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Protein C activity in babesiosis of dogs

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ABSTRACT

One of the numerous complications which could occur in babesiosis of dogs is disturbance in the coagulation system. The protein C pathway is one of the three major anticoagulant mechanisms and its activity was investigated in dogs with babesiosis, before and after therapy. We determined the decrease of protein C activity, which could be the consequence of inflammatory cytokines releasing, reduced synthesis in the liver, or increased erythropoietin production. Decrease in number of red blood cells and haematocrit could be due to mechanical, immuno-mediated or toxic-mediated haemolysis. Activity of aspartate-aminotransferase and alanine-aminotransferase and total bilirubin concentration was increased, indicating cellular damage of the liver.

Key words: protein C, coagulation, babesiosis, dog

Introduction

Canine babesiosis is caused by tick-borne haemoprotozoan parasites *Babesia canis* and *Babesia gibsoni*. In addition to fever and haemolytic anaemia, which are characteristic of babesiosis, other numerous complications occur in association with virulent parasites. One such is disturbance in the coagulation system (SCHETTERS et al., 1998).

Blood coagulation is a complex process of enzymatic interactions which finally results in clot formation by conversion of fibrinogen into insoluble fibrin. Haemostasis involves the concerted action of the vascular wall, platelets, coagulation and fibrinolytic pathways in the formation of a blood clot following vascular damage. Three major anticoagulant mechanisms appear to be involved: antithrombin/heparin, tissue factor pathway inhibitor (TFPI) and the protein C (PC) pathway (ESMON, 1999). When thrombin binds to

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thrombomodulin it undergoes a conformational change at its active site that converts it from a procoagulant enzyme into a potent activator of protein C. Activated protein C (APC) acts as an anticoagulant by proteolytically degrading and inactivating factors Va and VIIIa (ESMON and SCHWARTZ, 1995; MANN et al., 1998). It is well known that inherited deficiency of PC, antithrombin III (AT III) or protein S (PS) increases the risk of thrombosis, and the measurement of markers of activation of coagulation may correlate with the risk of developing thrombosis in these patients (HUMPHRIES, 1995). Animal models of thrombosis have played a crucial role in discovering and validating novel drug targets. These models have provided valuable information regarding the mechanisms of action of these agents and the interactions between antithrombotic agents that work by different mechanisms (LEADLEY et al., 2000).

Since publication of the determination of the clotting inhibitors of animal plasmas remains rare, the aim of the present study was to investigate protein C activity in dogs with babesiosis, before and after therapy.

Materials and methods

Blood samples were collected from 30 dogs infected with *Babesia* parasites. In all dogs, intraerythrocyte parasites were observed by microscopical examination in peripheral blood smears. Based on this finding, a diagnosis of canine babesiosis due to a large form, *Babesia canis*, was made.

Nineteen dogs with babesiosis were male and eleven were female, with ages ranging from 6 months to 14 years and represented various breeds. All dogs were individually owned and unrelated to each other. Twenty dogs had ticks attached to the skin. Duration of clinical signs ranged from 1 to 5 days prior to arrival at the Clinic. Clinical signs were as follows: depression (100%), anorexia (88%), pale mucous membranes (68%), fever (58%), splenomegaly (44%), water hammer pulse (24%), and icterus (10%). All dogs had been in good health three months prior to infection with parasites. The control group consisted of 30 clinically normal dogs of different breeds (17 males and 13 females) with a similar age distribution.

Measurements were made before treatment with Imizol (Imidocarb dipropionate - Schering Plough) and on the 2nd, 3rd, 5th and 10th days after therapy. Imizol (6 mg/kg) was applied subcutaneously once only. Blood was drawn into vacutainer (Becton Dickinson) tubes. PC analyses were performed with automatic coagulometer IL 9000 (Instrumentation Laboratory, Milan, Italy), using reagents of Dade Behring (Marburg Gmbh, Germany). This method is based on kinetic test (ESPANA et al., 1989). Biochemical profile was performed according to standard methods, using an automated biochemistry analyzer (Olympus AU 600, Olympus Diagnostica GMBH, Hamburg, Germany) with dedicated reagent kits.

The biochemistry panel included the following parameters: aspartate-aminotransferase (AST), alanine-aminotransferase (ALT) and total bilirubin concentration. Haematological analysis was assessed with automatic cell counter Serono Baker 9120 (Baker Diagnostics, Pennsylvania, USA). The following parameters were measured: number of erythrocytes (RBC), leucocytes (WBC), platelets (PLT) and haematocrit (PCV).

Statistica 5.0 for Windows was used for statistical analysis (Statistica for Windows computer program), Statsoft Inc. (1995). Obtained results were compared with those obtained from 30 clinically healthy dogs. Mann-Whitney U test was used for statistical analysis. Significance was set at $P<0.05$.

Results

Values of protein C activity and haematological parameters in dogs with babesiosis before and after therapy are presented in Table 1.

Table 1. Haemostatic and haematological parameters of control dogs and dogs with babesiosis (Mean ± SD; range; N)

Parameters	PC (%)	RBC ($\times10^{12}$/L)	PCV (%)	WBC ($\times10^{9}$/L)	PLT ($\times10^{9}$/L)
Control	37.7 ± 6.6	6.5 ± 0.8	50.1 ± 6.0	10.2 ± 2.3	259.5 ± 81.3
	21.2 - 56.9	5.2 - 7.8	38 - 63	5.3 - 13.4	137 - 487
	30	30	30	30	30
0. day	31.6 ± 11.0	5.1 ± 1.7*	42.0 ± 12.7*	8.1 ± 5.4*	17.6 ± 14.1*
	9.1 - 46.9	1.1 - 8.2	11 - 62	2.0 - 25.2	4 - 56
	30	30	22	30	26
2nd day	32.3 ± 10.3	5.0 ± 1.8*	38.6 ± 7.8*	12.5 ± 5.6	35.3 ± 35.4*
	16.5 - 50.3	1.0 - 7.8	22 - 50	3.4 - 27.6	4 - 30
	30	30	21	30	26
3rd day	33.6 ± 10.1	4.9 ± 1.7*	37.9 ± 8.5*	12.3 ± 5.6	59.5 ± 53.7*
	12.7 - 51.4	1.2 - 6.9	10 - 52	4.7 - 25.6	14 - 234
	30	25	19	25	24
5th day	28.5 ± 8.3*	4.9 ± 1.7*	39.3 ± 7.0*	15.4 ± 18.0	125.8 ± 90.5*
	12.2 - 45.5	1.2 - 6.8	16 - 46	4.7 - 26.2	16 - 339
	30	25	21	25	24
10th day	28.6 ± 5.7*	5.2 ± 1.3*	44.6 ± 7.3*	10.7 ± 2.6	224.0 ± 101.5
	17.2 - 36.0	1.5 - 7.0	22 - 51	5.4 - 18.1	13 - 456
	30	25	19	25	24

*P< 0.05 in comparison with control

Values of protein C activity are expressed as a percentage of the values of normal dogs. Normal plasma of healthy animals was pooled and served for determination of PC activity. Comparing PC activity in the blood plasma of dogs with babesiosis with healthy individuals, results were significantly lower on days 5 and 10. RBC and PCV were significantly lower during the measurement time. Leukopenia was significant prior to therapy. After therapy the leukocyte count showed no difference when compared with the control group. Thrombocytopenia was present until the 5th day of observation.

Biochemical parameters are presented in Table 2.

Table 2. Biochemical parameters in control dogs and dogs with babesiosis (Mean ± SD; range; N)

Parameters	AST (U/L)	ALT (U/L)	Bilirubin (μmol/L)
Control	17.3 ± 11.4	25.7 ± 9.2	3.3 - 2.3
	8 - 72	12 - 61	0.7 - 12
	30	30	30
0. day	110.8 ± 87.7*	103.7 ± 178.9*	36.3 ± 108.3*
	18 - 368	17 - 930	0.3 - 510
	22	24	22
2nd day	94.3 ± 79.7*	90 ± 155.1*	34.7 100.5*
	14 - 290	20 - 790	2.0 - 450
	23	23	20
3rd day	79.3 ± 50.5*	74.0 ± 132.9*	24.8 ± 65.7*
	14 - 189	15 - 679	2.7 - 300
	20	23	21
5th day	63.6 ± 54.4*	67.7 ± 111.4*	19.5 ± 48.2*
	12 - 233	12 - 578	1.3 - 220
	21	24	22
10th day	38.5 ± 31.0	58.5 ± 109.6*	6.1 ± 7.8*
	12 - 144	12 - 540	2.0 - 34
	19	22	19

*P<0.05 in comparison with control

Dogs with babesiosis showed significantly increased serum activity of AST and ALT, followed by hyperbilirubinemia. ALT activity and bilirubin concentration normalised on the 10th day of measurement.

Discussion

Canine babesiosis has been considered worldwide as a cause of haemolytic anaemia (BOOZER and MACINTIRE, 2003). In babesiosis the pathogens invade the RBCs of infected animals and cause severe destruction of the RBCs. In the present study, a significant decrease of RBC and PCV was present in measurements. Three mechanisms of haemolysis in canine babesiosis have been hypothesized: mechanical, immuno-mediated, toxic - caused by the production of the haemolytic factor of the parasites (BOURDEAU and GUELFI, 1995). It is well known that dogs with immune-mediated anaemia have an increased risk of thromboembolic disorders. Release of procoagulants from the lysed RBCs is the cause of this disorder (NELSON and COUTO, 1992). Total bilirubin increased during infection as a result of the erythrolysis provoked by the parasites. Most of the dogs with babesiosis showed signs of anaemia, and PC decrease could be due to the increased production of erythropoietin triggered by low erythrocyte count. Namely, erythropoietin has been shown to be the substance which causes PC decrease (KISAARSLAN et al., 1999). Pathology occasioned by *Babesia* infection might not necessarily be due to the direct effects of the parasite's toxins, or even its sequestration. Rather, it may be the response of the host by production of some molecules. Decrease of PC activity on the 5th and 10th days of measurement could be the consequence of inflammatory pathogenesis in babesiosis.

In the present study, all dogs had mild to severe thrombocytopenia before the treatment and until the 10th day of observation. These results indicate that babesiosis is an unlikely diagnosis in the absence of thrombocytopenia. Systemic disseminated intravascular coagulation (DIC), immune-mediated destruction, and platelet sequestration in the spleen are possible mechanisms of thrombocytopenia (BOOZER and MACINTIRE, 2003). VERCAMMEN et al. (1997) observed pronounced thrombocytopenia in dog babesiosis, which remained present until the end of a 12-week observation period. Leukocytosis, or leukopenia, has been previously described at different timings after the onset of infection (GUELFI and CANDEBAT, 1998). We have observed leukopenia in the acute phase, followed by normal leukocyte count one day after treatment. This is in agreement with the study of MATHE et al. (2006), who observed leukopenia, anaemia and thrombocytopenia in the acute phase of the disease.

Three major anticoagulant mechanisms appear to be involved: antithrombin/heparin, TFPI, and the PC pathway. The protein C pathway appears to be the primary target for cytokine action (ESMON, 1999). Since increased leukocyte count is a basic inflammatory parameter, the release of pro-inflammatory cytokines has also been described in *B. canis canis* infection (KRAJE, 2001). Release of inflammatory cytokines in babesiosis results in the adherence of infected erythrocytes (LUCAS et al., 1997; SCHETTERS et al., 1998).

In conclusion here, the decrease in PC activity could be the consequence of the release of inflammatory cytokines.

The significant increase in AST, ALT and total bilirubin may have been due to cellular damage of the liver by the parasites. Total bilirubin showed a rise as a result of the erythrolysis provoked by the parasite. In the majority of dogs with babesiosis, FURLANELLO et al. (2005) also noted mild elevation of AST, ALT and total bilirubin concentration. It is well known that the liver is the primary site for synthesis of activators and inhibitors of coagulation. The probability that PC decrease is altered during hepatic damage in babesiosis may be the result of lack of production or inhibition during specific types of liver injury. MOHANTI et al. (1997) suggest that reduction in the levels of protein C, protein S and AT III in human malaria, a disease which shares very similar complications with canine babesiosis, is the result of the consumption of these clotting factors due to microvascular thrombosis, rather than to reduced synthesis in the liver.

Preliminary results of HUMPHRIES (1995) suggest that correcting a specific deficiency of either AT III or protein C with a specific concentrate of AT III and PC can decrease this increased coagulation activity in humans. It would be worthwhile investigating whether exogenous PC therapy in babesiosis of dogs could counterbalance the coagulation abnormalities, and influence the outcome, in severe cases by preventing organ failure. Properly designed prospective studies will be required to determine whether assay techniques of anticoagulant components will assist in the identification of dogs predisposed to thrombotic events.

Finally, this study demonstrates that reduction in PC activity is present in naturally induced babesiosis of dogs. The prognostic value of this marker remains to be fully defined in future epidemiological and clinical studies.

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SAŽETAK

Jedna od mnogobrojnih komplikacija koja se može javiti kod babezioze pasa je poremećaj u sustavu grušanja krvi. Protein C je jedan od tri glavna čimbenika antikoagulacije, a njegova aktivnost je istraživana u pasa s babeziozom, prije i nakon terapije. Zabilježen je pad aktivnosti proteina C, što može biti posljedica oslobađanja upalnih citokina, smanjene sinteze u jetri ili povećane proizvodnje eritropoietina. Sniženje broja eritrocita i hematokrita može biti rezultat mehaničke ili imunološki posredovane hemolize ili hemolize uzrokovane oslobađanjem toksina. Aktivnost aspartat-aminotransferaze i alanin-aminotransferaze i koncentracija ukupnog bilirubina bila je povećana, što upućuje na oštećenja jetrenih stanica.

Ključne riječi: protein C, koagulacija, babezioza, pas