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Antithrombin III in healthy dogs and in dogs suffering from babesiosis

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ABSTRACT

Antithrombin III (AT III) was measured in 15 dogs with *Babesia canis* infection and in 15 healthy dogs. Blood samples for control group and patients were taken before the therapy with imidocarb dipropionat and on the 1st and 5th days after the therapy. Measurements of AT III activity in canine plasma with thrombin dependent chromogenic substrate assay using automatic analyzer showed a good within-run precision. The results were expressed as proportions of the norm. Value for AT III before the therapy was lower than in the control group. Activity of AT III was higher on the post-treatment day 5 than before the therapy. A survey of individual data demonstrated that in 2 dogs the pre-treatment AT III values were 0.66 and 0.79, respectively, showing consumption inhibitors of coagulation.

Key words: babesiosis, dog, antithrombin III

Introduction

Canine babesiosis is a tick-borne disease of worldwide importance, ranging in severity from relatively mild to fatal (LOBETTI, 1998). The causative organism of canine babesiosis is the intraerythrocytic protozoan parasite, either *Babesia canis* or *Babesia gibsoni*. Three subtypes or strains of *B. canis* are recognized, namely *B. canis canis*, *B. canis vogeli* and *B. canis rossi* (LOBETTI, 1998; SHAW et al., 2001).

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The pathophysiological mechanisms of canine babesiosis are very intricate (JACOBSON and SWAN, 1995). The acute-phase response is an early complex and non-specific body reaction to attacks of agents such as bacterial, viral, or parasitic infections (VAN LEEUWEN and VAN RIJSWIJK, 1994). The responses start locally and then become systemic. The secondary systemic reaction includes neurological, endocrine, and metabolic alterations such as fever, leucocytosis, increased hormone levels, activation of haemostasis and complement system, formation of kinines and rearrangement of the plasma protein pattern (PEREIRA and BURINI, 1992). The acute-phase response is triggered by the action of proinflammatory cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor (TNF-α), which are responsible for a series of metabolic changes that follow a pathogen invasion (WOLF and KEUSCH, 1999).

Intraerythrocytic parasitaemia causes both intravascular and extravascular haemolysis, which results in regenerative anaemia, haemoglobinaemia, haemoglobinuria and bilirubinuria. Babesiosis can cause severe tissue hypoxia, anaerobic metabolism and metabolic acidosis with consequent widespread tissue damage and a probable release of inflammatory mediators. Pyrexia that also develops is attributed to the release of endogenous pyrogens after erythrolysis, destruction of the parasite and/or activation of inflammatory mediators. The systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS) occur frequently in complicated canine babesiosis (WELZL et al., 2001). The systemic inflammatory response syndrome that precedes MODS is caused by excessive release of inflammatory mediators (LOBETTI, 1998).

Altered haemostasis associated with babesiosis has been reported in the past.

Activation of the fibrinolytic pathway co-occurs with the activation of the coagulation cascade. This results in fibrino- and fibrinogenolysis, the release of an increased quantity of fibrin/fibrinogen degradation products (FDP) into the bloodstream and a possibility of haemorrhage (SLAPPENDEL, 1988). The results (BARIĆ RAFAJ et al., 2001) confirm that Hageman's factor is activated in *Babesia canis* infection. Complications of babesiosis include cerebral involvement, renal failure, acute respiratory distress syndrome (ARDS), hepatic dysfunction, and so on. Many of these complications are believed to be at least related to the hypercoagulable state in this disease. Our study demonstrated an alteration in the platelet function and an increased procoagulant activity of parasitized RBC in *B. canis* infection. These factors are believed to be responsible for the hypercoagulable state in acute babesiosis, particularly in cases caused by *B. canis*. Hence, we decided to study antithrombin III (AT III), an inhibitor of blood coagulation, as it may help us understand the pathogenesis of various complications associated with babesiosis, and consequently develop a means of prevention or appropriate therapy for such complications.

Materials and methods

Fifteen dogs of both sexes (7 males and 8 females) and of different age groups (7 months to 9 years) were presented at the Clinic for Internal Diseases, Chair for Cynology, Faculty of Veterinary Medicine, University of Zagreb, with clinical signs of acute babesiosis and were prospectively studied. The control group comprised 15 healthy dogs of both sexes (7 males and 8 females) aged from 10 months to 8 years. The dogs were clinically normal, aparasitaemic, and came from the same area as the dogs with babesiosis.

Blood samples for control group and patients were collected from the cephalic vein into 3 ml EDTA tubes, 0.129 M sodium citrate tubes and serum tubes on three occasions: before treatment with imidocarb dipropionate (''Imizol'', Coopers Animal Health, Herts, England) and on post-treatment days 1 and 5. Plasma and serum were separated from the blood, centrifuged at 4 ˚C for 15 min (1500 g) within 30 minutes of collection, and then stored in polypropylene tubes at -70 ˚C until analysis. Thin blood smears were taken from the capillary bed in the cranial margin of the ear and stained with the May Grünwald-Giemsa method (SOFTIĆ, 1984). Babesiosis was diagnosed by finding the parasite *Babesia canis* in host erythrocytes.

For the study of plasma inhibitory activity, AT III was determined with an automated chemical analyzer using a chromogenic substrate, the Berichrom® Antithrombin III (A) test kit (DADE Behring, Marburg, Germany) at 37 ˚C. The assay is based on the inhibition of thrombin by its natural plasma-derived inhibitor, AT III, in the following manner: antithrombin III in the sample is converted into an immediate inhibitor by heparin and it then inactivates the present thrombin. The residual thrombin content is determined by a kinetic test which measures the increase in absorbance at 405 nm. The absorbance decreases linearly with the amount of antithrombin III present in the patient sample. The pooled plasma of 15 normal dogs was used to construct a 6-point standard curve. The results were expressed as proportions of the norm. The detection limit was set at 3.7% of the norm. Precision and accuracy were good: intra-assay coefficient of variation for normal samples ranged from 1.8 to 3.1%, and for pathological plasmas from 2.2 to 4.0%. The inter-assay for normal samples ranged from 2.4 to 4%. For pathological samples, a value of 3.2% was set. The normal values of AT III using 15 healthy (assessed through a complete clinical examination, haemogram and biochemical profile) adult dogs of different breeds and genders were initially established.

Haematological analysis (erythrocytes, haemoglobin, haematocrit, mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), and leukocytes) were performed on EDTA blood using an automatic haematology analyzer (Serono 9120; Serono Baker Diagnostic). Detailed microscopic smear examination was recorded manually. The serum collected after clot retraction and centrifugation was analysed for levels of urea, creatinine, bilirubin, aspartate aminotransferase (AST), and

alanine aminotransferase (ALT) using an automated analyser Olympus AU 600 (Olympus Diagnostica GMBH) with Olympus chemicals (Olympus Diagnostica GMBH).

Data were analysed using the software application Statistica 6.1. The presence of a statistically significant difference ($P<0.05$) between the study groups in a number of variables was tested using t-test and analysis of variance.

Results

The statistical analysis demonstrated that in dogs suffering from babesiosis before the therapy AT III is significantly lower than in the control group (Fig. 1).

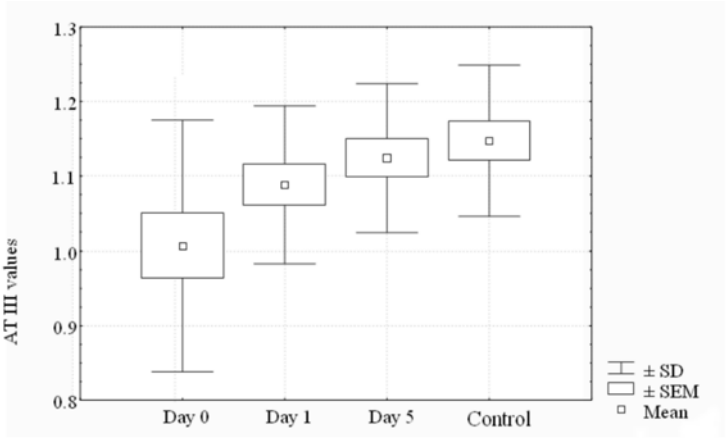

Fig. 1. AT III values in sick and healthy – control dogs

No differences were found between the control group and the sick dogs on post-treatment days 1 and 5. AT III values increased over the study period. In sick dogs, a significant increase in antithrombin III occurred between the pre-treatment measurement and the results obtained on post-treatment day 5. The pre-treatment AT III results were 1.01 ±0.16 (Table 1). Twenty-four hours later, the AT III values increased first to approximately 1.09 ±0.11, and finally to 1.12 ±0.10 on post-treatment day 5. Furthermore, it was noticeable that pre-treatment AT III results showed a large standard deviation, which was also confirmed by the minimum and maximum AT III pre-treatment results ranging from 0.66 to 1.29. A survey of individual data demonstrated that in 2 dogs the pre-treatment AT III values were 0.66 and 0.79, respectively.

Table 1. AT III activity in the control group and in dogs with *Babesia canis* infection measured before treatment and on post-treatment days 1 and 5 (P< 0.05)

	N	M	Min	Max	SD
AT III healthy	15	1.15	0.81	1.20	0.10
AT III 0. day	15	1.01*	0.66	1.29	0.17
AT III 1. day	15	1.09	0.91	1.30	0.11
AT III 5. day	15	1.12**	0.83	1.31	0.10

* Healthy control vs. 0 day, $p < 0.05$; ** 0 day vs. 5. day, $p < 0.05$

Table 2. Haematological and biochemical parameters in control group, and in dogs with *Babesia canis* infection before treatment (M ± SD) (P< 0.05).

Hameatological and biochemical parameters	Healthy control n = 15	Dogs with *Babesia canis* infection (0. day), n = 15
Erythrocytes ($\times 10^{12}$)	7.1 (0.98)	5.46* (1.77)
Haemoglobin (g/L)	165 (18.9)	126* (38.4)
Leukocytes ($\times 10^{9}$)	11.2 (2.0)	10.9 (8.8)
HMT (L/L)	0.54 (0.07)	0.43* (0.13)
Neutrophils (%)	49.7 (10.4)	67.4* (10.5)
Bands (%)	2.7 (2.1)	3.8 (6.6)
Lymphocytes (%)	39.1 (14.6)	23.3* (11.4)
Monocytes (%)	1.7 (0.4)	4.8 (6.6)
Eosinophils (%)	6.1 (4.9)	0.5* (1.4)
Basophils (%)	0	0
MCV (fl)	76 (3.4)	79 (5.4)
MCH (pg)	22 (1.5)	24 (1.8)
MCHC (g/dl)	32 (1.8)	29* (2.1)
Urea (mmol/L)	7.8 (2.1)	15.8 (22.7)
Creatinine (μmol/L)	111 (10.6)	144 (133.9)
Bilirubin (μmol/L)	2.6 (1.4)	7.1* (6.6)
AST (U/L)	17.8 (4.1)	100.9* (84.6)
ALT (U/L)	22.3 (8.2)	71.3* (34.2)

Haematological and biochemical results of the control group (healthy) dogs and of babesiosis patients before the treatment are presented in Table 2. The results show that erythrocytes, haemoglobin, haematocrit, segmented neutrophils, lymphocyte, eosinophils, MCHC, bilirubin, AST and ALT values were significantly lower pre-treatment than in the control group ($P<0.05$).

Discussion

This study demonstrates a significant alteration in haemostasis in babesiosis caused by *Babesia canis*. The alteration leaned towards the hypercoagulable state, which tended to revert back to normal after antibabesial treatment. Measuring AT III, an inhibitor of blood coagulation, should throw light on the pathophysiology of the hypercoagulable state seen in *Babesia canis* infection. AT III, a single-chain glycoprotein with a M of 58000 Da, produced by liver (systemic action in blood) and, to a lesser extent, by vascular endotelium (local action), is an endogenous inhibitor of blood coagulation, providing approximately 70-90% of the total plasma inhibitory activity (GREEN, 1984; VINAZZER, 1987; CALDIN, 1998). It inactivates thrombin (factor IIa) and other serine proteases, including factors XIa, IXa, Xa, XIIa, and kallikrein in a progressive, irreversible manner to form inactive complexes (VINAZZER, 1987). The rate of AT III-mediated inactivation clotting factor is markedly enhanced by heparin (GREEN, 1984).

The mean value of AT III in our control group was 1.14, with a range of from 0.81 to 1.2. According to MISCHKE (1998), the reference range of AT III in healthy dogs is from 0.82 to 1.18. For BATEMAN et al. (1999), physiological values of AT III in dogs are between 0.78 and 1.14, while KARGES et al. (1994) propose slightly higher values, ranging from 1.17 to 1.33. CALDIN et al. (1996) state that the mean values of AT III established on the sample of 50 healthy adult dogs were 115 ±7.5%. In relation to the control group, AT III activities fell to the lowest level before treatment ($P<0.05$). In two dogs, pre-treatment AT III activity was 0.66 and 0.79, respectively. The lack of decrease of AT III activities in dogs before treatment may have been a result of the ability of the liver to synthesize AT III faster than it is catabolized (BOUDREAUX et al., 1989).

Plasma AT III activity is a key test for the diagnosis and monitoring of DIC (BICK, 1988; BICK and BAKER, 1992; KIRBY, 2000). The activity of AT III declines early in the DIC process as this endogenous anticoagulant is consumed. Activity levels of less than 80% are presumed to be diagnostic of DIC in humans. When, in critically ill humans, antithrombin levels drop below 60%, a 96% mortality rate is expected (HELGREN et al., 1984). Clinical experience in dogs and cats has shown that AT III levels of less than 80% are indicative of DIC. Levels of less than 60% require replacement of AT III as part of the treatment plan (KIRBY, 2000). At the same time, AT III is a good prognostic indicator. The prognosis is guarded as much as the levels of AT III are reduced in blood (CALDIN, 1998).

The prognosis in the case of MODS corresponds to the degree of deficiency of inhibitors of coagulation, such as antithrombin III and protein C (HESSELVIK et al., 1989; FOURRIER et al., 1992; OWINGS et al., 1996). In experimental models of MODS that generally use infection or endotoxin as a stimulus, altering the haemostatic system to minimize fibrin accumulation has prevented progressive organ dysfunction and mortality (REDENS and EMERSON, 1989). According to HAIRE et al. (1998) AT III level is a marker of the severity of the inflammatory response.

However, some authors suggest that AT III might have an anti-inflammatory as well as anti-DIC activity (UCHIBA et al., 1995). Namely, AT III induces endothelial cell release of prostacyclin (PGI_2), which inhibits cytokine production and suppresses leukocyte and T-cell activation (KAINOH et al., 1990; JOCHUM, 1995).

A reduction in AT III activity as a result of renal insufficiency, lack of synthesis or increased consumption has been associated with thrombosis in several species (GREEN and KABEL, 1982; GREEN, 1984). According to JOHNSTONE et al. (1986), a decreased level of AT III activity may be associated with the possibility of increased intravascular thrombin production, which in turn stimulates an increased consumption of the natural inhibitor AT III. Lowered levels of AT III activity tilt the fine balance of the haemostatic mechanism towards fibrin and thrombin production, because the inhibitory mechanisms are weakened. AT III plasma half-time is 12 hours. It is possible that hepatocytes, which may be damaged in the course of babesiosis as indicated by the significantly higher AST and ALT values in sick dogs, reduce their synthesis and thus influence AT III activity. In addition to the disturbance in the hepatocyte function, haemolytic-uremic syndrome may be one of many causes of the acquired AT III deficiency. Levels of urea and creatinine in the pre-treatment dog sera were not significantly higher than the respective levels in the control group. We may therefore conclude that the renal function in dogs with babesiosis is preserved, and that it is not likely that dogs in our study excreted AT III by the renal route.

Twenty-four hours after treatment, AT III activity increased to reach the post-treatment day 5 value, a value significantly higher than those measured before treatment. A reason for this may be that AT III is at the same time an acute phase protein, and the level of acute phase proteins is increased in canine babesiosis (Matijatko-personal communication).

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SAŽETAK

Aktivnost antitrombina III (AT III) izmjerena je u 15 pasa zaraženih parazitom *Babesia canis* i u 15 zdravih pasa. Krv za pretrage, u kontrolnoj skupini i u pacijenata, uzeta je prije terapije imidocarb dipropionatom te prvi i peti dan nakon terapije. Aktivnost AT III određena je u plazmi kromogenom metodom upotrebom automatskog analizatora. Određivanje se pokazalo preciznim unutar serije. Rezultati su izraženi kao udio od normalnog. Vrijednosti za AT III prije terapije bile su niže nego u kontrolnoj skupini. Aktivnost AT III bila je viša petog dana terapije nego prije terapije. U dva psa aktivnost AT III prije terapije bila je 0,66 i 0,79 što upućuje na zaključak da je došlo do potrošnje inhibitora koagulacije.

Ključne riječi: babezioza, pas, antitrombin III