for samples collected with delay to anticoagulation, with or without vacuum, were outside our expected intervals for clinically healthy dogs in the direction of accelerated coagulation. Similarly, the median R value for samples collected with vacuum and delayed anticoagulation was lower than the expected interval in our clinically healthy dogs. This was not related to excessive imprecision of the non-activated method, as intra-assay variations were similar to those previously reported for both kaolin and tissue factor activated TEG.[10,17] Complete blood counts, biochemical profiles, and other hemostatic data were not generated for the 40 clinically healthy dogs in this study. It is therefore possible that some of these dogs had subclinical disease and hemostatic abnormalities that could have broadened the range of generated expected values. Despite this potential, some TEG parameters were still affected enough by collection method to be considered high or low.

In contrast to most coagulation instruments, the assay temperature of the Thromboelastograph Hemostasis Analyzer is readily adjustable, thus allowing assessment of hemostasis at a patient's body temperature. Although the difference between typical human and canine body temperatures is small, all canine samples in these studies were tested at 39 °C in an

attempt to better reflect in vivo conditions. Similarly, others have used 39 °C for TEG in rabbits,[24,25] and hypothermic human patients have been assessed at their subnormal body temperatures to assess the state of hemostasis present in the patient.[26] Although there is no comparable technique or standard for comparison of TEG results such that analytical accuracy of TEG can be verified at any temperature, our canine results at 39 °C are generally similar to those of canine samples tested without activators at 37 °C.[18,27] In addition, control samples consistently yield expected results and precision is comparable to that of other reports.[10,17] Therefore, although values may differ mildly from those that would have been generated at 37 °C, the temperature was consistent across all paired samples and the differences between groups reflect differences in collection method. Similar results would be anticipated with analysis at 37 °C.

TEG reference intervals generated without regard to collection method or generated with methods different from those used for patient samples may be misleading, and hemostatic abnormalities may be masked. Similarly, inconsistent sample collection may lead to inappropriate clinical decisions based on perceived changes in TEG that actually reflected variations in sample collection. To avoid this potential problem, results should be interpreted by using reference intervals based on the specific methods used to collect and analyze the patient samples.

In conclusion, sample collection method has a significant effect on TEG. Delay to sample anticoagulation and/or exposure to vacuum yield TEG results that reflect increased sample coagulability. Collection methods should be considered when reference intervals are

established and during interpretation of TEG tracings. Specific sample collection methods should be reported for all investigations involving TEG. Future studies are needed to determine the specific factors responsible for the different TEG results associated with different sample collection methods.

Table 1: Expected values for non-activated TEG using blood drawn into syringes containing sodium citrate. Intervals are the central 95% of values for 40 apparently healthy dogs.

R	7.4 – 24.3 min
K	2.9 – 21.0 min
α	12.8 - 53.7 °
MA	29.4 - 61.5 mm

Table 2: Collection method CVs (%) Collection methods are further defined in the captions for Figures 1-3. n = 12 dogs for each collection method
* Samples for the No Delay and No Delay/No Vacuum groups were collected and processed the same way but in different studies.

Collection Method	R	K	α	MA
No Delay*	7.0	12.2	9.5	6.5
Delay	2.3	7.7	3.0	4.9
No Vacuum	5.6	9.1	8.7	5.8
Vacuum	9.4	10.9	10.3	8.4
No Delay/No Vacuum*	9.0	15.5	10.2	8.9
Delay & Vacuum	3.9	8.3	2.7	7.2

Figure 1: Box and whisker plots comparing blood drawn directly into citrate (No Delay) versus blood drawn into a plain syringe followed by gentle transfer to citrate (Delay). Box represents 25^{th} – 75^{th} interquartile range; line inside box represents median; whiskers represent high and low values. (* = $p < 0.05$, n=12)

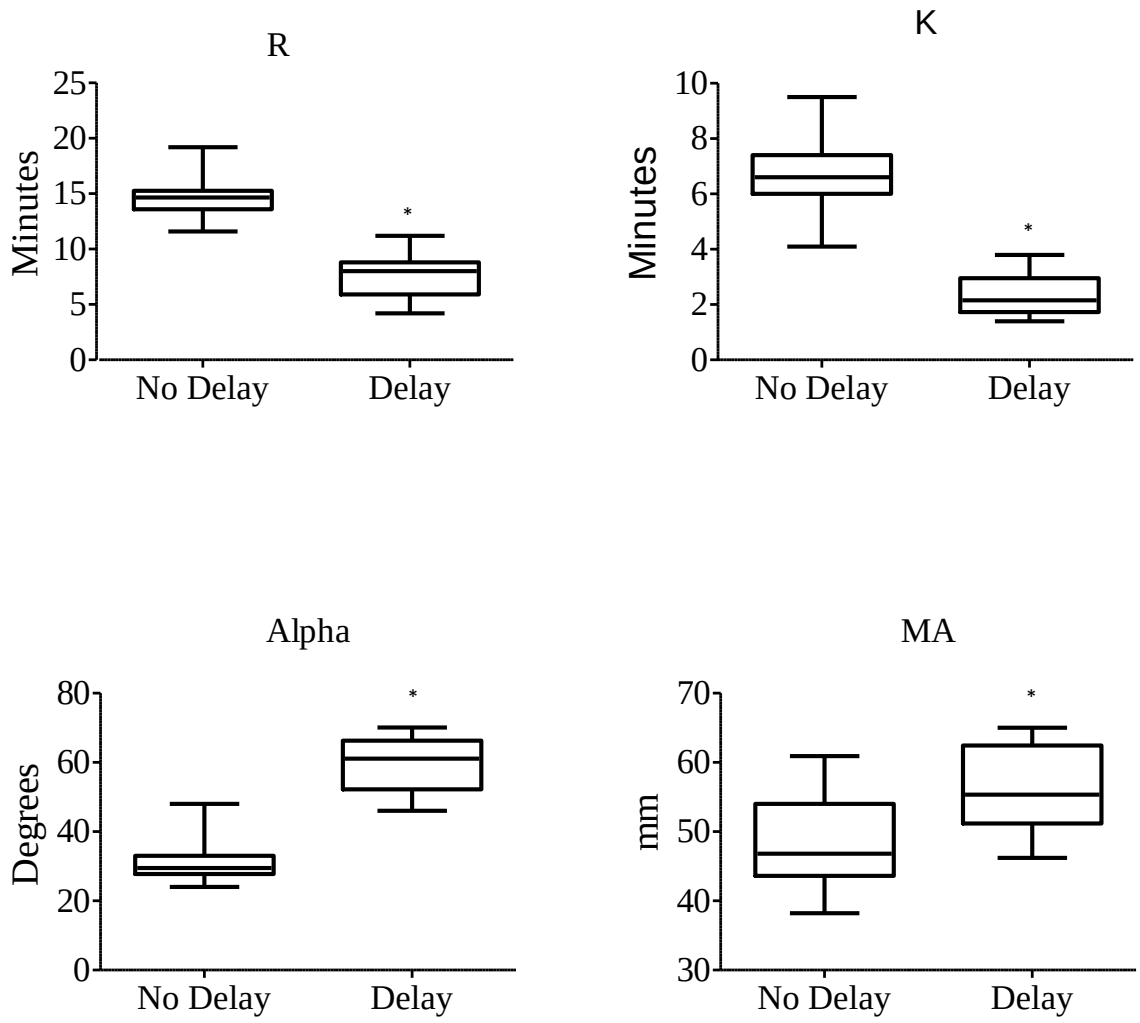


Figure 2: Box and whisker plots comparing blood drawn into citrate with gentle transfer to a plastic tube (No Vacuum) versus blood drawn into citrate with vacuum transfer to a plastic tube (Vacuum). Box represents $25^{th} - 75^{th}$ interquartile range; line inside box represents median; whiskers represent high and low values. ($* = p < 0.05$, n=12)

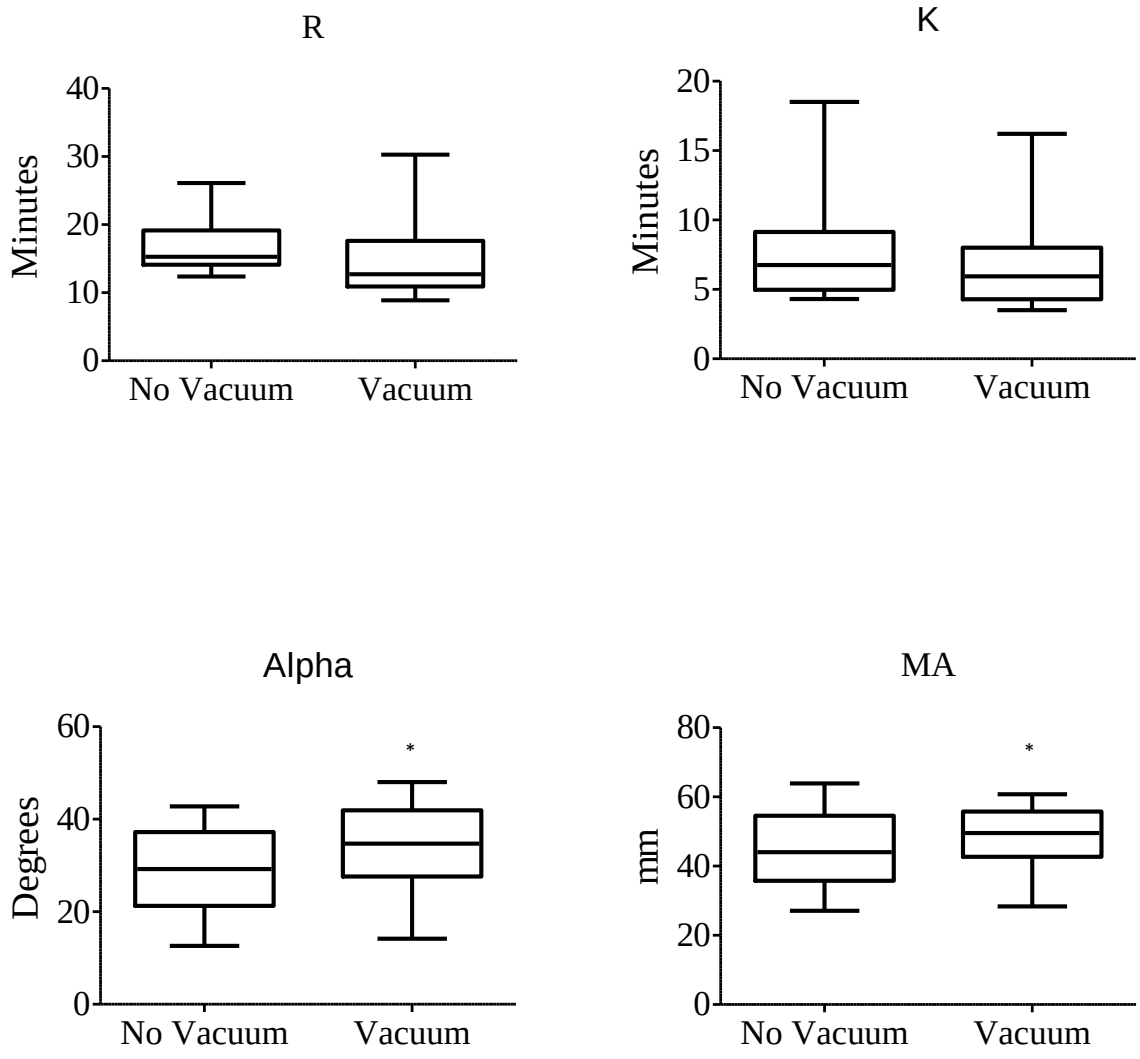
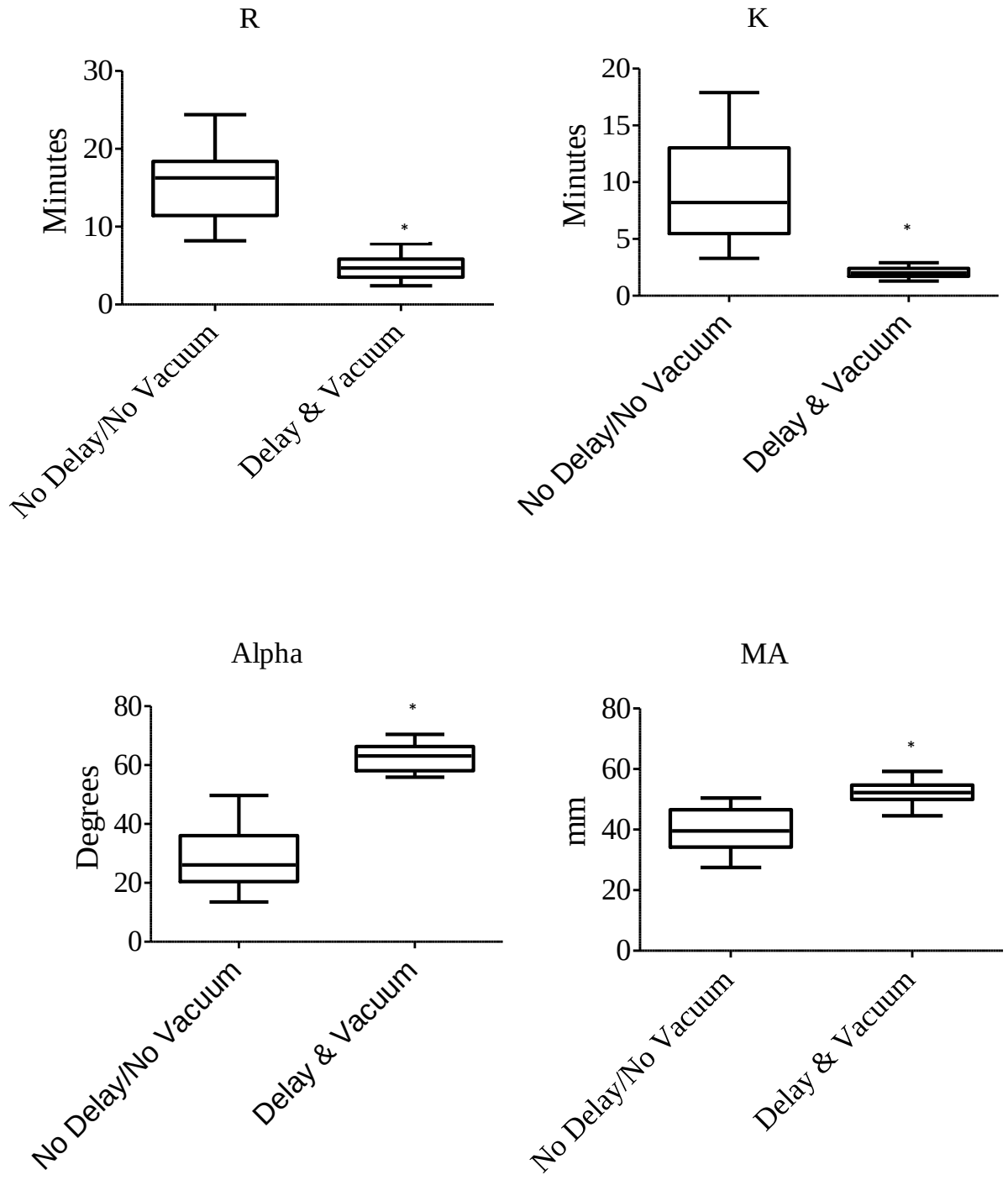


Figure 3: Box and whisker plots comparing blood drawn directly into citrate (No Delay/No Vacuum) versus blood drawn into a plain syringe followed by transfer with vacuum to citrate (Delay & Vacuum). Box represents $25^{th} - 75^{th}$ interquartile range; line inside box represents median; whiskers represent high and low values. (* = $p < 0.05$, n=12)



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CHAPTER THREE:

EVALUATION OF PLATELET ACTIVATION DURING BLOOD COLLECTION BY VARIOUS METHODS

Introduction

TEG differences seen among various sample collection methods may result from activation of platelets, coagulation factors, or a combination of the two. A delay to contact with the anticoagulant sodium citrate may result in activation of coagulation factors or platelets, as both are calcium-dependent processes. Exposure to vacuum during transfer to the vacuum tube results in blood being exposed to high sheer stress which can lead to platelet activation. Because platelets may become activated when exposed to either vacuum or a delay to contact with citrate, the role of platelet activation in the differences seen amongst various sample collection methods was investigated.

Flow cytometry allows for detection of individual cells and labeling of receptors in a complex mixture of cells such as whole blood. Flow cytometry has been used to detect activated platelets in various disease states in dogs including immune-mediated hemolytic anemia,[1,2] cardiac disease,[3] Scott syndrome,[4] and various inflammatory diseases.[5] Activation of platelets can be detected by using flow cytometry and markers for expression of P-selectin, platelet-leukocyte aggregates, and platelet microparticle formation.

P-Selectin Expression

P-selectin is a cellular adhesion molecule that is found Weibel-Palade bodies of endothelial cells and the α granules of platelets. It is also expressed on platelet-derived microparticles. Upon activation of platelets, P-selectin is redistributed to the platelet surface. On average, there are approximately 10,000 P-selectin molecules on each activated platelet.[6] Expression of P-selectin on platelets also plays a small role in platelet rolling and binding to endothelium. Additionally, P-selectin stabilizes platelet aggregates.[7] The binding of P-selectin to other platelets is thought to be a weak bond that requires platelets to be in close proximity as they are during aggregation.[8] This weak bond prevents binding of platelets to one another during flow states.

Activated platelets aid in the recruitment and margination of leukocytes through the binding of P-selectin on activated platelets to P-selectin glycoprotein ligand (PSLG-1) on most leukocytes. This binding leads to rolling and tethering of leukocytes to activated, adhered platelets.[6] The binding of leukocytes to platelets results in secretion of inflammatory mediators from both leukocytes and platelets. This interaction also results in tissue factor expression and expression of growth factors which together augment coagulation and wound repair. The interaction between P-selectin and leukocytes demonstrates the intimate relationship between the coagulation and inflammatory systems.

P-selectin expression has been extensively studied in human medicine as a marker of platelet activation and to assess thrombosis risk in various patient populations. Elevated P-

selectin has been demonstrated in prothombotic cancer patients,[9] diabetic patients,[10] in patients who are on dialysis,[11] and in many other conditions associated with a prothrombotic state. While it has been clearly documented in humans that P-selectin can be used as a marker of a prothrombotic state, the usefulness of P-selectin expression as a marker of antiplatelet therapy remains to be determined.[12]

In veterinary medicine, P-selectin has been used to evaluate platelet activation in various disease states. Thromboembolic complications are common in dogs with immune-mediated hemolytic anemia (IMHA), and two studies have shown that dogs with IMHA have increased P-selectin expression consistent with increased platelet activation.[1,2] Another study found that dogs with septic and non-septic inflammatory diseases also had increased P-selectin expression compared to controls.[5] P-selectin was also evaluated in dogs with cardiac disease and Scott syndrome, but elevated P-selectin expression was not found in either population.[3,4]

Platelet-Leukocyte Aggregrates

Compared to P-selectin expression, platelet-leukocyte aggregates are thought by some to be a more sensitive marker of human platelet activation.[13] Platelet-leukocyte aggregates form during activation of platelets primarily through binding of P-selectin to PSLG-1. Studies have shown that activated platelets can lose their P-selectin in vivo and therefore P-selectin levels may

not be elevated even though platelets have been activated.[14] Platelet-leukocyte aggregates form soon after platelet activation and persist longer in patients with platelet activation.[13]

Platelet-leukocyte aggregates have been evaluated in dogs. In one study, dogs with IMHA had fewer platelet-leukocyte aggregates than the control population.[1] This finding was unexpected because platelet-leukocyte aggregates are present at increased concentrations in human prothrombotic diseases. The authors attributed this finding to a strong correlation between platelet-leukocyte aggregates and platelet concentration.[1] Many patients with IMHA have a mild thrombocytopenia due to consumption. This, in theory, makes fewer platelets available for interaction with leukocytes. The authors suggested that the thrombocytopenia present in many of the IMHA dogs was the reason for decreased numbers of platelet-leukocyte aggregates.[1]

Platelet-Derived Microparticles

Microparticles were initially identified over 40 years ago and were described as platelet dust.[15] It is now known that microparticles can form from leukocytes, erythrocytes, endothelial cells, and platelets.[16] Platelet-derived microparticles are the most abundant type of microparticle in circulation. Microparticles can be formed following platelet activation using thrombin or collagen or during storage,[17] via apoptosis,[18,19] or with exposure to complement factors[20] or

shear stress.[21-23] Microparticles derived from platelets are thought to be primarily prothrombotic in nature. This is due in part to their anionic phospholipid membranes.

In veterinary medicine, the concentration of circulating microparticles has been shown to be elevated in dogs with IMHA.[1] In dogs with Scott syndrome, a disorder of platelets that predisposes affected dogs to bleeding, microparticle formation is impaired as it is in people with a similar syndrome.[4]

Conclusions

Flow cytometry allows for detection of activated platelets through identification of various markers including P-selectin, platelet-leukocyte aggregrates, and platelet-derived microparticles. Since these markers of platelet activation have been shown to be elevated in numerous human and veterinary diseases that are associated with a prothrombotic state, they may also be useful markers of platelet activation during the sample collection process. This is especially true when the ultimate goal is to identify hemostatic abnormalities in patients that may otherwise be masked by poor sampling technique.

While methods have been developed for detection of P-selectin, platelet-leukocyte aggregates, and platelet-derived microparticles, there are few reports evaluating platelet-leukocyte aggregates and microparticles in canine disease states. P-selectin has been shown to be elevated in dogs with prothrombotic conditions, and platelet activation during exposure to shear stress can result in P-selectin expression. Therefore we chose to evaluate P-selectin as a

marker of platelet activation in our different sample collection methods. We hypothesized that blood collection using a vacuum tube or with delays to anticoagulation result in more platelet activation than when blood is collected directly and without delay into a syringe containing sodium citrate.

Materials and Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee. For comparison of sample collection methods, blood was collected from twelve healthy dogs that were privately owned or part of a research breeding colony.

Sample Collection

Paired 2.7 mL blood samples were collected in random order (Microsoft Excel, Redmond, WA) from the right and left jugular veins of 12 dogs. One blood sample was drawn into a syringe containing 0.3 mL of 3.2% sodium citrate. The paired sample was drawn into an empty syringe and then transferred by vacuum within 5 seconds to a tube containing 0.3 mL of 3.2% sodium citrate (BD Vacutainer Plus Citrate Tubes, Franklin Lakes, NJ). All samples were collected with a 20 g one-inch needle into a 3 mL plastic syringe (BD Luer-Lok syringe with BD PrecisionGlide needle, Franklin Lakes, NJ). Samples were excluded if venipuncture was not successful on the first attempt or if any needle redirection was necessary.

Sample Preparation

Within 30 minutes of sample collection, 20 µL of citrated whole blood was diluted with 980 µL of phosphate buffered saline plus 4% bovine serum albumin (PBS), and 100 µL of this diluted whole blood was then placed in each of 4 plastic tubes (tubes 1-4). Tube 1 served as the sample tube, and 10 µL of PE-conjugated anti-CD 61 antibody (CD 61) (clone VI-PL2, BD Pharmingen, SanDiego, CA) was added to the diluted blood. Following a 15-minute incubation in the dark, 20 µL of FITC-conjugated anti-CD 62 (P-selectin) antibody (CD 62) (clone sc-19672 FITC, Santa Cruz Biotechnology, Santa Cruz, CA) was added. Tube 2 was a negative control for CD 62 non-specific binding; 10 µL of CD 61 was combined with the dilute blood followed by a 15 minute incubation period in the dark. Then 20 µL of FITC-conjugated isotype control was added (clone SC-2856, Santa Cruz Biotechnology). Tube 3 was the negative control for both CD 61 and CD 62. Dilute blood was combined with 10 µL of PE-conjugated IgG1 isotype control (clone MOPC-21, BD Pharmingen) and incubated for 15 minutes followed by addition of FITC-conjugated isotype control antibody. Tube 4 was the positive control. Platelets were activated using 10 µL of phorbol myristate acetate (PMA). After incubation for 20 minutes at 37 °C, 10 µL of CD 61 was added and incubated for 15 minutes, then 20 µL of CD 62 was added. All tubes were again incubated in the dark for 15 minutes (Figure 4). Just prior to analysis, 500 µL of FACS flow buffer was added to each tube to decrease coincidence.

Flow Cytometry

All samples were analyzed on one flow cytometer (BD FACSCalibur, BD Sciences, San Jose, CA). Prior to analysis of the samples, compensation was performed to ensure adequate separation of fluorescence events. All samples were analyzed within 30 minutes of the final antibody labeling. Platelets were gated based on known forward and side scatter properties. Proper identification of platelets was verified by gating on CD 61 positive events. Gates were set so that approximately 99% of the isotype control events were negative. A total of 10,000 CD 61-positive events were counted with simultaneous counting of CD 62-positive events. Results are expressed as the median fluorescence intensity of CD 62 events that occurred in the CD 61 positive population.

Statistical Analysis

Median fluorescence intensity for CD 62 was compared using a Wilcoxon Signed-Rank Test. $P < 0.05$ was considered significant.

Results

The median fluorescence intensity was mildly but significantly higher in the samples exposed to both vacuum and delay to anticoagulation compared to the samples that were collected directly into a syringe containing citrate (9.44 versus 9.14, respectively) (Figure 5). Additionally, when samples were stimulated with PMA, the median fluorescence intensity was

significantly higher in the samples exposed to both vacuum and delay to anticoagulation compared to the samples that were exposed to neither (38.89 versus 36.04, respectively) (Figure 6).

Discussion

In this study, there was a small but significantly higher expression of P-selectin in blood that had been exposed to both vacuum suction from the tube and a delay to contact with citrate compared to the blood that was not exposed to vacuum or a delay to contact with citrate. This difference persisted after stimulation with PMA indicating that the platelets are also more reactive.

Platelets can become activated through exposure to shear stress.[24,25] The blood that was transferred with vacuum to the tube was likely exposed to a greater shear stress than the blood that did not undergo vacuum transfer. The shear stress is likely a result of the pressure change across the syringe and tube. Additionally, platelet activation is inhibited by citrate due to the sequestration of calcium. A slight delay to contact with the anticoagulant citrate may have allowed some platelet activation in these samples.

The difference in P-selectin expression, while significantly different between the two groups, was not large. Activation of coagulation factors may play a greater role than platelet activation in the TEG differences seen between the two collection methods (Chapter 2). Alternatively, platelet activation may not have been detected well by P-selectin expression. Platelet–leukocyte aggregates may be a more sensitive marker of platelet activation in some

disease states.[13] Platelet activation could have also been evaluated using other methods such as aggregometery or the platelet function analyzer.

This study has several limitations including use of only one marker of platelet activation. Further studies are needed to fully assess platelet activation during different sample collection methods, and the role of coagulation factor activation during sample collection should be assessed.

In conclusion, platelets are more activated during blood collection when samples are exposed to both vacuum suction from the tube and a delay to contact with citrate. Due to this activation, it may be more ideal to collect blood directly into a syringe containing citrate for TEG analysis to minimize platelet exposure to vacuum suction and shear stress. Additionally, elimination of a delay to contact with citrate may also help prevent platelet activation. Further studies are needed to evaluate coagulation factor analysis during collection and to determine the optimal sample collection method for TEG.

Figure 4: Flow diagram showing sample preparation for flow cytometry. CD 61 is the antibody against glycoprotein IIIa which is expressed on all platelets and used to identify platelets. CD 62 is the antibody against P-selectin which is only expressed on activated platelets. PMA = phorbol myristate acetate.

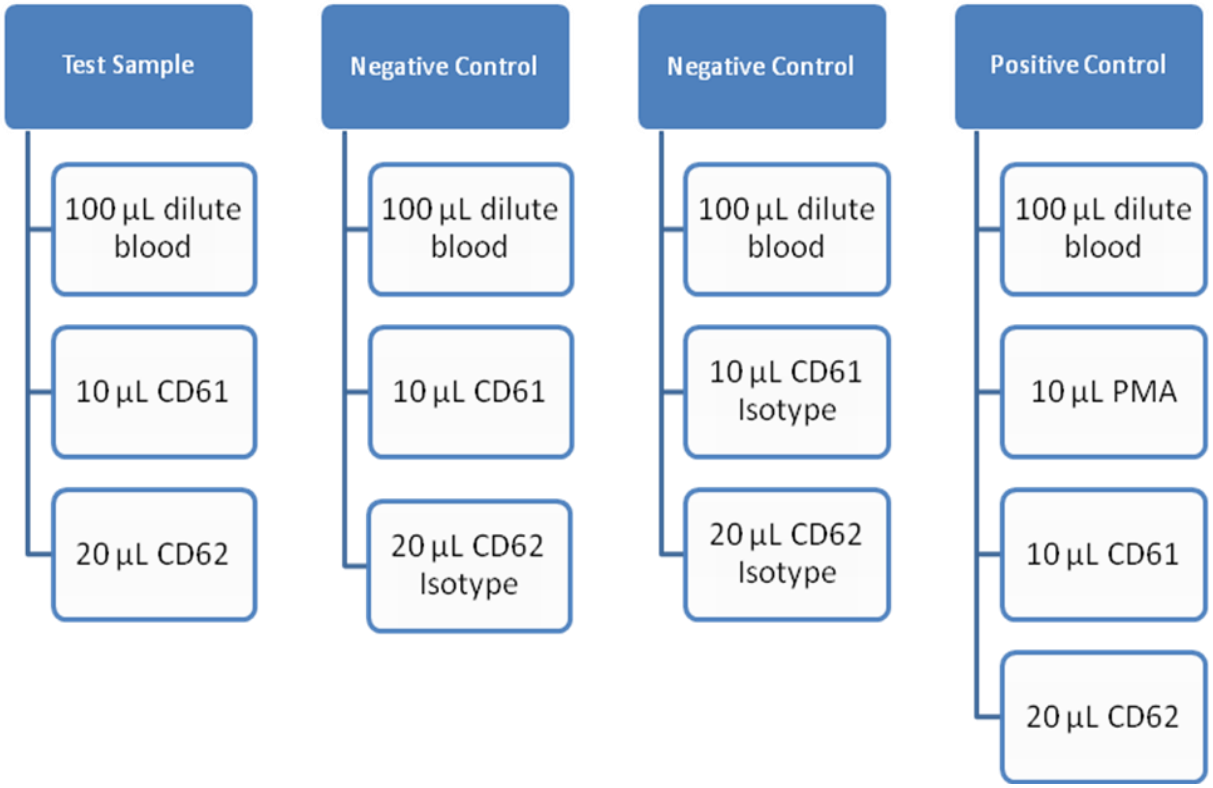


Figure 5: Box and whisker plots comparing median fluorescence intensity of blood drawn directly into citrate (No Delay/No Vacuum) versus blood drawn into a plain syringe followed by transfer with vacuum to citrate (Delay & Vacuum). Box represents $25^{th} - 75^{th}$ interquartile range; line inside box represents median; whiskers represent high and low values. ($* = p < 0.05$, n=12)

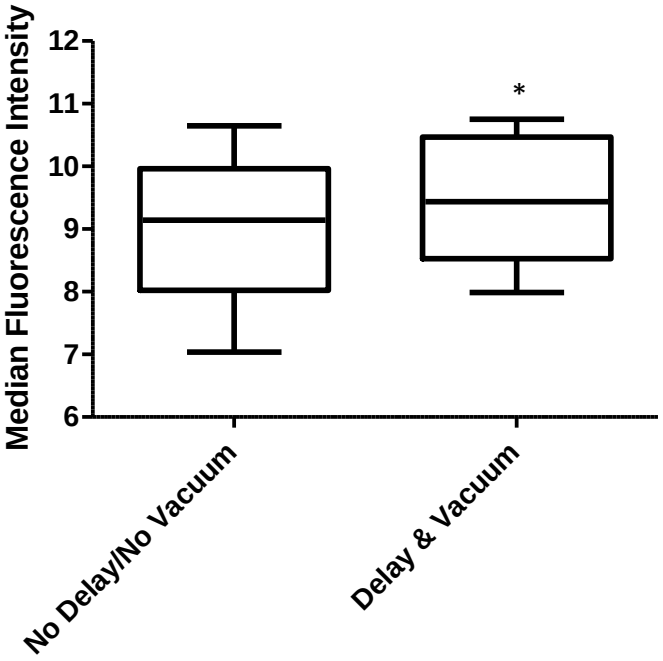
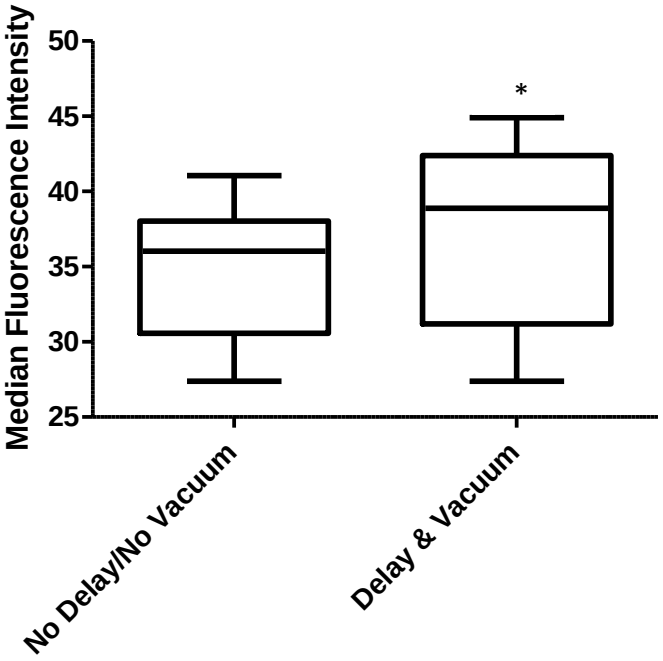


Figure 6: Box and whisker plots comparing median fluorescence intensity following activation with PMA of blood drawn directly into citrate (No Delay/No Vacuum) versus blood drawn into a plain syringe followed by transfer with vacuum to citrate (Delay & Vacuum). Box represents $25^{th} - 75^{th}$ interquartile range; line inside box represents median; whiskers represent high and low values. ($* = p < 0.05$, n=12)



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CHAPTER FOUR:

FUTURE DIRECTIONS

The two studies presented here demonstrate that sample collection methods have a significant impact on TEG results and that platelet activation is at least partially responsible for some of the difference. Further studies to assess platelet activation during sample collection, including assessment of platelet-leukocyte aggregates and formation of microparticles, are needed. Development of these assays will also allow for research in animals with clinical disease where platelet activation is thought to play a role in predisposing them to thromboembolism.

The activation of coagulation factors during sample collection should also be evaluated. Inclusion of corn trypsin inhibitor in a syringe during collection followed by transfer to citrate will help eliminate contact activation during sample collection and allow for the assessment of the degree of contact activation during a delay to contact with citrate. Additionally, by using cytochalasin D for platelet inhibition, TEG could be performed using the different sample collection methods to assess coagulation factor activation.

Furthermore, the role of activators in minimizing preanalytical variation should be explored. There is some evidence that activators may minimize preanalytical variation, but whether or not they also mask clinically relevant coagulation abnormalities needs to be determined. Evaluating coagulation in dogs with immune-mediated hemolytic anemia or sepsis using TEG with and without activators would be a first step in determining if activators mask coagulation abnormalities in clinical patients with coagulation abnormalities.

The overall future goal is to determine the optimal sample collection method for TEG that minimizes preanalytical variation while still allowing for detection of coagulation abnormalities in clinical disease.