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Markers of Coagulation Activation, Endothelial Stimulation, and Inflammation in Dogs with Babesiosis

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Background: Babesia infections in dogs can result in a wide range of clinical and laboratory presentations, including coagulopathy. Expression of intercellular adhesion molecule-1 (ICAM-1) and von Willebrand factor (vWF) in dogs with babesiosis is unknown.

Objectives: Whether inflammation in babesiosis triggers activation of ICAM-1 and the coagulation system.

Animals: Twelve and 10 dogs with naturally occurring babesiosis before and after antiparasitic treatment, respectively, were compared with 10 healthy dogs.

Methods: In this prospective study, diagnosis was made by blood smear examination and confirmed by PCR. C-reactive protein (CRP), soluble intercellular adhesion molecule 1 (sICAM-1), and von Willebrand factor (vWF) levels were measured by a canine ELISA kit, fibrinogen (FIB) and factor VIII activity levels were measured by coagulometric methods, and blood cell counts (WBC, RBC, PLT) were determined with an automatic analyzer.

Results: Compared to healthy dogs, the CRP, sICAM-1, and FIB concentrations were significantly increased before therapy and remained high for 3 days after therapy in dogs with babesiosis. vWF activity was significantly decreased in dogs with babesiosis before treatment. FVIII activity did not differ between dogs with babesiosis and healthy dogs. WBC; RBC and PLT were significantly lower before treatment and normalized by 3 days after treatment.

Conclusion and Clinical Importance: A proinflammatory condition in babesiosis appears to influence endothelial dysfunction and hemostatic activity. Although clearly beneficial for the parasite, sequestered blood cells can obstruct blood flow in small vessels, promote an inflammatory state, and could increase the severity of babesiosis.

Key words: Hemostasis; Inflammatory markers; Intercellular adhesion molecule; von Willebrand factor.

Babesia canis is an intraerythrocytic protozoan parasite that is transmitted by ticks and by transfusion to dogs. Babesiosis in dogs is a common disease with a worldwide distribution[1–4] and a high and increasing prevalence in Croatia.[5,6] In addition, babesiosis is becoming more frequently diagnosed in humans, as animal reservoirs and tick vectors have increased in number and humans have inhabited areas where reservoir and tick populations are high.[7,8] *Babesia* parasites in dogs can result in a wide range of clinical presentations that range from a mild, subclinical illness to complicated forms and death.[6,9]

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Abbreviations:

AvWS	acquired von Willebrand syndrome
CAM	cell adhesion molecule
CRP	C reactive protein
EC	endothelial cells
ELAM	endothelial leukocyte adhesion molecule
FIB	fibrinogen
FVIII	factor VIII
ICAM	intercellular adhesion molecule
PLT	platelets
pRBC	parasitized red blood cells
RBC	red blood cells
VCAM	vascular cell adhesion molecule
VWD	von Willebrand disease
vWF	von Willebrand factor
WBC	white blood cells

Hematologic changes play an important role in the pathogenesis of babesiosis,[5–8] which involves an acute-phase response that is triggered by the overproduction of inflammatory mediators.[10–12]

Coagulation and inflammation are closely related parts of the host defense mechanism, and inflammation is known to promote coagulation.[13,14] Loss of the structural and functional integrity of the endothelium leads to endothelial dysfunction, which reduces the endothelial capacity to maintain homeostasis and leads to the development of pathological inflammatory processes and vascular disease.[15] Endothelial dysfunction results in hemostatic activation, inflammation, and vasoconstriction.[16] Although previous studies have identified an association between C reactive protein (CRP) and endothelium-dependent vascular reactivity,

it remains unclear whether the association is a consequence of a primary pathological trigger or an endothelial disturbance.[16,17]

From a biological perspective, changes in cellular adhesive properties are important for *Babesia* parasites. Infected red blood cells (RBC) show increased adhesive properties for different cell types, including vascular endothelial cells, resulting in their accumulation in different organs, probably to avoid destruction in the spleen.[18–20] However, the precise molecular mechanisms of this process are still unclear. In addition, adhesion molecules likely play an important role in tissue damage secondary to the inflammatory response.[21,22] Few comparative studies have been performed on *Babesia* parasites, and the molecular mechanisms that underlie the pathogenesis of babesiosis in dogs remain virtually unknown.

Given previous observations of alterations of the inflammatory response and the hemostatic system during babesial infection,[3,23,24] we hypothesized that inflammation in babesiosis might be associated with the enhanced expression of cell adhesive molecules and endothelial activation. The aim of the present study was to assess the levels of inflammation, coagulation activation, and endothelial stimulation in dogs with babesiosis, before and after antiparasitic treatment, and to compare the results with healthy controls.

Materials and Methods

Dogs with babesiosis admitted to Sesvete Veterinary Station were eligible for prospective enrollment in the study. Samples of blood were obtained before (n = 12) and the 3rd day after (n = 10) intramuscular treatment with imidocarb dipropionate (6 mg/kg). The diagnosis of babesiosis infection was made by direct observation of large piroplasms in stained blood smears, which were recognized as large species. Polymerase chain reaction analysis confirmed the presence of *B. canis* subspecies in all dogs.[25] The number of parasitized RBCs (pRBCs) was determined by microscopy examination before and after treatment. Before the treatment, the average pRBC number was 71×10^9/L (range, 1×10^9/L to 378×10^9/L). After the treatment, parasites were not detectable in peripheral blood smears.

The control group consisted of 10 healthy dogs. At the time of enrollment, none of the healthy dogs had histories of previous clinically relevant illness. Routine hematologic and biochemical analyses were performed, and all of the obtained results were within reference ranges.

Blood was collected with an 18-g needle by jugular venipuncture, using the Vacutainer blood collection system.[a] Blood samples were drawn atraumatically and without stasis into EDTA, serum, and trisodium citrate (0.011 mol/L) tubes. Serum samples were centrifuged (1,500 × *g* at 4°C for 10 minutes), within 2 hours of collection. Citrated blood samples were centrifuged (2,000 × *g* at 4°C) and plasma and serum were stored at −80°C before analysis.

The hematologic parameters were analyzed on automatized hematology analyzer.[b] CRP and soluble intercellular adhesion molecule (sICAM) were measured in serum, using ELISA canine kit.[c] von Willebrand factor (vWF) detection in citrated plasma was carried out using the ASSERACHROM "STA LIATEST vWF immunoassay detection kit."[d] Standards, controls and samples are pipetted into the wells of a microtiter plate precoated with specific antibodies. After plate washing, an enzyme linked antibody was added, and the enzyme reaction was measured by optical density. All ELISA tests were performed in duplicate. We analyzed the multimeric structure of vWF in plasma by low resolution SDS-agarose gel electrophoresis,[e] followed by electroblotting on nitrocellulose and imunoenzyme luminiscence detection. The VWF multimers analysis was used for determination of vWF multimers in 3 healthy dogs and 3 dogs with the lowest vWF activity. The 1-stage clotting assay for factor VIII (FVIII) and fibrinogen were performed on the ACL 7000 analyzer[f] using reagents from that manufacturer, i.e., HemosIL SynthASil aPTT reagent, FVIII-deficient plasma and calibration plasma obtained from 10 healthy dogs. Fibrinogen concentration was determined using the thrombin clotting time, where the time taken for the patient's sample to clot is compared to a standard canine curve.

The Mann–Whitney test was used to identify statistically significant differences between the healthy and infected populations. Differences with a *P*-value <.05 were considered statistically significant. All statistical analyses were performed with the Statistica 8 for Windows software program.[g] The results in dogs infected with babesiosis, before (B0) and after treatment (B3) with imidocarb dipropionate, were compared with those in healthy dogs (controls). All levels of significance were $P < .01$, and are indicated in the relevant figures by *.

Results

Significantly increased concentrations of fibrinogen (mean 6.3 g/L, control 2.5 g/L), CRP (mean 154 mg/L, control 1.4 mg/L), and sICAM-1 (mean 0.450 mg/L, control 0.180 mg/L) were evident in dogs with babesiosis before the treatment as compared to healthy dogs. These concentrations remained significantly increased for 3 days after antiparasitic treatment, when parasites were not present in the circulation (mean fibrinogen 5.3 g/L, mean CRP 50 mg/L, and mean sICAM-1 0.238 mg/L) (Figs 1–3).

Before treatment, dogs with babesiosis showed significantly lower vWF activity levels (B0 mean 35%, control 59%) than those of healthy dogs. Although the vWF activity was still low at 3 days after treatment, there was no statistical difference compared to healthy dogs (B3 mean 44%, control 59%) (Fig 4). In the 3 healthy dogs that were tested, the high, middle, and low molecular weight (MW) forms of vWF were observed. In contrast,

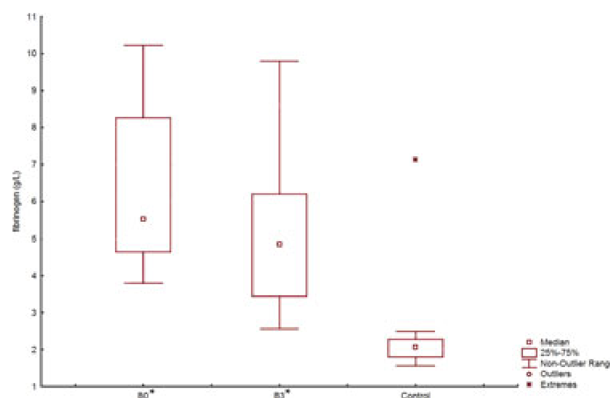

Fig 1. Concentrations of fibrinogen in babesiosis before treatment (B0) and on day 3 after treatment (B3) compared with control, healthy dogs (*$P < .01$).

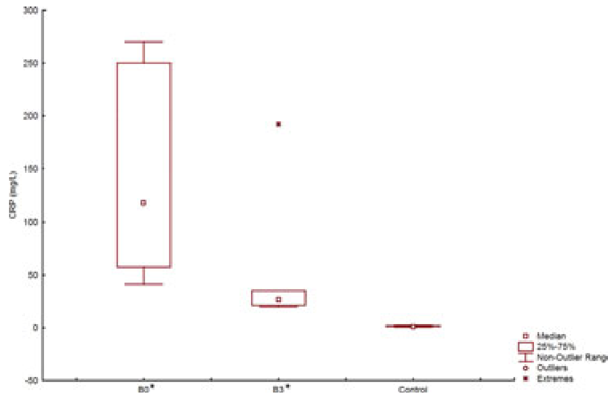

Fig 2. Concentrations of C reactive protein in babesiosis before treatment (B0) and on day 3 after treatment (B3) compared with control, healthy dogs (*$P < .01$).

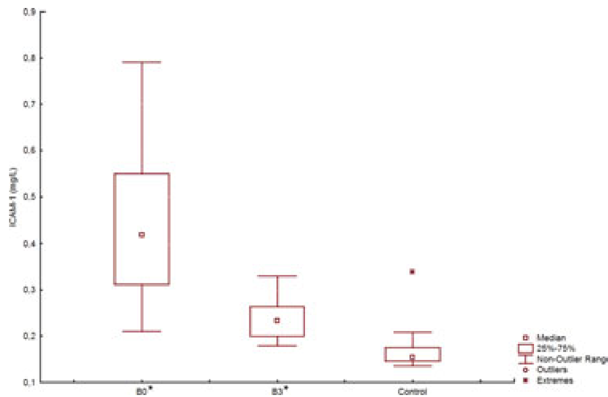

Fig 3. Concentrations of soluble intercellular adhesion molecule 1 in babesiosis before treatment (B0) and on day 3 after treatment (B3) compared with control, healthy dogs (*$P < .01$).

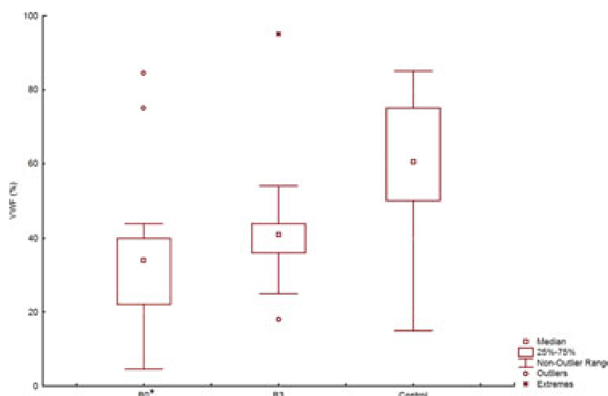

Fig 4. Activity of von Willebrand factor in babesiosis before treatment (B0) and on day 3 after treatment (B3) compared with control, healthy dogs (*$P < .01$).

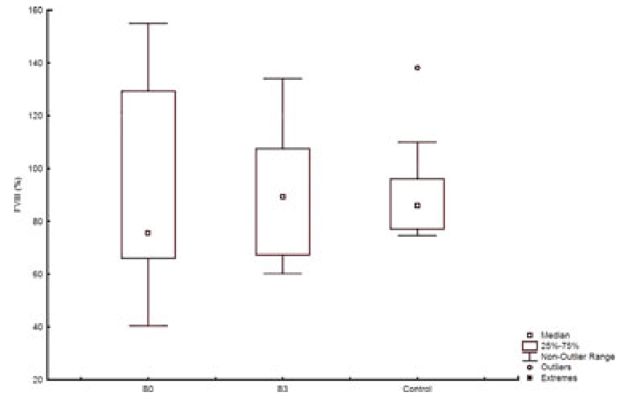

Fig 5. Activity of factor VIII in babesiosis before treatment (B0) and on day 3 after therapy (B3) compared with control, healthy dogs (*$P < .01$).

Table 1. WBC, RBC, and PLT in dogs with babesiosis before treatment (B0) and the 3rd day after treatment (B3) compared with control, healthy dogs.

	n	Mean	Median	Range
WBC ($\times 10^9$/L)				
B0*	12	6.2	5.4	2.9–10
B3	10	14	12	8.7–19
Control	10	10	11	6.1–16
RBC ($\times 10^{12}$/L)				
B0*	12	6.1	6.1	4.5–8.1
B3	10	7.2	7.2	5.3–8.4
Control	10	7.3	7.1	6.8–8.3
PLT ($\times 10^9$/L)				
B0*	12	99	95	59–145
B3	10	148	149	89–210
Control	10	207	171	121–368

WBC, white blood cells; RBC, red blood cells; PLT, platelets.
*$P < .01$.

the 3 dogs with the lowest vWF activity levels (15, 8, and 4%) showed a complete absence of high MW multimers. The FVIII activity did not differ between animals with babesiosis and healthy animals (B0 mean 86%, B3 mean 90%, control 91%) (Fig 5).

The numbers of RBCs, WBCs, and PLTs were significantly lower before treatment and normalized by 3 days after treatment (Table 1).

Discussion

Consistent with previous reports,[2,10,11] the dogs in this study displayed excessive proinflammatory activity with significantly increased concentrations of CRP and fibrinogen. The levels of these 2 proteins remained significantly high for 3 days after treatment. Numerous studies have reported the occurrence of hyperfibrinogenemia as an acute-phase protein reaction in dogs with babesiosis.[12,26–28] Increased fibrinogen concentration indicates activation of the coagulation system and potential fibrin

formation.[12] High fibrinogen might interfere with the antigen binding of antibodies,[29] which can suppress the immunologic system and prolong *Babesia* survival in the circulation of dogs.

C reactive protein has been reported to be a suitable but nonspecific marker of inflammation in dogs, which can be used as a predictive marker for risk of disease and to monitor the response to treatment.[30,31] Upon infection with *Babesia* parasite, the plasma concentration of CRP increased even before parasites were detected in the blood.[12] As proinflammatory factors, CRP and fibrinogen could lead to the upregulation of ICAM-1. CRP influences the activation of ECs and the expression of adhesion molecules, whereas fibrinogen promotes fibrin formation.[31,32] Fibrin and its cleavage products upregulate ICAM-1 production in ECs, providing a link between fibrin deposition and adhesion molecule expression, which might subsequently lead to leukocyte accumulation and transendothelial extravasation.[33–35]

Intercellular adhesion molecule-1 is cytokine-inducible glycoprotein belonging to the immunoglobulin superfamily. It is constitutively expressed on vascular ECs and is upregulated in response to various stimuli, including cytokines, during inflammation.[36,37] Cell-bound ICAM-1 can be released from the surface; therefore, increased serum concentrations of sICAM-1 indicate systemic endothelial activation.[37] In the present study, dogs with babesiosis before and 3 days after antiparasitic treatment showed significantly increased sICAM-1 compared to the healthy control dogs.

As a proinflammatory factor, CRP has direct effects on EC activation, causing the expression of adhesion molecules.[38] The results obtained in human studies support the hypothesis that the induction and interaction of adhesion molecules are the main mechanisms in CRP-induced chemokine secretion.[39] Increased body temperature in dogs with babesiosis can also induce the expression of ICAM-1.[40] The pathogenesis of human babesiosis is closely linked to the host response to infection and parasite-induced modifications in the erythrocyte membrane, where ICAM-1 is upregulated.[41] Some *Babesia* species export proteins to the surface of infected erythrocytes, resulting in the adherence of these erythrocytes to the vascular endothelium and in their delayed clearance by the spleen. This mechanism is widely considered to protect invaded cells from entrapment and destruction in the spleen, and to maintain the microaerophilic parasites in a relatively hypoxic environment.[1,18,42]

The adhesion of infected RBCs to the endothelium can lead to a blockade of the microcirculation and the local proliferation of parasites, which may further increase parasitemia.[1,8,43] Sequestered RBCs can obstruct blood flow in small vessels, resulting in a local increase in the inflammatory cytokine concentration and an increase in disease severity.[44] Despite intense research in human medicine, the role of adhesion molecules in the activation pathways of hemostasis and inflammation in dogs requires additional investigation.

The endothelial dysfunction, interaction of blood cells with the endothelium, and activation of the coagulation cascade secondary to an acute-phase response may serve as triggers of hemostasis disturbances.[11,12,27] As an essential part of primary hemostasis, vWF is a useful marker for EC activation, functioning as both a FVIII carrier and a PLT-vessel wall mediator in the blood coagulation system.[45–47] The vWF protein is secreted by 2 pathways: 1 pathway is continuous and does not require cellular stimulation, and the other is regulated and is responsive to different stimuli.[46,48] Several factors influence vWF activity, including the acute-phase response and enzymatic proteolysis. The inflammatory state results in an increase in plasma vWF activity.[46,49,50]

In contrast to our expectations, the vWF activity in dogs before antiparasitic treatment was significantly decreased compared to healthy dogs, even with evidence of an inflammatory state. In addition, in the 3 dogs with the lowest vWF activity, multimeric analysis showed a complete absence of large vWF multimers. The FVIII activity of the infected dogs did not differ from that of healthy dogs. Analyses of the vWF activity and the structure of the multimers in canine medicine are frequently performed to diagnose von Willebrand disease, which is characterized by an inherited quantitative or qualitative deficiency of vWF.[51,52] However, there is scant data concerning vWF activity in other canine diseases. In contrast to von Willebrand disease, which is an inherited disease, acquired von Willebrand syndrome describes any qualitative, structural, or functional disorder of vWF that is not inherited.[53,54] Various pathogenic mechanisms have been proposed as the cause of acquired von Willebrand syndrome in humans; these include the action of autoantibodies, increased vWF clearance from the plasma, or sequestration of high MW vWF multimers, which have been demonstrated in patients with hematologic disorders.[55–57] Inflammation and the release of cytokines during inflammation could influence the conversion kinetics of hyper-reactive, ultralarge vWF multimers to the smaller and less-active plasma forms of vWF.[50,58]

In addition, hypothyroidism could decrease synthesis of otherwise normal vWF and cause acquired von Willebrandt syndrome.[59] Decreased thyroxine concentration is recorded in dogs infected with *B. canis*,[60] so hypothyroidism could represent possible cause of acquired von Willebrand syndrome in babesiosis.

Of the 3 blood cell types evaluated in this study, PLTs were affected most dramatically by babesiosis infection. The PLT numbers were significantly lower before treatment and normalized by 3 days after treatment. Because nearly all tick-borne infections in mammalian hosts, including babesiosis, can result in thrombocytopenia,[61] the PLT count is routinely tested in dogs with babesiosis. If thrombocytopenia is not present in dogs, then a diagnosis of babesiosis is not probable.[27,62–64] There are several possible explanations for the observed decreased PLT count in dogs with babesiosis before treatment. Splenomegaly, which is associated with many tick-borne diseases, increases

PLT sequestration and destruction by splenic macrophages.[61] Consumptive coagulopathy is another possible explanation of thrombocytopenia in babesiosis.[3,26] Autoantibodies to phosphatidyl-serine may contribute to the thrombocytopenia seen in *B. bovis* infections.[65] In addition, PLT-expressed lymphocyte function-associated antigen (LFA)-1 can interact with ICAM-1 to promote PLT binding to ECs. The adhered PLTs can function as bridging molecules between the endothelium and the pRBCs.[36]

Inflammatory processes induce leukocytosis and the synthesis of acute-phase reactants. However, in this study, compared to the healthy controls, the babesiosis-infected dogs before treatment displayed a significantly decreased number of WBCs, which normalized by 3 days after treatment. Neutropenia was previously noted in babesiosis during the early phase of the disease before antiparasitic treatment.[3,9,66] One mechanism involved in leukopenia includes the ability of PLTs, which bind with activated ECs, to interact with leukocytes and induce their so-called "secondary capture." The subsequent neutrophil-endothelial interaction[67] could contribute to the initial decrease in WBC number before treatment and trigger vascular inflammation. Leukocytes have the capability to bind with ICAM-1 via receptors.[36,67] Thus, ICAM-1 overexpression could contribute to the initial decrease in the WBC count observed at the beginning of the disease and mediate leukocyte transendothelial migration as part of a cascade of molecular interactions.

We observed that the number of RBCs was significantly lower before treatment and normalized after treatment. Anemia, particularly hemolytic anemia, is frequently reported in canine babesiosis. In this process, erythrocytes are destroyed mainly because of the action of autoantibodies, macrophage activity, splenic sequestration, or as a result of glucose-6-phosphate dehydrogenase deficiency.[9,12,68] The binding of an antierythrocyte antibody can lead to intra- or extravascular hemolysis.[69] The cytoadherence of parasitized erythrocytes to EC receptors as a result of ICAM-1 overexpression could represent an additional mechanism for the fall in RBC number in *Babesia* infections. However, this influence is probably minor in babesiosis, given the very low number of circulating parasites.

Our study had several limitations. The investigators had no control over the disease process before the dogs presented to the hospital. Only a small number of animals were included in the study. Other markers of endothelial dysfunction, such as vascular cell adhesion molecule and endothelial leukocyte adhesion molecule, were not included in our analyses. Nevertheless, despite these limitations, our results indicate that an inflammatory response is associated with the endothelial disturbances and hemostatic imbalances that are observed in dogs with naturally occurring babesiosis. The acute-phase protein reaction could have direct effects on EC activation, causing the increased expression of ICAM-1, which can bind pRBCs, WBCs, and PLTs via their receptors. The endothelial dysfunction, interaction of blood cells with the endothelium, and activation of the coagulation cascade secondary to an acute-phase response may trigger hemostasis disturbances, contributing to the pathogenesis of babesiosis. Thus, vascular effects could be an important pathological mechanism in this disease. However, the results of this preliminary study should be verified by further research.

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Conflict of Interest: Authors disclose no conflict of interest.

Footnotes

[a] Becton, Dickinson and Co., Rutherford, NJ
[b] ABC Vet hematology analyzer; ABX Diagnostics, Montpellier, France
[c] USCN Life Science, Wuhan, China
[d] STA-R Diagnostica Stago, Parsippany, NJ
[e] Multiphor II system; Pharmacia Biotech Amersham, Uppsala, Sweden
[f] Instrumentation Laboratory, Bedford, MA
[g] StatSoft, Tulsa, OK

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