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Original Article

Development of a fibrinolysis assay for canine plasma

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ABSTRACT

Unbalanced coagulation and fibrinolysis leads to hemorrhage or thrombosis. Thromboelastography has been used to characterize hypo- and hyper-fibrinolysis in dogs, however the technique requires specialized instrumentation and proprietary reagents that limit its availability. The aim of this study was to develop a simple microplate method for assessment of fibrinolysis in canine plasma. Plasma from healthy dogs was mixed in a microwell plate with tissue factor, calcium, phospholipid and tissue plasminogen activator. Light absorbance was measured at regular intervals until return to baseline. Peak optical density (milli-absorption units, mAU), formation velocity (mAU/s), lysis velocity (mAU/s) and area under the curve (mAU.s) were calculated. The influence of potential interferents, variation in fibrinogen and ex vivo addition of heparin and aminocaproic acid on assay performance was determined. Inter-day coefficients of variation were ≤15% for all variables. Bilirubin ≤ 1.88 mg/dL and hemoglobin ≤ 0.09 mg/dL did not interfere with assay variables. Aminocaproic acid (40 μg/mL) and heparin (0.125 U/mL) caused almost complete inhibition of fibrinolysis and coagulation, respectively. All variables except lysis velocity ($R^2 = 0.08$) were associated with fibrinogen concentration ($R^2 > 0.8$). This assay showed acceptable performance characteristics for measurement of fibrinolysis in normal canine plasma. The assay utilizes small volume citrate plasma samples and readily available instrumentation and reagents, is not influenced by mild to moderate hemolysis or icterus and detects the presence of fibrinolysis inhibitors.

Introduction

Fibrinolysis is the plasmin-mediated proteolysis of fibrin to form fibrin degradation products (Harvey, 2012). Altered fibrinolysis occurs in many human bleeding and thrombotic syndromes; for example, hyperfibrinolysis often causes fatal hemorrhage in human beings with acute promyelocytic leukemia (Breen et al., 2012), whereas hypofibrinolysis is a well-established contributory factor to venous and arterial thrombosis (Meltzer et al., 2008; Guimaraes et al., 2009; Smalberg et al., 2011). Relatively little is known about the fibrinolytic status of clinically ill dogs, since assays capable of detecting altered fibrinolysis are not widely available. However, viscoelastic assays have shown hyperfibrinolysis occurs in dogs with bleeding tendencies, e.g. the hemorrhagic form of disseminated intravascular coagulation (DIC; Vilar-Saavedra and Hosoya, 2011). Hypofibrinolysis in dogs at risk of thrombosis has also been detected using a modified viscoelastic assay configured with tissue plasminogen activator (tPA) to induce conversion of endogenous plasminogen to plasmin (Spodsberg et al., 2013).

Turbidimetry has been widely used as a research technique to monitor plasma clot formation and lysis by changes in light scatter as fibrin fibrils form, aggregate and are subsequently degraded (Carr and Hermans, 1978; Hantgan and Hermans, 1979; Weisel and Nagaswami, 1992; van Geffen and van Heerde, 2012; Zucker et al., 2014). Turbidity is logarithmically related to light absorbance (Carr and Hermans, 1978). Therefore, commonly available microplate readers can be used to measure changes in the light absorbance of plasma, reflecting clot formation and break down (Wolberg et al., 2002). With modifications, light absorbance assays have the potential for broader application to assess fibrinolysis in veterinary species.

In contrast to viscoelastic techniques, microplate methods do not require expensive equipment or proprietary reagents. Furthermore, plasma-based assays are unlikely to be affected by fluctuating hematocrits or platelet counts, which complicate the interpretation of whole blood viscoelastic assays in anemic or thrombocytopenic animals (Brooks et al., 2014). In the current

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study, we set out to develop a light absorbance assay to monitor clot formation and lysis in canine plasma.

Methods

Study population

Plasma from staff and student owned dogs was used for assay development. Health of donor dogs was confirmed by history, physical examination, urinalysis, biochemistry and hematology. No dogs were receiving any medications other than routine anti-parasite prophylaxis. All samples were collected from 1 February 2014 to 31 August 2015 with owner consent and Iowa State University Institutional Animal Care and Use Committee approval (protocol number 12-13-7687-K; date of approval 19 December 2013).

Sample collection

A 21 G polypropylene butterfly catheter (Surflo Winged Infusion Set, Terumo) was inserted into a cephalic, jugular or saphenous vein and, after collection of a serum sample or allowing a few drops of blood to fall, blood was drawn into a syringe containing 3.8% sodium citrate, pH 8 (final citrate concentration 0.38%). Blood was immediately transferred to a polypropylene tube and mixed by gentle inversion. Within 30 min, samples were centrifuged at 2500 *g* for 20 min with no brake. The resulting platelet poor plasma was stored at −80 °C until analysed.

Fibrinolysis assay

Procedure

Plasma was thawed in a 37 °C water bath for 10 min prior to analysis. Plasma (75 μL) was mixed in a 96-well plate (Thermo Scientific) by pipetting with 5 μL of 1:100 rabbit brain thromboplastin (Pacific Hemostasis Thromboplastin D, Fisher Diagnostics), 20 μL phospholipid (final concentration 4 μM, MP Reagent, Diagnostica Stago), 10 μL tPA (final concentration 800 ng/mL, Cathflo Activase, Genentech) and 90 μL calcium chloride (final concentration 9 mM, Sigma–Aldrich). Dilutions were performed using 2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) saline (10 mM HEPES, 150 mM sodium chloride, pH 7.5; Sigma–Aldrich). Each sample was assayed in duplicate. Reagent concentrations were optimized based on titration experiments (see Appendix: Supplementary Fig. 1).

Immediately after mixing, optical density (OD) at 390 nm was measured every 8 s for 1 h by a multi-detection microplate reader (Synergy HT, BioTek Instruments). Wavelength was selected to maximize peak height in interference free samples (see Appendix: Supplementary Fig. 2).

In preliminary experiments, the observed peak OD for canine plasma samples were considerably lower than previously reported values for human plasma in similar assay configurations. To investigate this difference, pooled normal human plasma (George King Biomedical) was assayed as described for canine plasma. The peak OD for the human plasma was higher than for canine plasma and comparable to previous human reports (He et al., 2001; Goldenberg et al., 2005). Since these findings were consistent with a species difference rather than a failure of assay performance, no further modifications were made to the canine assay (see Appendix: Supplementary Fig. 3).

Assay variables

Assay variables were modified from a previous report (Zucker et al., 2014). Baseline was defined as the lowest value in the first 80 s of data collection and inflection point as the time at which absorbance increased from baseline by 10 milli-absorption units (mAU). Clot formation was assessed by the variables time to inflection (s), peak optical density (peak OD, mAU), clot formation velocity (FV, mAU/s), calculated by (Peak OD − 10 mAU)/(Time from inflection to peak), and area under the curve (AUC, mAU.s). Clot lysis was assessed by lysis velocity (LV, mAU/s) calculated by (Peak OD/2)/(Time from peak to 50% lysis) (Fig. 1).

Assay validation

Assessment of imprecision

Intra-day coefficient of variation (%) was calculated for aliquots of pooled normal dog plasma assayed six times (in duplicate) over three runs on a single day and duplicate determinations were performed 20 times in 10 runs over 4 days for calculation of the inter-day coefficient of variation. Due to the lack of an established gold standard for recognition of altered fibrinolysis, plasma was not available from dogs with known hyper- or hypo-fibrinolysis for assessment of coefficient variation for samples with abnormal fibrinolysis.

Assessment of interference

For assessment of interference from hemolysis, a hemolysate was prepared by subjecting washed canine erythrocytes to osmotic

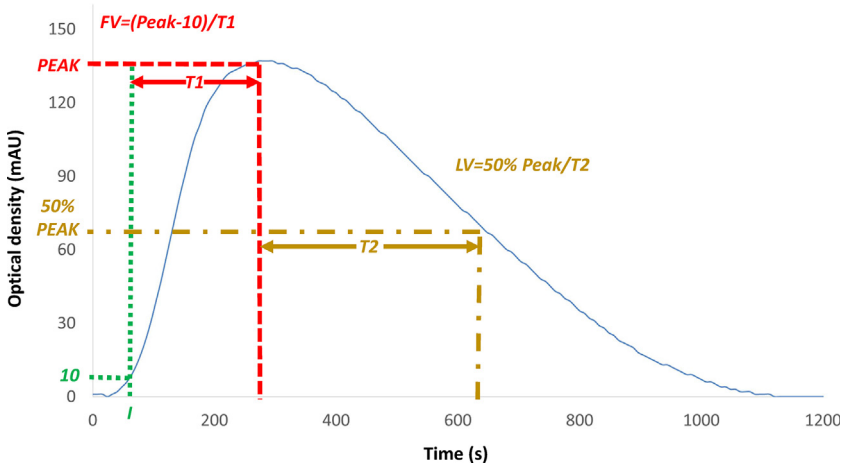

Fig. 1. Clot lysis parameters. FV, formation velocity; LV, lysis velocity; I, inflection time; mAU, milli-arbitrary units; T1, time from inflection to peak; T2, time from peak to 50% lysis.