Thromboelastometric evaluation of hemostasis in dogs infected with Leishmania infantum

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Abstract

Objective – Evaluation of hemostasis via thromboelastometry in dogs with leishmaniasis before and after treatment.

Design – Longitudinal observational study.

Setting – University Veterinary Teaching Hospital.

Animals – Eighty-four adult, client-owned dogs.

Measurements and Main Results –Whole blood samples for the coagulation profile were collected from symptomatic dogs with leishmaniasis (group S), asymptomatic dogs with leishmaniasis after treatment (group T), and a control group of healthy dogs (group H). Hemostasis was evaluated by means of standard coagulation profile (PT, aPTT and fibrinogen) and by thromboelastometry (ROTEM). PT and aPTT were within the upper reference range in all three groups. Comparison of the ROTEM variables between the three groups showed statistically significant differences between group S versus groups T and H, but remaining within the reference ranges. Statistically significant differences in hematocrit and fibrinogen concentrations were noted between groups (Group S vs. H: hematocrit P=0.001, fibrinogen P=0.002; Group S vs. T: hematocrit P=0.001, fibrinogen P=0.001). These variations have interfered with some parameters of the ROTEM profile.

Conclusions – This study showed normal standard coagulation profiles in all three groups. The ROTEM results did not fall outside of the maximum values of the reference ranges.

Key words: small animal, hemostasis, leishmaniasis, thromboelastometry.

aPTT activated partial thromboplastin time

CFT clot formation time

CT clotting time

Group H control group

Group S symptomatic dogs before treatment

Group T asymptomatic dogs after treatment

MCF maximum clot firmness

PT prothrombin time

TEG thromboelastography

TEM thromboelastometry

Introduction

Leishmaniasis is an infective zoonosis caused by a protozoan of the genus *Leishmania*. It is endemic to the Mediterranean basin and the dog is the primary reservoir of infection. A chronic disease with many and different clinical signs, canine leishmaniasis can stimulate the overproduction of antibody-forming immune complexes.[1,2] Their subsequent deposition in different tissues ensues in skin and ocular lesions, renal failure and vasculitis, and inflammatory cytokine production. [3-7] Other clinical conditions known to be associated with leishmaniasis, such as epistaxis, hematuria, thromboembolism and disseminated intravascular coagulation (DIC), are related to alterations in primary and secondary hemostasis.[8-11]

Several studies have signaled thrombocytopenia and a decrease in platelet function in canine leishmaniasis, [12-17] whereas investigation of secondary hemostasis and fibrinolysis has given discordant results. Indeed, some studies reported hypocoagulability due to an increase in activated partial thromboplastin time (aPTT), while others described compensated disseminated intravascular coagulation (DIC) due to an increase in fibrinogen degradation product (FDP) with normal fibrinogen levels. [13,14,18]

To the best of our knowledge, only three studies to date have evaluated the effects of treatment on hemostasis in dogs with leishmaniasis. Valladares and others (1998) evaluated the effects of therapy

with meglumine antimoniate on platelet, prothrombin time (PT), aPTT, fibrinogen and FDP in experimentally infected beagles. Alterations shown before treatment were a decrease in platelet count and aggregation and an increase of FDP. After treatment, platelet function an number normalized, while FDP decreased but then returned to within the normal range. [13]

Cortese and others (2008) studied the effects of prednisone on standard coagulation profile (PT, APTT and fibrinogen) and platelet aggregation in dogs treated with meglumine antimoniate and allopurinol. The dogs showed a decrease in platelet aggregation before treatment. A significant improvement in platelet aggregation was detected after treatment but the values remained lower than in the control group at the end of the study.[19] A later study by Cortese (2009), assessed hemostasis before and after treatment in dogs with *Leishmania infantum* or *Ehrlichia canis* or both. The dogs with single infection (leishmaniosis or ehrlichiosis) showed a decrease in platelet number and function that improved after treatment but did return to normal. In the dogs with double infection, platelet aggregation after treatment was still significantly lower than that in the healthy dogs. [20]

Previous studies have assessed hemostasis by means technologies that evaluate single steps of coagulation; hemostasis, however, is a dynamic process that involves plasmatic factors and cells (cell-based model of coagulation). [21] Viscoelastic techniques are recent methods for evaluating hemostasis. They rely on the use of whole blood to obtain a description of coagulation which more likely reflects in vivo hemostatic processes. Recently, thromboelastometry/thromboelastography have been used to identify hypocoagulability and hypercoagulability in dogs. [22-26]

Thromboelastometry measures clot formation kinetics, clot firmness and rate of dissolution (fibrinolysis); the results of the analysis are presented graphically.[27,28] For each sample, ROTEM generates different profiles, including: the in-TEM profile for the intrinsic pathway; the ex-TEM profile for the extrinsic pathway; and the fib-TEM profile correlated to functional fibrinogen levels. Hemostasis is assessed using whole blood, taking into account both the plasma and cellular components.

79 The first aim of this study was to evaluate hemostasis by means of a standard coagulation profile
80 and thromboelastometry (ROTEM) in dogs with untreated leishmaniasis. The second aim was to
81 determine whether the hemostatic alterations regressed in treated dogs. Our hypothesis was that in
82 symptomatic dogs the infection affects hemostasis and that the alterations regress after therapy.
83
84 **MATERIALS AND METHODS**
85 **Animals**
86 The study protocol was approved by the Bioethical and Animal Welfare Committee of our
87 institution. The dog owners were informed about the study protocol and gave their written consent
88 for participation in the study.
89 All dogs were patients admitted to the Veterinary Teaching Hospital because of suspected infection
90 of *Leishmania infantum* based on clinical signs. Physical examination, complete blood count [a], and
91 biochemical evaluation [b], serum protein electrophoresis, urinalysis [reactive strips [c] and sediment
92 analysis], protein/creatinine ratio, serological tests for *Leishmania infantum* [immunofluorescence
93 antibody test (IFAT) performed at the Istituto Zooprofilattico Sperimentale of Piemonte, Liguria
94 and Valle d'Aosta laboratory], and serological tests for *Ehrlichia canis* [d], *Borrelia burgdorferi* [d],
95 *Anaplasma phagocytophilum* [d] and *Dirofilaria immitis* [d] were performed. To establish the
96 diagnosis of leishmaniasis, cytology and/or PCR of lymph node samples obtained by fine needle
97 aspiration (PCR performed at the Istituto Zooprofilattico Sperimentale of Piemonte, Liguria and
98 Valle d'Aosta laboratory) were carried out. Dogs positive for *Leishmania infantum* on the basis of
99 cytology or PCR of lymph node aspirate were considered to be affected by leishmaniosis if they
100 presented clinical signs and laboratory tests suggestive of infection (Table 1). These dogs were
101 divided in two groups: group S (symptomatic dogs before treatment) included sick dogs with
102 clinical signs of *Leishmania* (stage C according to the guidelines);[29] group T (asymptomatic dogs
103 after treatment) included dogs treated with meglumine antimoniate and allopurinol or miltefosine
104 and allopurinol [according to the guidelines (Table 2)][30] and had completed therapy at least 2

105 months prior to enrolment in this study, did not present clinical signs compatible with
106 leishmaniasis, and had an albumin/globulin ratio (A/G) > 0.6.
107 The control group (group H) included dogs admitted to the Veterinary Teaching Hospital for routine
108 hematological control before spaying/castration or blood donation. The dogs were deemed healthy
109 on the basis of complete history and physical examination, CBC and biochemical analysis,
110 coagulation profile and not exposed or infected by *Leishmania* (serological tests [d], IFAT, cytology
111 and/or PCR analysis of lymph node).
112 Exclusion criteria were a positive result for *Ehrlichia canis*, *Borrelia burgdorferi*, *Dirofilaria*
113 *immitis* and *Anaplasma phagocytophilum*, and the administration of non-steroidal anti-
114 inflammatory drugs, corticosteroid or transfusion during the 2 months prior to enrolment in the
115 study. At enrolment, prophylactic treatment for sand fly was recommended.
116
117 **Hemostasis**
118 Samples of whole blood for the coagulation profile were collected by jugular venipuncture (20-
119 gauge needle) and placed into two tubes containing 3.2% trisodium citrate (1 part citrate: 9 parts
120 blood) [e]. Samples that were difficult to obtain (e.g., repeated venipuncture attempts, needle
121 repositioning or interruption of blood flow into the tube) were discarded and blood draws were
122 made from the contralateral jugular vein.
123 Secondary hemostasis was evaluated by means of standard plasma based assays (PT, aPTT and
124 fibrinogen)[f]. For thromboelastrometry with the ROTEM [g], whole blood samples were stored at 37
125 °C in 3.2% trisodium citrate tubes and analyzed 30 minutes after collection according to the
126 manufacturer's instructions; the analyses were run for 60 minutes. For each sample, in-TEM, ex-
127 TEM and fib-TEM profiles were performed to evaluate the intrinsic pathway (with activation by
128 ellagic acid) [h], the extrinsic pathway (with tissue factor activation) [i], and fibrinogen function
129 (platelets inactivated with cytochalasin D) [l], respectively. The following parameters were assessed
130 for each profile: clotting time ([CT], s); clot formation time ([CFT], s); maximum clot firmness

([MCF], mm); α angle (α, °); profiles are presented as reaction curves (Fig. 1). CT represents the first phase of fibrin formation, from activation of the test to a clot amplitude of 2 mm; this parameter is mainly affected by the concentration of plasma coagulation factors and coagulation inhibitors (e.g., antithrombin or drugs).[26,27] CFT expresses the velocity of clot formation and is affected predominantly by platelet count and function and by fibrinogen activity. MCF, the maximum firmness the clot reaches, is determined by both platelet count and function and fibrin formation in the presence of factor XIII.[26,27] The α angle corresponds to the slope of the tangent on the elasticity curve; it describes the kinetics of clot formation and is affected predominantly by platelet count and function and fibrinogen.[26,27]

Abnormal ROTEM tracings were defined as ROTEM results outside of the maximum or minimum values of our reference ranges (Table 3), hypercoagulable tracings when there was a decrease in CT and CFT and an increase in MCF, α angle or a combination thereof, and hypocoagulable tracings when there was an increase in CT and CFT and a decrease in MCF, α angle or a combination thereof.

Statistical analysis

The data were entered into an ad hoc database [n]. All coagulation variables are continuous values and were checked for normality by means of a test for normality based on skewness and another test based on kurtosis; the two tests were then combined into an overall test statistic.

To test the differences between the three groups (H, S, T), the continuous outcome variables were analyzed with ANOVA if they resulted normally distributed and were compounded by the Bonferroni correction to identify the ties of any differences, otherwise the Kruskall-Wallis test was performed. The two sample Mann-Whitney test was used to identify where the differences lay, as suggested by Altman (1991).[31] Variables such as age, platelet, fibrinogen, hematocrit, protein/creatinine ratio and creatinine were also compared.

Forward stepwise linear regression analysis was applied to the logarithmic transformation of the variables to determine whether any single ROTEM parameter correlated with the blood variables and resulted significantly different in the three groups. The significance level for addition to the model was fixed; terms with $P<0.2$ were eligible for addition. Significance was set at $P<0.05$.

Results

One hundred dogs were enrolled in the study: 45 in group S, 34 in group T and 31 in group H. Sixteen dogs in group S were excluded (infected but without clinical signs of leishmaniasis [n=5], neoplasia [n=3], positive for *Dirofilaria immitis* [n=4], and positive for *Ehrlichia canis* [n=4]) and 10 in group T (neoplasia [n=2] and A/G ≤0.6 [n=8]). Twenty-nine dogs were included in group S (Figure 2): 19 males (15 intact and 4 neutered) and 10 females (4 entire and 6 spayed), age range 1-14 years (mean age 6.1 ± 3.5; body weight 20.4 ± 13.3 kg). Twenty-four dogs were included in group T (Figure 3): 15 males (12 intact and 3 neutered) and 9 females (6 intact and 3 spayed), age range 2-14 years (mean age 5.8 ± 3.5; body weight 18.3 ± 3.5 kg). Finally 31 dogs were included in group H (Figure 4): 16 males (15 intact and 1 neutered) and 15 females (8 intact and 7 spayed), age range 1-18 years (mean age 5.6 ± 3.7; body weight 23.5 ± 8 kg).

In group S 20/ 29 dogs (69%) were anemic [median 32.9%, min 20, max 42], 2/ 29 (7%) were thrombocytopenic [median $261x10^3$/µL ($261x10^9$/L), min 107, max 670], 2/29 (7%) had a creatinine level outside of the upper reference range [median 0.82 mg/dL (7.,5 µmol/L), min 0.47, max 3.5], none had transaminase levels outside of the reference range, 20/ 29 (69%) had proteinuria [median 0.88, min 0.034, max 9.1], none had PT or aPTT outside of the upper reference range, and 13/29 (45%) had fibrinogen levels outside of the upper reference range [median 424 mg/dL (12.5 µmol/L), min 180, max 1094]. In group T 4/ 24 (17%) dogs were anemic [median 43.3%, min 31.6, max 56.4], none were thrombocytopenic, had creatinine levels outside of the upper reference range, or transaminase levels outside of the reference range, 2/24 (8%) had proteinuria [median 0.17, min

0.02, max 4.54], none had PT or aPTT outside of the upper reference range, and 1/24 (4%) had fibrinogen level outside of the upper reference range [median 273 mg/dL (8 µmol/L), min 150, max 926]. None of the dogs in group H presented alterations in CBC, biochemical profile, urinalysis, protein/creatinine ratio, serum protein electrophoresis or standard coagulation profile.

There was no significant difference in the mean age between the three groups.

The ROTEM results and their comparison are represented as box plots in Figure 5. Comparison of the thromboelastometric variables between groups S and H showed a significant decrease in CT on the ex-TEM profile (P=0.0002) and a significant decrease in CFT on the in-TEM (P=0.0002) and ex-TEM (P=0.00001) profiles for the symptomatic (group S) dogs; furthermore, there was an increase in MCF (in-TEM P=0.0116; ex-TEM P=0.0146; fib-TEM P=0.00001) and α angle (in-TEM P=0.0001; ex-TEM P=0.00001; fib-TEM P=0.001) on all profiles. Comparison of the ROTEM values between group S and group T revealed a significant decrease in CFT on the in-TEM (P=0.0064) and ex-TEM (P=0.00363) profiles and a significant increase in MCF on the in-TEM (P=0.00352) and fib-TEM (P=0.0009) profiles for group S; furthermore, there was a significant increase in α angle on the in-TEM (P=0.0179) and ex-TEM (P=0.0074) profiles. Finally, comparison between groups T and H showed no significant differences, except for a significant decrease in CT on the ex-TEM profile (P=0.0001) and a significant increase in MCF (P=0.00372) and α angle (P=0.0259) on the fib-TEM profile for group T.

Variables such as age, creatinine, protein/creatinine ratio, platelet, hematocrit and fibrinogen were compared. Some statistically significant differences emerged for hematocrit and fibrinogen concentration (Table 4).

Table 5 reports the results of the forward stepwise linear regression; the data were adjusted for group of belonging. Plasma fibrinogen concentration was significantly associated with all parameters of the ex-TEM profile, with CFT, MCF and α angle of the in-TEM profile, and with CT and MCF on the fib-TEM profile. The variations in hematocrit interfered with α angle on the ex-

TEM profile. Platelet concentration, though not significantly different in the three groups, was associated with MCF on both the ex-TEM and in-TEM profiles and with CFT on the in-TEM profile.

Discussion

This study evaluated hemostasis in dogs with *Leishmania infantum* and assessed whether the hemostatic differences regressed in the treated dogs.

Leishmaniasis is known to stimulate overproduction of antibodies, with nearly no cellular response, forming abundant circulating immune complexes that can deposit in various different tissue and cause inflammation and vasculitis. [3,32,33] A recent study compared severe visceral leishmaniasis with a systemic inflammatory response syndrome.[34] The cell-based model of coagulation allowed to identify a two-way interaction between hemostasis and inflammation; indeed, some prothrombotic activated factors, such as FXIIa, activate neutrophils and complement, stimulate monocytes and the release of inflammatory cytokines, and thrombin has a chemotactic action on neutrophils and macrophages.[35] The inflammation that attends the coagulation process alters the cell membrane (the surfaces develop a procoagulant activity), leading to the production of inflammatory cytokines, the expression of tissue factor and a decrease in the production of antithrombin and FXII in the acute phase.[36]

The standard coagulation profiles were normal in all three groups in our study. Previous studies reported normal PT and aPTT in Beagle dogs with experimentally induced leishmaniasis and in naturally infected dogs.[13,19,20] In contrast, an increase in aPTT was described in dogs with leishmaniasis that showed an increase in alanine aminotransferase, and prolongation of PT and aPTT was noted in a dog with DIC and nephrotic syndrome caused by leishmaniasis.[9,14,18] From these studies it follows that altered standard coagulation profiles were described in dogs with leishmaniasis and concomitant organ damage or dysfunction.

In contrast with previous studies reporting normal fibrinogen levels, we noted an increase in fibrinogen concentration in some group S dogs.[13,18-20] The body responds to injury and infectious agents with a complex series of events that activate the inflammatory response.[37] The increase in fibrinogen could reflect the inflammatory state related to this disease, and the discrepant results obtained in these studies might be due to differences in the populations investigated.

The ROTEM results in the group S dogs showed some statistically significant differences as compared to those of the groups T and H but none of the results fell outside of the maximum values of the reference ranges.

The dogs in group S showed abnormalities on blood and urine testing typical of dogs with leishmaniasis. Alterations of some hematological or biochemical parameters are known to influence the results of ROTEM/thromboelastography (TEG) analysis. [38]

A recent study found a correlation between haematocrit, platelet count, plasma coagulation factors and ROTEM results in canine whole blood samples.[38] Smith et al. (2012) reported that hematocrit was correlated with all parameters on the in-TEM and ex-TEM profiles, except for CT on the in-TEM profile.[38,39] Decreased hematocrit, despite the presence of red cell mass within the reference ranges, can cause a relatively hypercoagulable trend of ROTEM variables. Furthermore, some studies reported a correlation between platelet concentration and CFT, α angle and MCF on in-TEM and ex-TEM profiles, as well as a significant association between the thromboelastographic (TEG) values of global clot strength (G) and platelet count.[38,40] Additionalle, these studies showed that a reduction in platelet count corresponds to a hypocoagulable tendency in ROTEM and TEG variables. [38,40] In contrast, a recent study reported a relative hypercoagulable trend in thrombocytopenic dogs with uncomplicated babesiosis.[41]

Also, plasma fibrinogen concentration has been significantly correlated with CFT, α angle and MCF, regardless of the activator used.[38] An increase in fibrinogen concentration causes a hypercoagulable tendency, whereas a decrease leads to a hypocoagulable tendency in ROTEM variables.[42]

Other factors that influence ROTEM results are proteinuria and an increase in azotemia. A recent study identified hypercoagulability by means of TEG in 7 dogs with renal failure and in 11 dogs with severe proteinuria.[43]

In view of these findings, we applied forward stepwise linear regression to determine the influence these factors had on the ROTEM results. Factors such as protein/creatinine ratio, creatinine and platelet count did not differ between the three groups and did not affect the ROTEM variables on comparison among the three groups. However, platelet count did seem to influence ROTEM variables on intragroup comparison, being directly correlated with MCF on the ex-TEM and in-TEM profiles, and inversely correlated with CFT on the in-TEM profile. The ROTEM variables most affected by platelet count and function were CFT, MCF and α angle which, during a decrease in platelet function, could show a hypocoagulable trend. Although thrombocytopathy is reported in dogs with *Leishmania*, this condition was not found in this study.[14,16] These conditions might not have been detected for two reasons. First, ROTEM is not the most sensitive tool to assess platelet function, and second, the inflammatory condition of these dogs could have activated the coagulation process, by tissue factor-mediated thrombin generation, downregulation of physiological anticoagulant mechanisms and inhibition of fibrinolysis.[44] This hemostatic activation might have balanced the effect of a decrease in platelet function, precluding the detection of a hypocoagulable trend.

Hematocrit and fibrinogen concentrations differed across all three groups. However, among group comparison, hematocrit was inversely correlated only with α angle on the ex-TEM profile, whereas fibrinogen concentration was correlated with CFT, MCF and α angle on the in-TEM profile, with all parameters on the ex-TEM profile, and with CT and MCF on the fib-TEM profile. Then, statistically significant differences between groups S versus H and T were due in part to the increase in fibrinogen, minimally to the decrease in hematocrit,: thus, *Leishmania* infection and inflammation may play a role in activating the coagulation system. [36,44]

Comparison between groups H and T showed few statistically significant differences, limited to a decrease in CT on the ex-TEM profile and an increase in MCF and α angle on the fib-TEM profile. Since there was no difference in fibrinogen concentration between groups H and T, the persistence of infection and some degree of inflammation might explain the alterations of ROTEM variables.

The present study has several limitations. First, the small number of dogs enrolled limits generalization of the results. Second, we did not investigate of inflammatory proteins and mechanisms of inflammation-induced coagulation and fibrinolysis activation (e.g., protein C, protein S, antithrombin, tissue factor patway inhibitor and plasminogen activator inhibitor type 1). Third, because the study was conducted in a reference hospital, it was not possible to follow up the same symptomatic dogs over the course of their treatment. Such a study design (paired data) could have allowed us to obtain stronger and more generalizable results. Finally, none of the symptomatic dogs in our population presented important organ injury/failure related to leishmaniasis, which might explain the few hemostasis alterations identified.

In conclusion, this study showed normal standard coagulation profiles in all three groups. Comparison of the ROTEM variables showed statistically significant differences between group S versus groups T and H, while remaining within the reference ranges. These differences could have partially due to an increase in fibrinogen concentration and minimally to a decrease in hematocrit. In group T most of the hematological, biochemical and urinalysis parameters returned to within the normal ranges and only a few ROTEM variables differed from those of group H. The ROTEM results did not fall outside of the maximum values of the reference ranges and the clinical relevance of this finding is questionable.

Footnotes

[a] ADVIA 120 Hematology, Siemens Healthcare Diagnostics, Tarrytown, NY, USA.

[b] ILAB 300 plus, Clinical Chemistry System, Instrumentation Laboratories, Milan, Italy.

[c] Multistix 10 SG Reagent Strips, Siemens Healthcare Diagnostics, Tarrytown, NY, USA.

[d] Snap 4 Dx, IDEXX Laboratories, Westbrook, ME, USA.

[e] Venosafe 3.8% buffered sodium citrated, Terumo, Leuven, Belgium.

[f] Coagulometer StART, Diagnostica Stago, New York, USA.

[g] ROTEM, Tem International GmbH, Munich, Germany.

[h] Star-TEM 10 (0.2 mol/l CaCl2 in HEPES buffer pH 7.4 and 0.1% sodium azide in glass vials), Tem International GmbH, Munich, Germany.

[i] In-TEM (partial thromboplastin phospholipid made of rabbit brain (chloroform extract), ellagic acid, buffer, preservatives in small glass vials), Tem International GmbH, Munich, Germany.

[l] Ex-TEM (recombinant tissue factor and phospholipids, heparin inhibitor, preservatives and buffer in small glass vials), Tem International GmbH, Munich, Germany.

[m] Fib-TEM (Cytochalasin D / DMSO solution 0.2 mol/l CaCl2 in HEPES buffer pH 7.4, preservative in glass vials), Tem International GmbH, Munich, Germany.

[n] Stata Statistical Software: Release 11. StataCorp LP, College Station, TX, USA.

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Table 1: Guidelines for diagnosis and clinical classification of leishmaniasis in dogs.[29,30]

Stage of leishmaniasis	Features
A: Exposed	Includes dogs with negative cytologic, histologic, parasitological and molecular findings and low antibody titers against *Leishmania*. Dogs are clinically normal or have signs associated with other diseases.
B: Infected	Includes dogs in which parasites have been detected through direct diagnostic methods (eg. microscopic evauation, organism culture, or PCR assay) and with low antibody titers against *Leishmania*. Dogs are clinically normal or have signs associated with other diseases.
C: Sick (clinically evident disease)	Includes dogs with positive cytologic results regardless of serologic results, dogs with high antibody titers against Leishmania spp, and rarely infected dogs. One or more clinical signs common to leishmaniasis are present. Given the varied clinical manifestations of the disease, observed signs suggestive of disease can differ from the common clinical signs, as long as they can be clearly associated with ongoing infection. When physical examination does not reveal clinical signs, dogs in this category should still be defined as sick when hematologic, biochemical and urinary alterations common to leishmaniasis are detected. Laboratory changes other than those considered common can also be indicative of disease, provided that they are associated with the infection.

Table 2: Current treatment protocols for canine leishmaniosis recommended by the Canine Leishmaniasis Working Group.[30]

Drugs	Dosages
Meglumine antimoniate	100 mg/Kg SC, once a day for 4 weeks or 50 mg/Kg SC, twice a day for 4-8 weeks
Allopurinol	10 mg/Kg PO, twice a day for 6-12 months
Miltefosine	2 mg/Kg OS, once a day for 28 days
Allopurinol	10 mg/Kg PO, twice a day for 6-12 months

Table 3:Institutional reference intervals for ROTEM tests obtained in a group of 45 healthy dogs. [45]

Test	CT s	CFT s	MCF mm	α °
in-TEM	126– 363	47-224	50-75	55-81
ex-TEM	29-92	54-275	36-73	47-79
fib-TEM	14-102	na*	6-26	40-78

* not applicable;

Values are expressed as 5^{th}-95^{th} percentile (95% confidence intervals).

CT, clotting time; CFT, clot formation time; MCF, maximum clot firmness; alpha, angle α

Table 4: Hematocrit and fibrinogen concentrations in groups S, T and H.

Variable	Group S	Group T	Group H
Hematocrit L/L	0.33 (0.2-0.42) [32.9% (20-42)] *P=0.001 # P=0.001	0.43 (0.32-0.56) [43.3% (31.6-56.4)] £ P=0.16	0.45 (0.38-0.56) [44.8% (37.6-56)]
Fibrinogen µmol/L	12.45 (5.3-32.2) [424 mg/dL (180-1094)] *P=0.002 # P=0.001	8 (0.29-27.2) [273 mg/dL (150-926)] £ P=0.099	6.8 (3.2-14.2) [230 mg/dL (133-484)]

Data are expressed as median, minimum and maximum.

* statistically significant differences between group S and group H, # statistically significant differences between group S and group T, £ statistically significant differences between group T and group H ($P < 0.05$).

Table 5: Results of forward stepwise linear regression (P<0.05). All variables that differed between the groups were analysed.

Dependent variables	Independent variables	Log estimate	95% Confidence Interval	Standard Error	P value
in-TEM CFT	Fibrinogen	-0.414	-0.624 - -0.205	0.105	**0.01**
	Platelet	-0.356	-0.584 - -0.128	0.114	**0.003**
	Hematocrit	0.021	-0.485 - -0.528	0.254	0.93
in-TEM MCF	Fibrinogen	0.103	0.020 - 0.186	0.041	**0.015**
	Platelet	0.107	0.014 - 0.2	0.046	**0.025**
in-TEM α	Fibrinogen	0.107	0.047 – 0.168	0.030	**0.001**
	Platelet	-0.051	-0.195 - -0.092	0.072	0.475
	Hematocrit	0.056	-0.008 - -0.121	0.032	0.09
ex-TEM CT	Fibrinogen	-0.166	-0.324 - -0.009	0.079	**0.038**
	Hematocrit	0.279	-0.113 - -0.672	0.197	0.161
ex-TEM CFT	Fibrinogen	-0.518	-0.713 - -0.323	0.097	**0.001**
	Hematocrit	0.191	-0.310 - -0.693	0.252	0.449
ex-TEM MCF	Fibrinogen	0.113	0.028 - 0.198	0.042	**0.01**
	Platelet	0.103	0.008 - 0.199	0.047	**0.033**
ex-TEM α	Fibrinogen	0.147	0.100 - 0.193	0.023	**0.001**
	Hematocrit	-0.168	-0.283 - -0.053	0.057	**0.005**
fib-TEM CT	Fibrinogen	-0.211	-0.391 - -0.031	0.903	**0.022**
	Hematocrit	-0.224	-0.661 - -0.212	0.219	0.31
fib-TEM MCF	Fibrinogen	0.642	0.489 - 0.795	0.076	**0.001**
	Platelet	0.168	-0.676 - 0.083	0.190	0.124
	Hematocrit	-0.296	-0.006 - -0.342	0.087	0.059
	UPC	0.03	-0.018 - 0.078	0.024	0.22
fib-TEM α	Fibrinogen	0.177	-0.031 - 0.385	0.104	0.09
	Platelet	0.191	-0.049 - 0.431	0.120	0.118

CT, clotting time (sec); CFT, clot formation time (sec); MCF, maximum clot firmness (mm); ALPHA, angle α (degree); UPC, protein/creatinine ratio

522 Figure legends

523 **Figure 1**: Example of a normal thromboelastogram (ex-TEM profile).

524 CT clotting time, CFT clot formation time, MCF maximum clot firmness.

525 **Figure 2**: Pie chart of breed distribution in group S (symptomatic dogs before treatment).

526 Others breed: Great Dane, Pug, Dobermann, Galgo, Siberian husky, and English Mastiff.

527 **Figure 3**: Pie chart of breed distribution in group T (asymptomatic dogs after treatment).

528 Others breed: Great Dane, Galgo, Labrador retriever and English setter.

529 **Figure 4**: Pie chart of breed distribution in group H (control group).

530 **Figure 5**: Box plots representing the significant differences in the ROTEM parameters between the

531 three groups. Outliers have been excluded ($P < 0.05$).

532 Group S symptomatic dogs before treatment, Group T asymptomatic dogs after treatment, Group H

533 control group. CT clotting time (sec); CFT clot formation time (sec); MCF maximum clot firmness

534 (mm); ALPHA alpha angle (degree).

535 ***** statistically significant differences between group S and group H. In-TEM profile: CFT

536 ($P=0.0002$); MCF ($P=0.0116$); α angle ($P=0.0001$). Ex-TEM profile: CT ($P=0.0002$); CFT,

537 ($P=0.00001$); MCF ($P=0.0146$); α angle ($P=0.00001$). Fib-TEM profile: MCF ($P=0.00001$); α

538 angle ($P=0.001$).

539 **#** statistically significant differences between group S and group T. In-TEM profile: CFT

540 ($P=0.0064$); MCF ($P=0.00352$); α angle ($P=0.0179$). Ex-TEM profile: CFT ($P=0.00363$); MCF

541 ($P=0.0146$); α angle ($P=0.0074$). Fib-TEM profile: MCF ($P=0.0009$).

542 **£** statistically significant differences between group T and group H. Ex-TEM profile: CT

543 ($P=0.0001$); Fib-TEM profile: MCF ($P=0.00372$); α angle ($P=0.0259$).

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