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Thromboelastometry assessment of mycophenolate mofetil effect on thrombotic risk in dogs with primary and secondary immune-mediated hemolytic anemia and by *Erhlichia canis* infection

[*Avaliação por tromboelastometria do efeito do micofenolato de mofetila no risco trombótico em cães com anemia hemolítica imunomediada primária e secundária à infecção por* Erhlichia canis]

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ABSTRACT

This study aimed to identify, by means of thromboelastometry assessment, altered thrombotic risk in dogs with primary and secondary IMHA by *E. canis* infection after initiating the immunosuppressive therapy with mycophenolate mofetil. The animals' screening was based on complete blood count (CBC), biochemical and urine tests. Dogs with moderate to severe anemia (hematocrit ≤ 25%) which showed symptoms of immune-mediated hemolysis, such as spherocytosis, positive saline agglutination, bilirubinuria and/or hemoglobinuria, were included. Blood and urine samples were collected at two different moments. The first sample (M1) was collected at the time of diagnosis, when hematocrit was lower or equal to 25% before treatment with mycophenolate mofetil (Accord ®); the second sample (M2) was collected after treatment with mycophenolate mofetil, when hematocrit was greater or equal to 30%. Five out of the twelve animals selected died before the end of the study. No reduction in thrombotic risk was observed in the animals treated with mycophenolate mofetil. The animals that presented hypocoagulation at the time of diagnosis showed the worst prognosis, and their reticulocyte count displayed a better prognostic value than their erythrocytes count at the time of diagnosis.

Keywords: hemoparasitosis, hemostasis, immunosuppression, prognosis

RESUMO

O objetivo deste estudo foi esclarecer se há alteração do risco trombótico em cães com anemia hemolítica imunomediada primária e secundária a E.canis*, avaliado por meio da tromboelastometria, após início de tratamento com micofenolato de mofetila. A seleção dos animais foi baseada na avaliação de hemograma, exame bioquímico e urinálise. Cães com anemia moderada a severa (hematócrito ≤ 25%), com sinais de hemólise imunomediada, como esferocitose, aglutinação em salina positivo, bilirrubinúria e/ ou hemoglobinúria, foram incluídos. As amostras de sangue e urina foram coletadas em dois momentos diferentes. A primeira amostra (M1) foi coletada no momento do diagnóstico, quando o hematócrito era igual ou inferior a 25%, sem fazer uso do micofenolato de mofetila (Accord®), e o segundo momento (M2), após tratamento com micofenolato de mofetila, quando o hematócrito era igual ou maior que 30%. Doze animais foram selecionados, cinco morreram antes do término do estudo. Não houve diminuição do risco trombótico entre os animais tratados com micofenolato de mofetila; os animais que apresentaram menor coagulabilidade apresentaram pior prognóstico, e a contagem de reticulócitos apresentou melhor valor prognóstico do que a contagem de hemácias no momento do diagnóstico.*

Palavras-chave: hemoparasitose, hemostasia, imunossupressor, prognóstico

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INTRODUCTION

Immune-mediated hemolytic anemia (IMHA) is the most prevalent immune disorder in dogs. This condition is characterized by type II hypersensitivity and lead to premature destruction of erythrocytes (Balch and Mackin, 2007; McAless, 2010). Primary IMHA cannot be associated with an obvious predisposition. However, it is breed related, so it is an exclusion diagnosis. Secondary IMHA is related to several agents, and the ehrlichiosis stands out among the infectious ones (Giger, 2005; Balch and Mackin, 2007). Its treatment commonly involves immune response suppression by means of glucocorticoids and mycophenolate mofetil (Giger, 2005; Wang *et al*., 2013).

Among its main complications, the hypercoagulability - which predisposes dogs to disseminated intravascular coagulation and pulmonary thromboembolism - is the most important and leading cause of death of over 80% of cases (Carr *et al.*, 2002; Fenty *et al.*, 2011). The mechanism by which IMHA leads to hypercoagulability remains unclear, though the release of thromboplastin from lysed erythrocytes, platelet activation, hypoxia, inflammatory mediators´ response, endothelial injury, and altered blood viscosity are all believed to contribute to that process. The use of corticosteroids has also been suggested to be a potential cause of hypercoagulability (Fenty *et al*., 2011).

The hypercoagulation diagnosis is made through thromboelastometry(ROTEM®)/thromboelastography (TEG®), by assessing the blood hemostatic function as a whole using an *in vitro* technique which is able to diagnose both hyper and hypocoagulation before their clinical manifestations (Sinnott and Otto, 2009; Kol and Borjesson, 2010). The thrombin generation assay can also be used to detect hypercoagulation. However, it is not available for clinical use, as it still requires standardization and validation (Duarte *et al*. 2017).

Since the secondary hemostatic complications are the main IMHA-related death cause, this work aims to evaluate, by using thromboelastometry, the thrombotic risk on canine patients with primary and secondary immuno-mediated hemolytic anemia by *E. canis* infection before and after treatment with the immunosuppressive mycophenolate mofetil, in addition to assessing possible prognostic indicators. We hypothesized that the animals had a higher coagulation state at the time of diagnosis, and it decreased after treatment.

MATERIALS AND METHODS

This study was approved by the Animal Ethics Committee (protocol no. 194/2016) of FMVZ-UNESP/ Botucatu. The dog's owners were provided with an informed consent form for the blood and urine sampling procedures. Twelve animals of mixed genders (eight females and four males) with clinical signs of immune-mediated hemolytic anemia, aged between 11 months and 14 years old (mean four years; median three years), and weighting between 3.5 and 18.5kg (mean 8.9kg; median 9.5kg) were selected from the FMVZ-UNESP Veterinary Hospital. One of the females had been spayed.

Animal screening was based on CBC, biochemical and urine tests. Dogs with moderate to severe anemia (hematocrit $\leq$ 25%) and clinical signs of immune-mediated hemolysis, such as spherocytosis, positive saline agglutination, bilirubinuria and/or hemoglobinuria (identified by urine discoloration, positive urine test strip and absence of hematuria), were included. Dogs presenting other comorbidities, such as dermatitis, chronic kidney disease, cardiac and pulmonary diseases, endocrine disorders, joint diseases, and any kind of neoplasia were excluded. These comorbidities were detected through detailed anamnesis, physical examination, serum biochemical evaluation (creatinine, urea, alanine aminotransferase, alkaline phosphatase, gamma glutamyl transferase, total protein and albumin) and abdominal ultrasound. Moreover, animals that were positive for babesia on polymerase chain reaction (PCR) and leptospirosis serology reactive were also excluded.

Blood and urine samples were collected at two different moments. The first sample (M1) was collected at the time of diagnosis, with hematocrit lower or equal to 25% before treatment with mycophenolate mofetil (Accord ®); and the second sample (M2) was collected after treatment with mycophenolate mofetil (Accord ®), when hematocrit was greater or equal to 30%. M0 was considered the time of hospital admission (Figure 1).

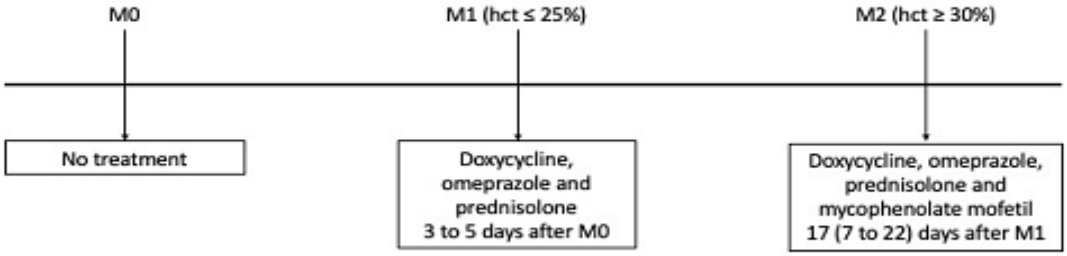

Figure 1. Scheme of the moments and treatments with respect to time in dogs with primary and secondary immune-mediated hemolytic anemia by Erhlichia canis infection.

The animals met the inclusion criteria at M1, when the tests results were compiled, the diagnosis was established, and inadequate responses to the following therapeutic protocol was verified: omeprazole 1mg/kg, every 24 hours; doxycycline 15mg/kg, every 24 hours, and prednisolone 2mg/kg, every 12 hours; for three to five days. The protocol performed at M1 was maintained at M2 and mycophenolate mofetil (Accord ®) 10mg/kg was added every 12 hours for seven to 22 days (mean of 17 days), until the hematocrit reached a value greater or equal to 30%. As for the deceased animals, the samples were collected only at moment M1, as they did not survive until M2. All animals of this study remained in their owners´ house during the treatment which had been recommended based on previous studies on ehrlichiosis and IMAH.

Two blood samples - one from each jugular vein (right and left) - were collected by syringe. 2.0mL of blood from the first syringe was placed in tubes containing 7.2% EDTA K3 anticoagulant for complete blood count, absolute reticulocyte count, saline agglutination test, and PCR of *Ehrlichia* sp., *E canis*, and *Babesia canis*. The remaining volume was placed in tubes without anticoagulant for *Leptospira* spp. serology prior to treatment. This sample was centrifuged at 3,000 rpm after clot formation for 5 minutes, to obtain serum. It was discarded 1.0mL from the second blood sample, and the remaining blood was immediately transferred into two tubes of 1.8mL, with 3.2% sodium citrate in a strict 1:9 ratio for thromboelastometry, prothrombin time (PT), and activated partial thromboplastin time (aPTT), according to the Partnership on Rotational ViscoElastic Test Standardization - PROVETS (Goggs *et al.*, 2014). Fifteen minutes after collection, a thromboelastometric analysis was performed in the first tube. The second tube was centrifuged, and the plasma was frozen and stored at -80°C until PT and aPTT were dosed.

A total volume of 10mL of urine was collected via catheterization or cystocentesis. Urinalysis was performed immediately after collection. Complete blood count was performed at the Veterinary Clinical Laboratory of UNESP/ Botucatu using a hematological counter (Poch 100iV Diff - Roche ®). A differential leukocyte count was determined for 100 cells, along with erythrocyte, leukocyte, and platelet morphology evaluations from blood smears stained with Diff-Quick (Laborclin®). For the reticulocyte count, a solution of blood and new methylene blue (1:1 ratio) was incubated in water bath at 37ºC for 15 minutes. Then, blood smear was performed in each sample and reticulocyte count was made in 1,000 erythrocytes using a manual technique.

The results were reported as absolute reticulocyte count (reticulocytes/uL). 10µL of whole blood were mixed in 50uL of saline solution in a slide at room temperature for the saline agglutination test. The slide was placed in a humid chamber for 15 minutes following microscopic evaluation. The results were reported as negative or positive according to the presence of agglutination. Urinalysis was performed at the Veterinary Clinical Laboratory of UNESP/ Botucatu and consisted of physical, chemical (Combur-Test® - Roche) and sediment microscopy in an optical microscope (40x). The urinary specific gravity was obtained by refractometry (Atago® T2-NE -

Japan). The PCRs for *Ehrlichia* sp. and *Babesia canis* were sent to the Molecular Veterinary Diagnostic Laboratory of UNESP/ Botucatu. Animals that were positive for *Ehrlichia* sp. were tested for *E. canis*.

Leptospirosis serology was carried out at the FMVZ UNESP/ Botucatu Zoonoses Diagnostic Laboratory, by means of the microscopic serum agglutination method with the following serovars: *L. australis; L. bratislava; L. autumnalis; L. canicola; L. cynopteri; L. djasiman; L. grippotyphosa; L. copenhagen; L. icterohaemorraghiae; L. pomona; L. pyrogenes; L. hardjo; L. canicola botucatu*.

Thromboelastometry was performed with a ROTEM® device (Delta, Pentapharm, Munich, Germany), which was maintained at 37°C. Two channels were used simultaneously; 300µL of recalcified citrated whole blood and 20µL of 0.2 M calcium chloride (startem®) were added to both of them, and clotting activation was performed separately in each channel by adding INTEM® reagents (20µL, 26 partial thromboplastin phospholipids, Pentapham, Munich, Germany) for the intrinsic pathway evaluations, and EXTEM® reagents (20µL, 26 tissue thromboplastin phospholipids, Pentapham, Munich, Germany) for extrinsic pathway evaluations. The reference interval was established by the FMVZ hemostasis laboratory, through a robust method involving 20 healthy animals and the following parameters: median, maximum, and minimum values, with a confidence interval of 90% (Friedrichs *et al.*, 2012).

Prothrombin time (PT) and activated partial thromboplastin (aPTT) were performed using a semi-automatic coagulometer (ClotTimer® Laser Sensor, Sorocaba, Brazil) in triplicate, using their corresponding test kits (Clot®, Sorocaba, Brazil). Abdominal ultrasound examination was carried out using the Esaot Mylab 70 Vet® imaging unit from the FMVZ-UNESP imaging service. For sample dimensioning, the two evaluation moments were compared considering the repeated measure method applied to the same animal. A minimum of seven animals were used to achieve a 5% significance value for comparison and 80% power for the statistical tests.

The response variable did not adhere to a normal distribution by the Shapiro-Wilk test. Therefore, the median, first quartile, and third quartile of each variable were considered. The Willcox nonparametric test was used to compare the two moments of the surviving group, and the nonparametric Mann-Whitney test was applied to compare the moment 1 of surviving and the death groups (Norman and Streiner, 2008). Analyses were made by using Graph Pad Prism 6 ® software, and the results were discussed at the 5% significance level.

RESULTS

Five out of the twelve animals selected (Figure 2) died (5/12 or 41.6 %) before the end of the study. They were all females, with a mean age of 3 years (11 months to 7 years). Two of them were of mixed breed - a boxer and a miniature pinscher - and one was a Brazilian terrier. Two females were diagnosed with primary IMHA and three with IMHA secondary to ehrlichiosis. The average survival was seven days (2-14 days) after the beginning of the treatment (Figure 2).

Among the seven survivors (7/12 or 58,3 %), four were males and three were females, with a mean age of 5 years (8 months to 13 years), six were mix breed, and one was a poodle. Of these, five presented IMHA secondary to ehrlichiosis and two exhibited primary IMHA. Four of the evaluated animals were negative for *Ehrlichia* sp. and eight were positive for *E. canis*. One animal was excluded from the study, as it was positive for *E.* sp. and negative for *E. canis*. The most frequent manifestations were: ixodidiosis 66.6%, vomiting 58.3%, pale mucosas 58.3%, hyperthermia 58.3%, jaundice 41.6%, lymph node enlargement 41.6%, dehydration 33.3% and diarrhea 33.3%. Four animals (4/12) received red blood cell transfusion at the beginning of treatment, after moment 1 (M1). Three of them died before moment 2 (M2). The complete blood count, reticulocyte count, and clotting times of both survivors and death groups are shown in Table 1. The thromboelastometric evaluation is displayed in Table 2.

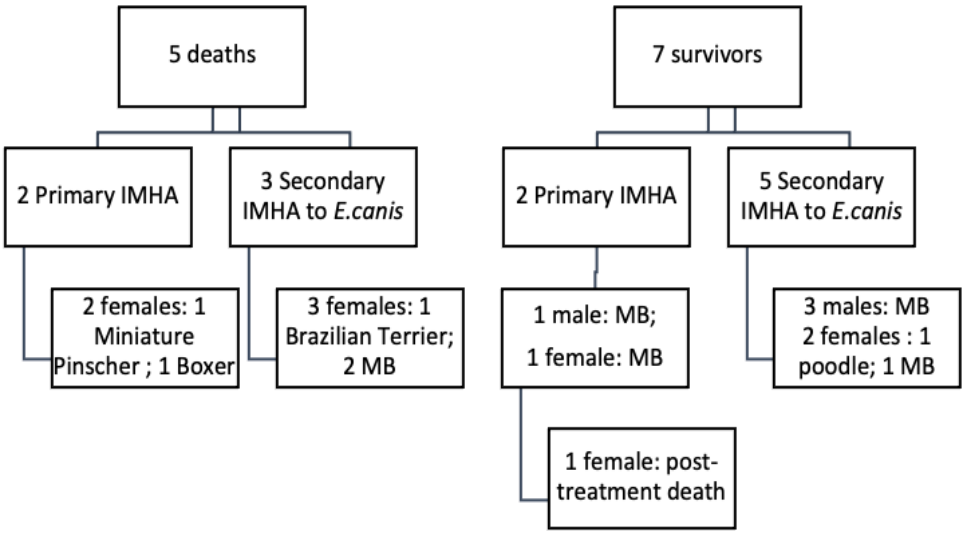

Figure 2. Distribution of death and survivor dogs with immune-mediated hemolytic anemia, according to their disease, sex and breed.

Table 1. Summary measures of complete blood count, absolute reticulocyte count (/uL), PT (Protrombin time) (s) and aPTT (activate partial thromboplastin time) (s) in dogs with primary and secondary immune-mediated hemolytic anemia by Erhlichia canis infection under treatment with mycophenolate mofetil, and their reference intervals

	Survivors M1 Median (1q - 3q)	Survivors M2 Median (1q - 3q)	Death Median (1q - 3q)	Reference
Erythrocytes(x10^6/µL)	1,780(1,300–2,200)*	4,200(3,610–5,400)	2,780(1,890–3,755)*	5,5–8,50
Hemoglobin (g/dL)	5.100(3.800–6,100)	10.800(9.700–12.200)	6.400(4.650–7.700)	12.0–18.0
Hematocrit (%)	17(15-19)	33(32-35)	20(15-23)	37.0–55.0
Platelets (/µL)	27,775(10,000–228,000)	374,000(290,000–59,800)	65,650(15,000–193,500)	160,000–430,000
Leukocytes (/µL)	40,600(11,500–67,600)	13,100(6,300–24,400)	17,900(6,500–44,245)	6,000–17,000
Reticulocytes (/µL)	185,320(56,710–194,000)	84,400(3,620–127,000)	41,000(7,450–236,835)	Regeneration
PT (s)	8.3(6.9–9.0)	7.9(7.4–8.5)	7.1(6.9–7.4)	6.4to7.4
aPTT (s)	12.5(11.6–13.5)	13.6(12.2–14.6)	29.9(11.7–53.0)	9to11

1q= first quartile; 3q= third quartile; M1= moment 1; M2 = moment 2. Reference value: Veterinary Clinical Laboratory of UNESP/ Botucatu. * Variable that showed statistical difference (P<0.05) between survivors and death group at M1; other variables did not reveal significant statistical differences.

Concerning the urinalysis, eight (8/12) animals showed moderate to severe bilirubinuria, and eleven (11/12) displayed occult blood and presence of erythrocytes in the urine. Six patients were evaluated by abdominal ultrasonography; three died before the test, and three did not return on the day scheduled for evaluation. Among the patients examined, three were diagnosed with IMHA and three with *E. canis*. All animals presented splenomegaly and alteration in echogenicity of hepatic parenchyma. One of them showed hepatomegaly. No patient displayed relevant changes to neoplasia in their abdominal organs.

DISCUSSION

Four dogs - three females and one male - were diagnosed with primary IMHA (33%). This result is consistent with IMHA higher prevalence in females (Miller *et al*., 2004). The mean age of the affected dogs was 4.4 years. Eight dogs were diagnosed with IMHA secondary to ehrlichiosis (67%). The large number of dogs with IMHA secondary to ehrlichiosis might be due to the fact that this study was performed in Brazil, a region with high average temperature throughout the year, apart from high rate of vectors´ reproduction and dissemination. Furthermore, the municipality of Botucatu is endemic for hemoparasites.

Therefore, this result is discrepant from the ones found in studies held in other countries, where primary IMHA is more common than the secondary one (Carr *et al.*, 2002; Giger, 2005; McAless, 2010). Among the animals diagnosed with primary IMHA, 75% (3/4) died.

Table 2. Summary measures of median, first quartile and third quartile of CT (clotting time) (s), CFT (clot formation time) (s), α angle (°) and MCF (maximum clot firmness) (mm) in dogs with primary and secondary immune-mediated hemolytic anemia by Erhlichia canis infection under treatment with mycophenolate mofetil, and their reference intervals

	INTEM®				EXTEM®			
Parameter	Surv. M1	Surv. M2	Death	Reference	Surv. M1	Surv.M2	Death	Reference
CT	94(66-129)	102(70-119)	219(85-466)	141(119;158)	18(14-24)	13(9-24)	13(11-42)	58(42;99)
CFT	38(34-105)	56(29-68)	39(0-137)	91(81;114)	35(19-103)	60(39-67)	14(6-623)	99(75;121)
α angle	82(81-84)*	79(76-84)	74(32-80)*	73(69;75)	83(82-87)	78(78-83)	83(75-87)	72(68;76)
MCF	67(58-76)	69(68-77)	42(15-72)	59(56;62)	68(53-77)	74(73-79)	54(19-77)	62(58;64)

INTEM® = Contact Activator; EXTEM® = Tissue Factor Activator; M1= moment 1; M2 = moment 2; Surv. = survivor. Reference value: Veterinary Clinical Laboratory of UNESP – Botucatu. * Variable that showed statistical difference ($p<0.05$) between survivor and death groups at M1 in the intrinsic pathway; other variables did not reveal significant statistical differences.

Among those with secondary IMHA, 37.5% (3/8) deaths were observed. Thus, in this study, the animals with IMHA secondary to hemoparasites presented a better prognosis than the ones with primary IMHA. In contrast to the expected results, the erythrocytes count was higher in the dead animals than in the surviving ones at the first moment, as it was demonstrated by the significant statistical difference ($P<0.05$). Nevertheless, when comparing the reticulocyte counts between the groups, although no statistical significance was found, a higher median was observed in the group of survivors M1, suggesting that reticulocytosis could be more relevant than the anemia intensity at diagnosis. The reticulocyte count can predict the appropriate response to anemia. In addition, a better regenerative response was observed among the survivors despite the anemia intensity, hence indicating its prognostic value.

The thromboelastometry results showed a hypercoagulability associated with decreased clotting time (CT) and clot formation time (CFT), besides increased α angle and maximum clot firmness (MCF). Hypocoagulability, on the other hand, was characterized by the opposite findings, i.e., prolongation of CT and CFT and decreased angle α and MCF (Goggs *et al*., 2014). In both intrinsic and extrinsic pathways of the survivors at M1 and M2, there was a decrease of CFT and an increase of α angle and MCF, which are characteristic of hypercoagulability.

When comparing the α angle in the intrinsic pathway of survivors and death groups at M1, a significant difference was noted, which shows the dead patients had a lower coagulation profile at the time of diagnosis. In addition, although not significant ($P>0.05$), a higher median CT and a lower median maximum clot firmness (MCF) were observed in the death group, which reinforces our finding that the animals displaying lower clotting at moment 1 (M1) had the worst prognosis. The same was observed by Goggs and collaborators (2012), who concluded that hypocoagulability at hospitalization of dogs with IMHA was a negative factor. This also supports the hypothesis that consumption coagulopathy is responsible for unfavorable prognosis.

Plasma clotting tests at PT and aPTT correlating with extrinsic and intrinsic pathways respectively did not show significant differences. Despite that, the higher aPTT values in the death group compared to those of M1 survivors are consistent with the Rotem results, which indicate hypocoagulation. A mild to moderate prolongation of the aPTT and a normal PT were common findings in cats with disseminated intravascular coagulation (DIC) (Perteson *et al*., 1995). The same occurred among dogs with DIC,

which is also frequently observed in IMHA. This may be due to the fact that the main coagulation mechanism for fibrin generation is the intrinsic pathway. Thus, once coagulation is initiated, most parts of the clot propagation are supported by this pathway (Brainard, 2014). However, other studies suggested that the inflammatory response reflected by an elevated level of C reactive protein, might interfered with the aPTT assay, leaving a false prolongation (Cheng *et al.*, 2009; Van Rossum *et al.*, 2012).

When comparing the survivor group at M1 and M2 moments in both intrinsic and extrinsic pathways of PT, aPTT and thromboelastometry, no significant differences were observed. This lack of significance can be explained by the small sample size. A similar study by Goggs and collaborators (2012) evaluated 30 dogs with IMHA through thromboelastograms (TEGs) and found an increase in hypercoagulability throughout the treatment. However, the therapeutic protocol of Goggs *et al*. (2012) was not standardized, as proposed by the present study, and the therapies were individualized for each patient using the following drugs: prednisolone or dexamethasone associated with cyclosporine, azathioprine, or mycophenolate mofetil. Furthermore, the animals were evaluated three times between admission and the fifth day of treatment, rather than after hematocrit increase, when there is a reduction in thromboplastin release from lysed erythrocytes, platelet activation, hypoxia, release of inflammatory mediators and endothelial lesion, the main factors involved in hypercoagulability, as per the present study.

The use of corticosteroids may also alter the thrombotic state of healthy patients. In a study conducted by Rose *et al.* (2011), the thrombotic risk was evaluated in six healthy beagles that received oral administration of prednisone at doses of 1mg/kg and 4mg/kg once a day for two weeks. Evidence of hypercoagulability was observed by thromboelastogram, independent of the dose.

The animals of this study maintained their hypercoagulability state after the treatment, which was evidenced by the absence of a statistical difference between M1 and M2 measurements. However, the small sample size and prolonged use of corticosteroids - which lead to hypercoagulation - may have interfered with those results. Therefore, further studies that follow standardized treatments with larger sample size are needed to clarify whether hypercoagulability changes throughout the treatment.

CONCLUSIONS

Under the conditions of this study, it is possible to conclude that there was no decrease in thrombotic risk among animals treated with mycophenolate mofetil. The animals that exhibited hypocoagulation at the time of diagnosis showed worse prognosis. Furthermore, reticulocyte count has a better prognostic value than erythrocytes count at the time of diagnosis.

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